Health effects of quercetin:
from mechanism to nutraceutical

© Agnes W. Boots, Maastricht, 2006

ISBN 10: 90-5278-586-4

ISBN 13: 978-90-5278-586-8

Cover design and figures: Harald Moonen

Layout: Tiny Wouters

Production: Datawyse | Universitaire Pers Maastricht

Printing of this thesis was financially supported by GlaxoSmithKline, ZonMw, Child care foundation, Dr. Ir. Van de Laar Stichting, Zambon Nederland BV.

nutrim

The studies presented in this thesis were funded by the Netherlands Organisation for Health Research and Development (project no. 014-12-012) and performed within the Nutrition and Toxicology Research Institute Maastricht (NUTRIM) which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

Health effects of quercetin: from mechanism to nutraceutical

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. mr. G.P.M.F. Mols,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 21 december 2006 om 14.00 uur

door

Agnes Wilhelmina Boots

Promotor

Prof. dr. A. Bast

Copromotor

Dr. G.R.M.M. Haenen

Beoordelingscommissie

Prof. dr. C.D.A. Stehouwer (voorzitter)

Prof. dr. W.A. Buurman

Prof. dr. M. Drent

Prof. dr. J.C.S. Kleinjans

Prof. dr. ir. I.M.C.M. Rietjens (WUR)

*“Life is what happens to you
while making other plans”*

John Lennon

Contents

Abbreviations	9
Chapter 1 General introduction	11
Chapter 2 Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants	49
Chapter 3 Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation	61
Chapter 4 No role of DT-diaphorase (NQO1) in the protection against oxidized quercetin	77
Chapter 5 The reversibility of the glutathionyl-quercetin adduct spreads oxidized quercetin-induced toxicity	93
Chapter 6 The quercetin paradox	109
Chapter 7 The anti-inflammatory activity of quercetin	125
Chapter 8 Antioxidant status associated with inflammation in sarcoidosis: a potential role for antioxidants	143
Chapter 9 Quercetin reduces markers of oxidative stress and inflammation in sarcoidosis	161
Chapter 10 Summary and general discussion	177
Samenvatting en algemene conclusies	187
Dankwoord	199
List of publications	207
Curriculum Vitae	213

Abbreviations

4-MC	4-methylcatechol
Ag	antigen
AP-1	activator protein-1
APC	antigen presenting cell
ATBC study	alpha-tocopherol-beta-carotene study
ATP	adenosine 5'-triphosphate
BAL(F)	bronchoalveolar lavage (fluid)
BSA	bovine serum albumin
BSO	buthionine sulfoximine
CDNB	chlorodinitrobenzene
cGMP	cyclic guanosine monophosphate
COPD	chronic obstructive pulmonary disease
DHA	dehydroascorbic acid
DLCO	diffusing capacity of the lung for carbon monoxide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTD	DT diaphorase
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
EDRF	endothelium-derived relaxing factor
EDTA	ethylene diamine-tetraacetic acid
ELF	epithelial lining fluid
ELISA	enzyme linked immune sorbent assay
ESR	electron spin resonance
FAD ⁺	flavin adenine dinucleotide
FEV ₁	forced expired volume in one second
FVC	forced vital capacity
GPx	glutathione peroxidases
GSH	glutathione
GSQ	glutathionylquercetin adducts
GSSG	glutathione disulphide
GST	glutathione transferase
HCl	hydrochlorous acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IFN- γ	interferon-gamma
I κ B α	inhibitory part of nuclear factor-kappa B
IKK	I κ B α kinase
IL	interleukin
IPF	idiopathic pulmonary fibrosis
LDH	lactate dehydrogenase

LPS	lipopolysaccharide
MDA	malondialdehyde
MSH	2-mercaptoethanol
MSQ	MSH adduct with oxidized quercetin
NAC	N-acetylcystein
NAD ⁺	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NBT	Nitro Blue Tetrazolium
NF- κ B	nuclear factor kappa-B
PBS	phosphate buffered saline
PUFA	poly-unsaturated fatty acid
QQ	quercetin quinone
RLE cells	rat lung epithelial cells
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
SD	standard deviation
SEM	standard error of the means
SERCA	sarco- and endoplasmatic reticulum calcium ATPase
SOD	superoxide dismutase
SSA	sulfosalicyclic acid
TCA	trichloric acid
TEAC	trolox equivalent antioxidant capacity
TGF- β	transforming growth factor beta
Th1	T-helper 1
TLR	Toll-like receptor
TNF α	tumour necrosis factor alpha

1

General introduction

Introduction

1.1 Free radicals and reactive species

1.1.1 *Origin and terminology*

Free radicals are reactive molecules due to the presence of one or more unpaired electron(s). They are formed in the human body either as an essential mediator in vital processes including neurotransmission and inflammatory reactions, or as a byproduct that does not have a role in the actual process. In aerobic life forms, the reduction of oxygen is of special interest. This reduction comprises binding of most of the oxygen to hydrogen to give water, a process involved in the oxidative phosphorylation. However, a small part of the oxygen (approximately 1-3%) is only partly reduced during this redox reaction (1). As a result, free radicals or reactive species, that can either oxidize other compounds or easily form radicals, will arise. These partly reduced forms of oxygen are collectively described as reactive oxygen species (ROS). Similarly, reactive nitrogen species (RNS) can be produced. Physiological important ROS include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), ozone (O_3) and hypochlorous acid ($HOCl$). Examples of physiological important RNS are nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^{\bullet}$). An overview of reactive oxygen and nitrogen species is given in Table 1.1 (2). It should be noted that not all ROS and RNS are equally reactive. Some compounds, such as H_2O_2 , $O_2^{\bullet-}$ and $NO^{\bullet}$ react *in vivo* relatively selectively with only a few biological molecules, whereas for example the radical $OH^{\bullet}$ is a very reactive ROS that will react instantaneously with virtually any molecule it encounters (3). The reactivity of the other ROS and RNS lies in between these extremes (4-6). Another difference between various ROS comprises the site of their reactivity; free radicals will react almost instantaneously at the site of their formation, whereas non-radical ROS such as H_2O_2 might also pass biological membranes and in that way spread their reactivity and possible toxicity.

1.1.2 *Beneficial effects*

As mentioned above, in the human body ROS and RNS are produced that display several crucial physiological functions, including smooth muscle relaxation, metabolism of xenobiotics and the respiratory burst to kill invading micro-organisms (7-10) .

Table 1.1 Typical physiological reactive oxygen and nitrogen species.

Reactive Oxygen Species (ROS)	
Radicals	Non-radicals
Superoxide anion, $O_2^{\bullet -}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\bullet}$	Hypochlorous acid, $HOCl$
Peroxyl, $RO_2^{\bullet}$	Ozone, O_3
Alloxyl, $RO^{\bullet}$	
Reactive Nitrogen Species (RNS)	
Radicals	Non-radicals
Nitric oxide, $NO^{\bullet}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\bullet}$	Nitrosyl cation, NO^+
	Nitroxyl anion, $NO^{\bullet -}$
	Peroxynitrite, $ONOO^{\bullet -}$
	Alkyl peroxynitrites, $ROONO$

Smooth muscle vasodilatation depends on the release of a relaxing factor from the endothelium of blood vessels. This factor, initially referred to as the endothelium-derived relaxing factor (EDRF), appeared to be $NO^{\bullet}$ (11). This radical is produced by nitric oxide synthases (NOS) out of *l*-arginine. $NO^{\bullet}$ will activate guanylate cyclase that forms cyclic guanosine monophosphate (cGMP), which may lead to muscle relaxation (10,12,13). Muscle relaxation can be stopped by the reaction of $NO^{\bullet}$ with $O_2^{\bullet -}$, that is formed locally in the endothelium of the blood vessels (14).

Numerous compounds are metabolized in the liver into more polar compounds by cytochrome P-450 (9). The reactive oxygen generated in the active site of this enzyme can oxidize virtually any endogenous compound or xenobiotic, including chemically very inert compounds as benzene (9,15).

During infections, ROS such as $O_2^{\bullet -}$ and $HOCl$ are used as lethal weapon to kill invading micro-organisms. The explosive production of ROS by phagocytic leukocytes (i.e. neutrophils, eosinophils, monocytes and macrophages) is called the respiratory burst (8,16). Important enzymes that are involved in this inflammatory reaction include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the inducible form of NOS and myeloperoxidase.

NADPH oxidase is dormant in resting phagocytic cells but comes into action when the cell is activated by invading micro-organisms (17). This enzyme initiates the oxidant generation of the respiratory burst by forming $O_2^{\bullet -}$. The inducible form of NOS is expressed on phagocytes and, upon stimulation, will form $NO^{\bullet}$ (18-21). $O_2^{\bullet -}$ and $NO^{\bullet}$ themselves are not very reactive towards micro-organisms. On the contrary, the product formed out

of these radicals, i.e. $\text{ONOO}^\cdot$, is very cytotoxic (22). Furthermore, the bactericidal activities of the phagocytes are boosted by the formation of the highly reactive HOCl out of H_2O_2 and chloride, a reaction catalyzed by the enzyme myeloperoxidase (23,24). The H_2O_2 necessary for this reaction is formed by the dismutation of superoxide anion, either spontaneously or catalyzed by the enzyme superoxide dismutase (25,26).

1.1.3 *Damaging effects*

ROS and RNS react readily with practically all bio-molecules, including DNA, RNA, proteins, carbohydrates and lipids, thereby damaging the attacked molecule (27). The reaction with these molecules often starts with the subtraction of a hydrogen atom from the attacked molecule, thereby converting the unpaired electron into a more stable electron-pair. Alternatively, an electron instead of a hydrogen atom might be transferred. From an electrochemical point of view, the hydrogen or electron donating molecule is oxidized. Consequently, free radicals and reactive species are often called (pro)oxidants.

An important target of oxidation by ROS and RNS are the poly-unsaturated fatty acids (PUFAs) present for example in the cell membrane (28). Initiation of this reaction involves the subtraction of a hydrogen atom from the attacked PUFA, thereby leaving an unpaired electron on the lipid. This newly-formed lipid radical undergoes molecular rearrangement to increase its stability (29) and will then rapidly react with oxygen, thereby creating a peroxy radical. Subsequently, this peroxy radical will create a lipid hydroperoxide as well as a new lipid radical by subtracting a hydrogen atom from a second PUFA. After molecular rearrangement and the reaction with oxygen and a third PUFA, a second lipid hydroperoxide and a third peroxy radical are generated. Propagation of this chain reaction, referred to as lipid peroxidation, takes place by continuously passing the unpaired electron from one molecule to another (28). Termination of this chain reaction may occur upon (i) the consumption of one of the two reactants, i.e. the PUFAs or the oxygen, (ii) the formation of a relatively unreactive radical or (iii) the reaction of two radicals that will combine to form a non-radical pair. Products formed during lipid peroxidation include 4-hydroxy-2-alkenals and the three-carbon compound malondialdehyde (MDA) (28). By reacting with DNA bases, MDA can cause mutagenic lesions that may be involved in the pathology of various diseases (30). MDA is widely used as an index of the occurrence of lipid peroxidation in the biological science, although its use is debated (28,31,32).

The initiation and propagation of lipid peroxidation are schematically depicted in Figure 1.1 and illustrate how free radicals may damage bio-molecules.

Figure 1.1 Process of lipid peroxidation, started by the radical $R^\bullet$.

Oxidative damage caused by ROS and RNS will lead, among others, to DNA lesions (33-35), function loss of enzymes (36,37), increased cell permeability (38,39), disturbed signaling over the cell (38-40) and eventually even necrotic cell death or apoptosis (27,39-41). Consequently, damage induced by reactive species is often suggested to play a role in the pathophysiology of various diseases, including diabetes (42), cancer (43), chronic obstructive pulmonary disease (44), sarcoidosis (45,46) and idiopathic pulmonary fibrosis (47).

1.1.4 Link between reactive species and inflammation

Besides directly or indirectly damaging various bio-molecules, reactive species are also involved in inflammation. The inflammatory response is designed to recognize, attack and kill invading pathogens (48,49). In vertebrates, this response is subdivided into two parts determined by the speed and specificity of the reaction, i.e. the innate and the adaptive immune response.

The innate immune response mediates the direct protection against infections, either by preventing its occurrence (via epithelial barriers) or by eliminating microbes (via phagocytes, natural killer cells or the complement system) (49,50). The adaptive immune response does not develop instantaneously and is mediated by lymphocytes and their products. B-lymphocytes will produce antibodies that are capable of counteracting infections and eliminating microbes, whereas T-lymphocytes will destroy the microbes directly (48,49).

To offer maximal protection against invading pathogens, cooperation between the innate and adaptive immunity exists. An example of this

interaction is that maturation of dendritic cells, controlled by the innate response, will lead to migration of these cells to lymphoid organs where they can activate the adaptive response by priming T-lymphocytes (51).

A key factor in both the innate and adaptive immune response is the production of cytokines, i.e. glycoproteins secreted by various immune cells including macrophages, neutrophils and helper T lymphocytes (48,52). Cytokines may exert either pro- or anti-inflammatory activities. One of the most prominent pro-inflammatory cytokines in both inflammatory responses is the tumour necrosis factor alpha ($\text{TNF}\alpha$), produced by e.g. macrophages upon the activation of the transcription factor nuclear factor kappa-B ($\text{NF-}\kappa\text{B}$) (53,54).

The production of various cytokines via activation of transcription factors such as $\text{NF-}\kappa\text{B}$ and activator protein-1 (AP-1) can also be induced by ROS (45,55-57). *In vitro* studies, using both macrophages and alveolar and bronchial epithelial cells, have demonstrated that oxidants can initiate the production of inflammatory mediators like interleukin (IL)-8 and NO^* (58). The activation of $\text{NF-}\kappa\text{B}$ by ROS is mediated by the breakdown of its inhibitor part, i.e. $\text{I}\kappa\text{B}\alpha$, that causes the normally inactive transcription factor to become active. This activation appears to be due to the phosphorylation and the subsequent degradation or displacement of $\text{I}\kappa\text{B}\alpha$, catalysed by various subforms of the $\text{I}\kappa\text{B}\alpha$ kinase (IKK) (56,59-61). However, some studies have suggested other plausible mechanisms behind this activation (62).

Contradictory, a few studies have reported that (cytokine-induced) $\text{NF-}\kappa\text{B}$ activation can also be inhibited by pre-treatment with or simultaneous exposure, either acute or chronic, to a specific ROS, i.e. H_2O_2 (62-65). Inhibition by H_2O_2 could be adaptively, resulting in a reduced ROS-forming activity that is not capable of effective phosphorylation of $\text{I}\kappa\text{B}$ (63). Alternatively, this inhibition may be associated with a promotion of apoptosis resulting from too high levels of damage, induced by the presence of both elevated ROS and inflammatory cytokines levels (65). This would be in line with the fact that only intermediate levels of oxidative stress are capable of activating $\text{NF-}\kappa\text{B}$, whereas high levels will induce apoptosis (66). This apparent paradox can be seen as a biphasic response of $\text{NF-}\kappa\text{B}$ to oxidative stress.

In summary, it can be stated that, although some studies display ambiguous results, ROS appear to induce $\text{NF-}\kappa\text{B}$ activation. Different outcomes of studies regarding this induction may be the result of variables including the cell type, culturing conditions, anti-oxidant defence present, the level of oxidative stress or other inducers and time frames involved (65).

As stated previously, an important cytokine induced by ROS via NF- κ B is TNF α . On its turn, TNF α itself can also mediate the activation of NF- κ B by e.g. increasing the activation of IKKs (61,67,68) as well as the production of ROS (69). The latter occurs via stimulation of the radical production during both the mitochondrial oxidative phosphorylation (59,70,71) and the inflammatory burst (69,72,73). Furthermore, TNF α can also enhance the transcription of other cytokines, including IL-8 (61,74). This feed forward mechanism, depicted in Figure 1.2, amplifies both the TNF α - and the NF- κ B-mediated effects. Final result of this amplification might be cytotoxic levels of cytokines and ROS, resulting in apoptosis.

Figure 1.2 Interplay between ROS and inflammation. NF- κ B=nuclear factor kappa-B; ROS=reactive oxygen species; TNF α =tumour necrosis factor alpha; RNS=reactive nitrogen species; IL-8=interleukin-8; iNOS=inducible nitric oxide synthase.

1.2 Antioxidants

Fortunately, the human body comprises an elaborate antioxidant defense system to protect cellular compounds from damage induced by free radicals, ROS and other reactive species. An antioxidant has been defined as “any

substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate” (75,76). The antioxidants that react directly with radicals or other reactive species to prevent cellular compounds from becoming oxidized, can be subdivided into enzymatic and non-enzymatic antioxidants (Table 1.2).

Table 1.2 Enzymatic and non-enzymatic antioxidants.

Enzymatic antioxidants	
Enzyme	Reaction
Superoxide dismutases (SOD)	$2 \text{O}_2^{\bullet-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$
Catalases	$2 \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2 \text{H}_2\text{O}$
Glutathione peroxidases (GPx)	$2 \text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2 \text{H}_2\text{O}$
	$2 \text{GSH} + \text{ROOH} \rightarrow \text{GSSG} + \text{ROH} + \text{H}_2\text{O}$
Non-enzymatic antioxidants	
Hydrophilic	Hydrophobic
Glutathione (GSH)	α -Tocopherol (vitamin E)
Ascorbate (vitamin C)	Carotenoids
Uric acid	Ubiquinol-10

1.2.1 Enzymatic antioxidants

Enzymatic antioxidants react with reactive species and are subsequent efficiently recycled. In fact, the enzyme functions as a catalyst. Consequently, only small amounts of these enzymes are needed to offer protection. Important antioxidant enzymes are superoxide-dismutases, catalase and glutathione peroxidases (27).

Superoxide dismutases (SOD), the first group of enzymes that was discovered with a free radical as substrate (77), catalyze the dismutation of $\text{O}_2^{\bullet-}$ to H_2O_2 and oxygen (O_2). Uncatalyzed, this dismutation of $\text{O}_2^{\bullet-}$ is relatively slow due to the required encounter of two $\text{O}_2^{\bullet-}$ radicals that is hampered by the fact that both radicals are negatively charged and will therefore repulse each other (78). SOD will considerably increase the rate of this dismutation. There are several forms of SOD that all share the feature of containing a transition metal ion, i.e. copper (Cu^{2+}), manganese (Mn^{2+}), iron (Fe^{3+}) or nickel (Ni^{2+}) in their catalytic center. These transition metal ions are capable of catalyzing electron transfer due to their positive charge and their ability to switch to another stable valence or oxidative state (25,79,80). SOD isoforms are distributed throughout the body, including in the mitochondria and cytosol (77,81-83).

Catalase, an enzyme present in all tissues but mostly in the liver and erythrocytes, catalyses the dismutation of hydrogen peroxide into water and

oxygen (78,84). Uncatalyzed, this dismutation is relatively slow due to the fact that H_2O_2 is a weak reductant. Together with most of the enzymes capable of generating H_2O_2 , catalase is predominantly located in the peroxisomes of the cell (78,84). Interestingly, catalase is also present in significant, but small, amounts in the mitochondria (85,86).

The selenium-containing glutathione peroxidases (GPx), enzymes distributed in almost all tissues but predominantly in the liver, catalyze the conversion of H_2O_2 into water (78,87-89). GPx can also reduce other peroxides, including lipid hydroperoxide, into alcohol (78,90). In contrast to the dismutases SOD and catalase, GPx is a reductase that consumes a cofactor, i.e. glutathione (GSH). During the conversion of each molecule of H_2O_2 , two molecules of GSH become oxidized into one molecule of glutathione disulphide (GSSG) (88,89). Since the activity of GPx requires a constant availability of GSH, GSSG will be reduced by the enzyme glutathione reductase. Conceivably, the tissue distribution of glutathione reductase is similar to that of GPx (91). In the reduction of GSSG, NADPH is oxidized and therefore, activity of GPx will lead to energy loss.

1.2.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants can be divided into hydrophilic and hydrophobic antioxidants. Hydrophobic antioxidants include α -tocopherol (vitamin E), carotenoids, and ubiquinol-10 and are mostly present in lipoproteins and membranes. Hydrophilic scavengers include GSH, ascorbate and uric acid. They can predominantly be found in cytosolic, mitochondrial and nuclear aqueous compartments (78). The most important endogenous hydrophilic antioxidants that contribute to the total antioxidant defense are GSH, ascorbic acid and uric acid.

GSH is a versatile antioxidant, present in all tissues in high intracellular concentrations ranging from 1 to 10 mM (92,93). Contradictory, extracellular concentrations are significantly lower, i.e. approximately 1 μM in plasma and 100 μM in epithelial lining fluid (ELF) of the human respiratory tract (94). Synthesis of GSH from glutamic acid, cysteine and glycine occurs in many different cell types and is catalyzed by the enzymes γ -glutamylcysteine synthase and glutathione synthase (95,96). Consequently, GSH is not required in the human diet nor can it significantly be taken up in the gastro-intestinal tract (97). GSH represents at least 90% of total low molecular weight non-protein thiols present in cells (96). Free radical scavenging properties of GSH are due to its thiol moiety and result in the formation of a detoxified reactive species and thiyl radicals ($\text{GS}^\bullet$). The latter can generate GSSG (27). Furthermore, GSH can also exert antioxidant activities by binding free metals through its cysteine thiol group since this will decrease the reaction among

these compounds and oxygen, i.e. the Fenton reaction, that results in the formation of ROS (96,98).

Ascorbate (vitamin C) is an excellent antioxidant, also present in all tissues in high intracellular concentrations (78). Extracellular concentrations are significantly lower, i.e. approximately 60 μM in plasma and 40 μM in ELF of the human respiratory tract (94). In contrast to glutathione and uric acid, ascorbate cannot be synthesized *in situ* in humans. The human inability to make ascorbic acid is due to a lack of L-gulonolactone oxidase, which catalyses the final step in the biosynthetic pathway (99-101). Consequently, ascorbate is required in the human diet. (102-114). During its scavenging activities, ascorbate becomes oxidized into dehydroascorbic acid (DHA) (78,95). The conversion of DHA into ascorbic acid is shown to be rapidly. This regeneration of ascorbate out of DHA can occur by GSH, suggesting that GSH can boost the function of ascorbic acid (105,106).

Uric acid is a metabolic endproduct of purine metabolism in humans, produced by the enzyme xanthine oxidoreductase (100,107,108). Additionally, uric acid levels are also influenced by diet and will for example increase with enhanced intake of meat or alcohol (109,110). Uric acid is present in all extracellular fluid compartments, but displays the highest levels in plasma (approximately 400 μM) and the epithelial lining fluid of the respiratory tract (approximately 200 μM) (94,107). Uric acid is proven to be a selective antioxidant that is especially capable of scavenging $\text{OH}^\bullet$ (111). During these scavenging activities, uric acid becomes oxidized into a urate radical anion that, in the absence of redox-partners such as ascorbate, will subsequently be converted into e.g. allantoin and urea (107).

1.2.3 Antioxidant interplay

In contrast to the anti-oxidative enzymes, non-enzymatic antioxidants act by directly scavenging free radicals. During this process, the antioxidants donate an electron or proton to a radical, thereby forming a relatively stable product out of the scavenged radical. Consequently, the antioxidant itself becomes oxidized during this reaction (112,113). Since only the reduced form of the antioxidant can exert scavenging capacities, the oxidized form of the antioxidant, generated during its protective actions against free radicals, has to be converted back into its reduced status. Moreover, the oxidized form of the antioxidant often comprises a radical that, due to some residual activity of its parent compound, might still cause damage to vital cellular targets (112,113). Therefore, the body contains a distinct network of antioxidants that can chemically reduce each other, thereby diminishing the reactivity of the formed antioxidant radical and regaining the reduced antioxidant for the defense against reactive species. In this way, antioxidants act in synergy to

destroy reactive species (78,114,115). A typical example of this antioxidant interplay is shown in Figure 1.3.

Figure 1.3 Typical example of the antioxidant interplay. LOO•=lipid peroxyl radical; LOOH=lipid hydroperoxide; DHA=dehydroascorbic acid; GSH=reduced glutathione; GSSG=oxidized glutathione.

As depicted in Figure 1.3, the lipid peroxyl radical (LOO•), formed during lipid peroxidation, becomes scavenged preferably by vitamin E. During this reaction, vitamin E becomes oxidized into vitamin E•. Vitamin E• is toxic and as antioxidant useless to the antioxidant defense system. The reaction of vitamin E• with for example GSH results in the reduction of vitamin E• and the concurrently oxidation of GSH into GSSG. In this way, vitamin E is preserved for the defense system at the expense of GSH. Alternatively, ascorbate is also known to regenerate vitamin E out of its radical (116). Recycling GSH or ascorbate for the network may occur by a reaction of their oxidized forms, i.e. GSSG or DHA, with another antioxidant, for example dihydro-lipoate.

This interplay is of special importance since each antioxidant exerts preferentially scavenging activities towards specific free radicals or reactive species at specific compartments. It is known, for example, that LOO• is preferably scavenged by vitamin E while HOCl reacts predominantly with GSH. The interplay between all antioxidants provides an intricate shield that offers optimal defense by the appropriate antioxidant against a particular reactive species at any site throughout the body (78,116).

1.3 Oxidative stress

In normal situations, the endogenous antioxidant network as described in 1.2.3 provides sufficient protection against reactive species such as ROS and RNS (7). However, when an imbalance between the production of and the protection against reactive species occurs in favor of the production, a situation called oxidative stress arises (Figure 1.4).

Figure 1.4 Imbalance between production of reactive oxygen species (ROS) and protection against ROS by antioxidants (AOX) results in the occurrence of oxidative stress.

Oxidative stress may result in increased oxidative damage and can be caused either by an overproduction of free radicals and ROS or by an impairment of the endogenous antioxidant defense system (78,117). Due to the intricate antioxidant network, as described earlier, a deficiency in one compound may also affect the efficacy of others. This could result in a greater loss of protection against reactive species than would be expected based on the deficiency of only one antioxidant.

Oxidative stress is associated with the pathophysiology of various diseases including sarcoidosis (46,118,119), idiopathic pulmonary fibrosis (IPF) (45,120,121) and chronic obstructive pulmonary disease (COPD) (44,122). As could be anticipated, due to the link between ROS and inflammation, in most of the diseases associated with oxidative stress elevated inflammation is also implicated.

1.4 Sarcoidosis

Sarcoidosis is a multi-systemic disorder, meaning that it can involve any organ in the body and that its clinical presentation is highly variable. Nevertheless, in 90% of all sarcoidosis cases the lungs are affected (123-125). It is difficult to give a concise definition of sarcoidosis, due to the fact that its exact cause is still unknown, but generally the disease can be described as a systemic, granulomatous and antigen-driven disorder (125-128).

1.4.1 Epidemiology and Prognosis

Sarcoidosis has been known for more than 100 years; it was first described by the dermatologist Hutchinson (1869) and several years later by two other dermatologists, Besnier and Boeck (1888) (124). The disease occurs worldwide and affects both genders and all ages and races. The incidence of the disease varies among different countries over the world. In Scandinavian

countries the incidence is higher compared to more southern countries (123). In the Netherlands and Germany it is estimated that the incidence is approximately 20-25 per 100.000 inhabitants (123).

The clinical symptoms of sarcoidosis patients include, besides fatigue and sometimes other symptoms related to extra-thoracic manifestation, various respiratory symptoms such as dyspnea, coughing and chest pain. Sarcoidosis is a benign disorder with a beneficial prognosis; a large percentage of individuals affected with the disease does not develop clinical symptoms and up to 30% shows spontaneous remission (123). A chronic course, that can result in a significant impairment of lung function, occurs in 10 to 30% of all sarcoidosis patients, whereas the mortality rate is 1 to 6%. In severe clinical cases, sarcoidosis can shift towards another interstitial lung disease, i.e. IPF, due to increased formation of fibrotic tissue in the lungs (129).

1.4.2 Granuloma formation

Main characterization of sarcoidosis is the formation of granuloma, i.e. any small nodular delimited aggregation of mononuclear inflammatory cells. As schematically depicted in Figure 1.5, granuloma formation in sarcoidosis is hallmarked by a highly polarized T-helper 1 (Th1) immune response to unknown endogenous or exogenous antigens (130). This Th1 immune reaction involves the infiltration of CD4⁺ lymphocytes, following recognition, entrapment and presentation of the unknown exogenous or endogenous antigen by antigen-presenting cells (APC) (129).

The result of the union between CD4⁺ lymphocytes and antigen-presenting cells is a coordinated release of chemokines and cytokines, predominantly interferon- γ (IFN- γ) and IL-2 respectively. These compounds activate macrophages to release more chemokines and cytokines, predominantly IFN- γ and IL-12, IL18 and TNF α . Subsequently, these chemokines and cytokines will recruit more inflammatory cells (lymphocytes, macrophages and fibroblasts), thereby inducing an amplification pathway that mounts a granulomatous response dominated by IFN- γ , IL-2, IL-12, IL-18 and TNF α (126,131-133). Indeed, the chronic overexpression of TNF α and IFN- γ is suggested to induce persistent inflammation and subsequent tissue damage during sarcoidosis (134,135). Furthermore, the individual capability of a patient to release TNF α is suggested to be linked to the progression of the disease, thereby associating this cytokine to the pathogenesis of sarcoidosis (136).

If the antigen(s) are removed or destroyed, immuno-suppressive cytokines such as transforming growth factor β (TGF- β) will down regulate the immune response and subsequently the granulomatous response will subside; however, persistence of the antigenic stimuli results in chronic disease (128,133,137). Moreover, the development and maintenance of

granuloma may be enhanced in sarcoidosis since sarcoid T-lymphocytes exhibit resistance to apoptosis (138). This is possibly due to a changed expression of molecules which modulate cell survival and death (130). In a small number of patients this inflammatory process will be shifted towards the formation of fibrotic tissue by the overexpression of profibrotic cytokines like $\text{TNF}\alpha$ and $\text{TGF-}\beta$ (133).

Figure 1.5 A schematic presentation of granuloma formation in sarcoidosis. Ag=antigen; APC=antigen presenting cell; IFN- γ =interferon-gamma; IL=interleukin; Th1=T-helper 1; TNF α =tumour necrosis factor alpha; TGF- β =transforming growth factor beta (adapted from Moller (139)).

1.4.3 Aetiology

Although it is known that a highly polarized Th1 immune response is involved in the pathogenesis of sarcoidosis, the exact aetiology of the disease remains indefinite. However, there is little doubt that environmental and occupational exposure to all kind of triggers may determine the course of the disease (125). Some of the proposed triggers are viruses, mycobacteria, organic agents, including pine tree pollen and cotton dust and inorganic agents like metals (140) and talc (141). Additionally, genetic factors and oxidative stress are suggested to play an important role in the genesis of sarcoidosis (125,126).

Various genetic analyses of families with several members affected by sarcoidosis have revealed that there might be a genetic susceptibility, but that this heritable risk is complex and polygenic (141). One of the genes of which it is suggested that it comprises a polymorphism that could be involved in the aetiology of sarcoidosis is the TNF α gene (136,142,143). Seitzer *et al.* have shown that the more uncommon TNF α 2 allele, associated with higher levels of TNF α production, displays a higher frequency in patients with Lofgren syndrome, a benign form of acute sarcoidosis (136). Newly developed molecular genotyping techniques are now being applied to explore more genetic aspects of the disease.

A pivotal role for oxidative stress in the aetiology of sarcoidosis has also been proposed (46,144-146). It has been shown that products of lipid peroxidation, i.e. ethane and 8-isoprostane, are elevated in the exhaled breath condensate of sarcoidosis patients (46,147,148). Additionally, oxidized proteins (149,150) are elevated in the bronchoalveolar lavage fluid (BALF) of sarcoidosis patients. Another consequence of the occurrence of oxidative stress is a reduced redox state, as is reported in the erythrocytes of female sarcoidosis patients (118). Furthermore, the transcription factor NF- κ B, of which it is known that it is activated by radical damage, is increased in alveolar macrophages and mononuclear blood cells of active sarcoidosis patients compared to those of healthy controls (119,151). Interestingly, glucocorticoids appeared not to be capable of reducing this increased NF- κ B activation in sarcoidosis (119).

1.4.4 Treatment

Conventional treatment is focused on attenuating granuloma formation with antimalarial drugs, that exert anti-inflammatory activities by inhibiting antigen presentation, or with nonspecific anti-inflammatory agents such as glucocorticosteroids, methotrexate, or azathioprine (128). However, these treatments fail to be completely efficacious (152,153). Recently, anti-TNF- α agents such as infliximab have shown some success in sarcoidosis (128,

133,137,154). The design of future therapies depends, among others, on the improved knowledge of the aetiology as well as of the critical immunological processes operative in different stages of the disease (133).

1.5 Antioxidant therapy

Due to their ability to scavenge free radicals and reactive species, thereby reducing oxidative stress and associated damage, various health claims have been made regarding the use of exogenous, dietary antioxidants (27,155). As a result, numerous studies have been performed to examine the possible beneficial health effects of antioxidant supplementation. However, most of these studies have been conducted with healthy volunteers, i.e. people with a sufficient antioxidant shield and no substantial oxidative stress. Therefore, it is not surprising that the outcome of these studies was often rather disappointing (156-160). Additionally, more beneficial health effects of antioxidant supplementation can be expected in patients suffering from a disease that is actually associated with increased levels of oxidative stress, such as sarcoidosis. In these patients, antioxidant levels are impaired and empowering their antioxidant shield via supplementation could, therefore, result in reduction of the elevated oxidative stress present and thus in improvement of their clinical status.

Moreover, free radicals and ROS are also involved in various other processes such as inflammation (45,55), cell-to-cell communication (161), atherosclerotic plaque formation (162,163), angiogenesis (161,164), impairment of receptor functions (165) and DNA lesions (35,164). Therefore, it can be expected that reducing the reactive species load, by strengthening the antioxidant defence with an exogenous antioxidant, will also mitigate these processes. This might be of especial importance in diseases of which the pathology is linked to various of these other processes as well, including sarcoidosis, atherosclerosis and cancer. In such diseases, tackling only one of the underlying processes will most likely not result in an optimal treatment, as can be exemplified by the fact that treatment of sarcoidosis with only immuno-suppressive agents like glucocorticoids fails to be completely efficacious (119). More effect can be expected from treatment with multi-target compounds, such as antioxidants, that are capable of tackling various processes at the same time. Consequently, the use of exogenous antioxidants to support the treatment of these diseases, or maybe even cure them, has gained a lot of interest recently (115). However, most studies regarding the use of antioxidants in chronic diseases have measured the effect of the supplementation on markers of oxidative stress or of one of the other processes mentioned earlier. Only in a relatively few studies the effects of antioxidant supplementation on the clinical status of patients has been

investigated so far. Table 1.3 highlights a selection of these studies, performed in various chronic diseases associated with elevated oxidative stress.

Table 1.3 A selection of clinical studies investigating the health beneficial effects of antioxidant supplementation in chronic diseases.

Anti-oxidant	Dosing regime	Disease	End point(s)	Antioxidant effect(s)	Ref.
NAC	12 weeks, 3x600 mg/day; combined with low maintenance corticosteroids	Pulmonary fibrosis	Pulmonary function tests	Improvement of pulmonary function tests	120
NAC	24 months, 3x600 mg/day; added to standard therapy	IPF	Lung functions including FEV ₁ and DLCO	Slower deterioration of lung functions	121
lycopene	1 week, 30 mg/day	Exercise-induced asthma	Post-exercise reduction of lung function FEV ₁	Protection against this post-exercise reduction in FEV ₁	167
garlic pearls	2 months, 250 mg/day	Essential hypertension patients	Oxidative stress parameters and BP	Reduction of oxidative stress parameters, reduction of BP	168
NAC	72 mg/kg i.v. as a bolus, later 72 mg/kg over 12 h	Pretreatment of cardiac surgery patients	Neutrophil-mediated inflammation and lung injury	Reduced neutrophil influx and elastase activity, possible protection against lung injury	169
α -lipoic acid	3 days, 600 mg/day	Diabetes	Oxidative stress in plasma and NF- κ B activity	Reduction of both parameters	170
flavonol-rich cocoa	6 weeks, 444 mg/day	Coronary artery diseases	Vascular function parameters	No effect	171
NAC	10 weeks, 600 mg/day	COPD	Lung functions including FEV ₁ and inflammatory parameters including IL-8	No effect on lung functions, reduction of some inflammatory parameters including IL-8	172
polyphenols	5 weeks, 2.66 g/day	COPD	Clinical symptoms and respiratory function tests	No effect	173

NAC=N-acetyl cystein; FEV₁=forced exhaled volume in 1 sec; DLCO=diffusing capacity of the lung for carbon monoxide; BP=blood pressure; NF- κ B=nuclear factor kappa-B; COPD=chronic obstructive pulmonary disease; IL-8=interleukin-8.

As can be seen, in pulmonary fibrosis, supplementation for 12 weeks (600 mg/day, combined with a low maintenance prednisolone concentration) with N-acetyl cystein (NAC), an antioxidant and precursor of GSH, caused an improvement of pulmonary function tests (120). More recently, supplementation for 24 months (600 mg 3 times a day, added to standard therapy with prednisone plus azathioprine) with NAC resulted in a significant slower deterioration of two lung functions in IPF, i.e. the diffusing capacity of the lung for carbon monoxide (DLCO) and the forced expiratory volume in one second (FEV₁) (121). In exercise-induced asthma, supplementation for 1 week (30 mg/day) with the antioxidant lycopene prevented post-exercise impairment of FEV₁ in 55% of all patients (166). In essential hypertension

patients, supplementation for 2 months (250 mg/day) with garlic, a compound containing anti-oxidative capacities, caused a mild blood pressure reduction (167).

In cardiac surgery patients, NAC-pretreatment (72 mg/kg i.v. as a bolus, later 72 mg/kg over 12 h) prevented possible lung injury by reducing the neutrophil influx and elastase activity (168). In diabetes, supplementation for 3 days (600 mg/day) with the antioxidant α -lipoic acid decreased the occurring oxidative stress and NF- κ B activity but, unfortunately, no clinical parameters were evaluated (169).

Contradictory to the above mentioned studies, some clinical studies failed to show beneficial health effects of antioxidant supplementation. In coronary heart diseases, flavonol-rich cocoa for 6 weeks (444 mg/day) did not modify vascular function (170) and in COPD, either NAC supplementation for 10 weeks (600 mg/daily) nor polyphenol supplementation for 5 weeks (2.66 gr/day) had an effect on lung function (171,172).

Important aspects that should be considered in the use of exogenous antioxidants include their scavenging capacities, their possible place in the antioxidant network and their bio-availability. In other words, the effectiveness of antioxidant therapy relies on whether the appropriate scavenger will attain intracellular concentrations that are high enough to fill the existing defect in the endogenous antioxidant network. Moreover, the possible toxicity of antioxidants, when administered in high levels that exceed their daily intake by far, have to be considered too, as can be derived from the toxic effects of high doses of antioxidant β -carotene found in two clinical trials with heavy smokers and asbestos workers (173-175). Clearly, further research is needed to optimize the use of specific antioxidants in diseases associated with oxidative stress

1.6 The flavonoid quercetin

1.6.1 *Origin, structure and intake*

Flavonoids are a class of naturally occurring polyphenolic compounds, ubiquitously present in photosynthesising cells (176,177). Over 5000 different naturally occurring flavonoids have already been identified and the list is still growing (178,179). Flavonoids are present in fruits, vegetables, nuts and plant-derived beverages such as tea and wine (180,181). Additionally, many antioxidant supplements as well as herb-containing medicaments contain high doses of flavonoids.

Most flavonoids share a common three-ring structure, depicted in Figure 1.6, of which ring A and B are aromatic and ring C is heterocyclic. The variation in the heterocyclic C ring forms the basis of the division of the

flavonoids in various subclasses, i.e. the flavones, isoflavones, flavonols, flavanols, flavanones, anthocyanidins and chalcones (182).

Figure 1.6 Structures of the major classes of flavonoids.

The total amount of flavonoids consumed in the Netherlands is estimated at several hundreds of mg per day (183). The Dutch intake of flavones and flavonols is determined as 23-24 mg per day and quercetin, the main flavonol present in our diet, represents 70% of this intake (183,184). The molecular structure of quercetin is given in Figure 1.7. Quercetin, commonly found in apples, onions and green tea (183), occurs mainly as glycoside, i.e. a sugar group such as glucose, galactose, rhamnose, rutinose or xylose is bound to one of the hydroxyl groups of the flavonol (179,185).

Figure 1.7 Molecular structure of quercetin.

1.6.2 Absorption, metabolism and bio-availability

Because of the hydrophilic character of its glycosides, only quercetin without a sugar group, i.e. the aglycon, was initially suggested to be taken up in the gastro-intestinal tract by passive diffusion (181,186). However, a study with human ileostomy volunteers showed not only that quercetin glycosides can indeed be absorbed in the small intestine, but also that this absorption surpasses that of the aglycon by far, i.e. 52% of the glycosides was absorbed versus 24% of the aglycon (187). Other studies have confirmed that the absorption of quercetin is considerably enhanced by its conjugation with a sugar group (188,189). A possible explanation for this increased absorption, resulting in a higher plasma peak concentration and an increased bio-availability, is the facilitation of glycoside absorption by either deglycosylation (190,191) or carrier-mediated transport (192,193).

Deglycosylation of quercetin glycosides involves β -glucosidases that are capable of liberating the aglycon for passive diffusion (190,191). Especially the intestinal lactase phlorizin hydrolase (LPH) displays a specific activity towards flavonoid glycosides (194) and its role in quercetin glycoside absorption has been confirmed in an *in situ* rat small intestine perfusion model (195). Carrier mediated transport of quercetin glycosides may involve the sodium-dependent glucose transporter-1 (196,197), but a role for other transporter proteins such as the multidrug resistance protein 2 (198) has also been suggested. After their facilitated uptake by means of carrier mediated transport, quercetin glycosides often become hydrolysed by intracellular β -glucosidases (191).

After absorption, quercetin becomes metabolised in various organs including the small intestine, colon, liver and kidney (199). Metabolites formed in the small intestine and liver are mainly the result of phase II metabolism by biotransformation enzymes and therefore include the methylated, sulphated and glucuronidated forms (200,201). Moreover, bacterial ring fission of the aglycon occurs in both the small intestine and colon, resulting in the breakdown of the backbone structure of quercetin and the subsequent formation of smaller phenolics (199,202).

Normally, human quercetin plasma concentrations are in the low nanomolar range, but upon quercetin supplementation they may increase to the high nanomolar or low micromolar range (203,204). Recently, a study regarding the tissue distribution in rats and pigs has shown that, upon quercetin supplementation, the highest accumulation of the flavonoid and its metabolites are found in (rat) lungs and (pig) liver and kidney. Unfortunately, pig lungs were not analysed (205). It has been shown that the half lives of the

quercetin metabolites are rather high, i.e. 11 to 28 hours. This indicates that, upon repeated quercetin supplementation, they could attain a considerable plasma level (188,206).

1.6.3 Beneficial effects

Epidemiological studies

Various epidemiological studies, using data on fruit and vegetable consumption to calculate the mean flavonoid intake, have been performed to provide support for the alleged beneficial health effects of a high intake of flavonoids such as quercetin. A protective effect of flavonole and flavone intake has been found regarding (i) the risk on fatal or non-fatal coronary artery diseases (183,207,208), (ii) the risk on lung cancer (209,210), (iii) the incidence of asthma (210) and (iv) the impairment of pulmonary functions in COPD (211). However, not all epidemiological studies regarding the association between flavonoid intake and the risk on various diseases, including a variety of cancers, were able to confirm this protective effect. Moreover, the alpha-tocopherol-beta-carotene (ATBC)-prevention study showed a borderline positive association between the flavonoid intake and a reduced risk for colorectal cancer (209). A possible explanation for this discrepancy could be due to insufficient correction for differences in smoking and dietary behavior. Smoking is associated with unhealthy behavior, including a higher intake of energy, alcohol and fat and a lower intake of fruits and vegetables (212,213). Additionally, a high consumption of fruits and vegetables, i.e. important sources of flavonoids, is associated with a more healthy behavior (214,215). Therefore, if not corrected for properly, such differences could result in confounders giving false associations.

Overall, epidemiological studies performed so far support a beneficial role for flavonoids in the lung, but they do not provide conclusive evidence for beneficial health effects of a high flavonoid or quercetin intake in diseases concerning other organs, including various forms of cancer. Consequently, more accurate epidemiological research is necessary to elucidate the overall beneficial health effects of flavonoids.

In vitro and in vivo studies

Quercetin has been shown to be an excellent *in vitro* antioxidant. Within the flavonoid family, quercetin is the most potent scavenger of ROS, including $O_2^{\bullet-}$ (216,217), and RNS like $NO^{\bullet}$ (218,219) and $ONOO^{\bullet-}$ (220,221). These anti-oxidative capacities of quercetin are attributed to the presence of two antioxidant pharmacophores within the molecule that have the optimal configuration for free radical scavenging, i.e. the catechol group in the B ring and the OH group at position 3 of the AC ring (222). Moreover, quercetin is

suggested to substantially empower the endogenous antioxidant shield due to its contribution to the total plasma antioxidant capacity which is 6.24 times higher than the reference anti-oxidant trolox, whereas for example the contribution of both vitamin C and uric acid virtually equals that of trolox (223).

Until now, very few studies have been performed to examine the *in vivo* anti-oxidative effects of quercetin. *In vivo* quercetin supplementation for 28 days (1 gram a day) in healthy volunteers resulted in a significantly increased plasma quercetin concentration, but did not show any beneficial health effects regarding risk factors for coronary diseases (203). This lack of effect might be explained by the fact that this study was performed in healthy volunteers who display only relatively low levels of oxidative stress and are, therefore, not in need of extra anti-oxidative defense. Another quercetin supplementation in healthy volunteers (11 mg/day for 28 days via a quercetin-rich fruit juice) also increased the plasma quercetin concentration as well as the total plasma antioxidant capacity (224). However, no *in vivo* oxidative stress markers or other parameters to measure *in vivo* health effects were included in this study. Interestingly, the fact that this supplementation did cause a 41% reduction of *ex vivo*-induced oxidative damage ($P < 0.07$) might contribute to the rationale for supplementing antioxidants only in situations of elevated oxidative stress.

In vitro, it has been shown that quercetin also possesses anti-inflammatory (225,226), immuno-suppressive (227,228), anti-fibrotic (229), anti-coagulative (230), anti-bacterial (216), anti-atherogenic (231,232), anti-hypertensive (231,233) and anti-proliferative properties (228,234,235). Furthermore, quercetin is reported to directly modulate the gene expression of enzymes involved in biotransformation (236-239) and to inhibit cell proliferation by interacting with estrogen binding sites (240,241). Altogether, these *in vitro* studies indicate that quercetin may exert health beneficial capacities via various damage modulating effects. However, most of these studies have been performed with immortalized or cultured cell lines only and are thus not easy to extrapolate to the *in vivo* human situation. Therefore, whether *in vivo* quercetin supplementation has beneficial health effects remains to be confirmed.

1.6.4 Toxic effects

It is well documented that during its anti-oxidative activities, quercetin becomes oxidized into various oxidation products (Figure 1.8). The two electron oxidation of quercetin yields the oxidation product quercetin-quinone, denoted as QQ, that has four tautomeric forms, i.e. an *ortho*-quinone and three quinonemethides (Figure 1.8) (242-244).

Figure 1.8 Oxidation of quercetin, followed by the possible reaction of its major oxidation product QQ with glutathione (GSH).

It has been well described that oxidation products like semiquinone radicals and quinones display various toxic effects due to their ability of arylating protein thiols (245-248).

Indeed, QQ is very reactive towards thiols and can instantaneously form an adduct with GSH, the most abundant endogenous thiols (243,249). This adduct is called GSQ. When no GSH is present, QQ can react with other thiols, e.g. protein thiols. This reaction may lead to toxic effects such as increased membrane permeability (250) or, as previously shown for other quinones (244,245), altered functioning of enzymes that contain a critical SH-group. Such QQ-induced toxicity has been shown in various *in vitro* studies but the *in vivo* formation, and possible toxicity, of QQ has not been demonstrated yet.

Quercetin has also been reported to display genotoxic effects *in vitro*. Interestingly, these mutagenic effects of quercetin are predominantly shown in bacteria and are suggested to require quinone formation as mediators as well (251-254). In mammalian cells and experimental animals, conflicting data regarding the capacity of the flavonoid to induce DNA lesions and mutations are reported.

On the one hand, induction of chromosomal aberrations and single strand breaks combined with point mutations are shown in respectively hamster ovary (252) and mouse lymphoma cells (255). On the other hand, quercetin supplementation in either mice (256) or rats with aorta restriction (257) could protect against benzo[a]pyrene-induced DNA damage and was suggested, respectively, to protect against the development of lung cancer (256) and to attenuate cardiac hypertrophy (257). Clearly, more research is required to explain the discrepancy observed between the *in vitro* and *in vivo* quercetin genotoxicity studies in order to further elucidate the possible genotoxic effects of the flavonoid for man.

1.7 Aim and outline of the thesis

This thesis focuses on the possible health-beneficial effects of the flavonoid quercetin. Especially the relation between the *in vitro* and *in vivo* behavior of quercetin is explored. Furthermore, the possible use of this flavonoid in sarcoidosis, an inflammatory disease, is examined.

Firstly, the behavior of quercetin during oxidative stress was evaluated *in vitro*. **Chapter 2** describes the protection against lipid peroxidation offered by catechol-containing antioxidants such as quercetin. The effects of its oxidation products, formed during this protection, are discussed. In **chapter 3** the reaction of the main oxidation product of quercetin, i.e. QQ, with thiols is further examined. Especially the reactions of QQ with GSH or with vitamin C are evaluated and compared. To investigate the reaction of QQ with DT-diaphorase (NQO-1), the experiments described in **chapter 4** are performed. The possible role of this enzyme in the protection against QQ-induced toxicity is examined. Since the QQ-toxicity appears to be focused on its reaction with thiols, **chapter 5** focuses on the fate of the adducts formed between QQ and various thiols, including GSH, the most abundant endogenous thiol. The *in vitro* work was concluded by investigating the protective effect of quercetin in relation to the potential toxic effects of its oxidation products in a more integrated cell system, using rat lung epithelial cells (RLEs) as described in **chapter 6**.

Secondly, the *in vitro* and *ex vivo* anti-inflammatory as well as the *in vivo* anti-oxidative effects of quercetin have been examined. **Chapter 7** presents the effects of a four-weeks comprising quercetin intervention on the antioxidant status as well as on the basal and *ex vivo*-induced inflammation in healthy volunteers. The potential toxicity of QQ is also discussed. The *ex vivo* anti-inflammatory effect of quercetin has further been examined in both sarcoidosis patients and healthy controls in **chapter 8**. Moreover, the antioxidant status of both the patients and their matched controls has been quantified.

Thirdly, the *in vivo* anti-oxidative and anti-inflammatory effects of quercetin supplementation in sarcoidosis patients are reported in **chapter 9**. Implications for the use of antioxidant supplementation, with for example quercetin, in the treatment of this disease are given.

Finally, the results and impacts of our findings are summarized in **chapter 10**. Implications for further research are also given.

References

1. Magder S. Reactive oxygen species: toxic molecules or spark of life? *Crit Care* 2006;10:208.
2. Kohen R and Nyska A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol* 2002;30:620-650.
3. Galli F, Piroddi M, Annetti C, Aisa C, Floridi E and Floridi A. Oxidative stress and reactive oxygen species. *Contrib Nephrol* 2005;149:240-260.
4. Halliwell B. Antioxidants in human health and disease. *Annu Rev Nutr* 1996;16:33-50.
5. Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet* 1994;344:721-724.
6. Sies H. Oxidative stress: oxidants and antioxidants. *Exp Physiol* 1997;82:291-295.
7. Bast A, Haenen GR and Doelman CJ. Oxidants and antioxidants: state of the art. *Am J Med* 1991;91:2S-13S.
8. Baldrige CW and Gerard RW. The extra respiration of phagocytosis. *Am J Physiol* 1933;103:235.
9. Bast A. Is formation of reactive oxygen by cytochrome P450 perilous and predictable? *Trends Pharmacol Sci* 1986;266-270.
10. Moncada S, Palmer RM and Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991;43:109-142.
11. Moncada S, Palmer RM and Higgs EA. Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochem Pharmacol* 1989;38:1709-1715.
12. Miki N, Kawabe Y and Kuriyama K. Activation of cerebral guanylate cyclase by nitric oxide. *Biochem Biophys Res Commun* 1977;75:851-856.
13. Arnold WP, Mittal CK, Katsuki S and Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. *Proc Natl Acad Sci U S A* 1977;74:3203-3207.
14. Gryglewski RJ, Palmer RM and Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* 1986;320:454-456.
15. Gillette JR, Brodie BB and LaDu BN. Oxidation of drugs by liver microsomes: role of reduced triphosphopyridine nucleotide (TPNH) and oxygen. *J Pharmacol Exp Therap* 1957;119:532-540.
16. Babior BM. The respiratory burst of phagocytes. *J Clin Invest* 1984;73:599-601.
17. Babior BM. Phagocytes and oxidative stress. *Am J Med* 2000;109:33-44.
18. Stuehr DJ, Kwon NS and Nathan CF. FAD and GSH participate in macrophage synthesis of nitric oxide. *Biochem Biophys Res Commun* 1990;168:558-565.
19. Marletta MA. Nitric oxide: biosynthesis and biological significance. *Trends Biochem Sci* 1989;14:488-492.
20. Weinberg JB, Misukonis MA, Shami PJ, Mason SN, Sauls DL, Dittman WA, Wood ER, Smith GK, McDonald B, Bachus KE et al. Human mononuclear phagocyte inducible nitric oxide synthase (iNOS): analysis of iNOS mRNA, iNOS protein, biopterin, and nitric oxide production by blood monocytes and peritoneal macrophages. *Blood* 1995;86:1184-1189.
21. Hevel JM, White KA and Marletta MA. Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J Biol Chem* 1991;266:22789-22791.
22. Huie RE and Padmaja S. The reaction of NO with superoxide. *Free Radic Res Commun* 1993;18:195-199.
23. Hampton MB, Kettle AJ and Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* 1998;92:3007-3017.
24. Klebanoff SJ. Myeloperoxidase: friend and foe. *J Leukoc Biol* 2005;77:598-625.
25. McCord JM and Fridovich I. Superoxide dismutase: the first twenty years (1968-1988). *Free Radic Biol Med* 1988;5:363-369.
26. Fridovich I. The trail to superoxide dismutase. *Protein Sci* 1998;7:2688-2690.

27. Diplock AT, Charleux JL, Crozier-Willi G, Kok FJ, Rice-Evans C, Roberfroid M, Stahl W and Vina-Ribes J. Functional food science and defence against reactive oxidative species. *Br J Nutr* 1998;80 Suppl 1:S77-112.
28. Halliwell B and Chirico S. Lipid peroxidation: its mechanism, measurement and significance. *Am J Clin Nutr* 1993;7155-7255.
29. Gutteridge JM. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin Chem* 1995;41:1819-1828.
30. Spiteller G. Lipid peroxidation in aging and age-dependent diseases. *Exp Gerontol* 2001;36:1425-1457.
31. Guillen-Sans R and Guzman-Chozas M. The thiobarbituric acid (TBA) reaction in foods: a review. *Crit Rev Food Sci Nutr* 1998;38:315-330.
32. Griffiths HR, Moller L, Bartosz G, Bast A, Bertoni-Freddari C, Collins A, Cooke M, Coolen S, Haenen GR, Hoberg AM, Loft S, Lunec J, Olinski R, Parry J, Pompella A, Poulsen H, Verhagen H and Astley SB. Biomarkers. *Mol Aspects Med* 2002;23:101-208.
33. Spencer JP, Whiteman M, Jenner A and Halliwell B. Nitrite-induced deamination and hypochlorite-induced oxidation of DNA in intact human respiratory tract epithelial cells. *Free Radic Biol Med* 2000;28:1039-1050.
34. Knaapen AM, Seiler F, Schilderman PA, Nehls P, Bruch J, Schins RP and Borm PJ. Neutrophils cause oxidative DNA damage in alveolar epithelial cells. *Free Radic Biol Med* 1999;27:234-240.
35. Waris G and Ahsan H. Reactive Oxygen Species: Role in the development of cancer and various chronic conditions. *J Carcinog* 2006;5:14.
36. Haenen GR, Tai Tin Tsoi JN, Vermeulen NP, Timmerman H and Bast A. 4-Hydroxy-2,3-trans-nonenal stimulates microsomal lipid peroxidation by reducing the glutathione-dependent protection. *Arch Biochem Biophys* 1987;259:449-456.
37. Sastre J, Pallardo FV and Vina J. Mitochondrial oxidative stress plays a key role in aging and apoptosis. *IUBMB Life* 2000;49:427-435.
38. Yorimitsu M, Muranaka S, Sato EF, Fujita H, Abe K, Yasuda T, Inoue M and Utsumi K. Role of alpha-tocopherol in the regulation of mitochondrial permeability transition. *Physiol Chem Phys Med NMR* 2004;36:95-107.
39. Kim R, Emi M, Tanabe K, Murakami S, Uchida Y and Arihiro K. Regulation and interplay of apoptotic and non-apoptotic cell death. *J Pathol* 2006;208:319-326.
40. Shen HM and Liu ZG. JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species. *Free Radic Biol Med* 2006;40:928-939.
41. Stangel M, Zettl UK, Mix E, Zielasek J, Toyka KV, Hartung HP and Gold R. H₂O₂ and nitric oxide-mediated oxidative stress induce apoptosis in rat skeletal muscle myoblasts. *J Neuropathol Exp Neurol* 1996;55:36-43.
42. Mehta JL, Rasouli N, Sinha AK and Molavi B. Oxidative stress in diabetes: a mechanistic overview of its effects on atherogenesis and myocardial dysfunction. *Int J Biochem Cell Biol* 2006;38:794-803.
43. Valko M, Rhodes CJ, Moncol J, Izakovic M and Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* 2006;160:1-40.
44. Boots AW, Haenen GR and Bast A. Oxidant metabolism in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 2003;46:14s-27s.
45. MacNee W. Oxidative stress and lung inflammation in airway diseases. *Eur J Pharmacol* 2001;429:195-207.
46. Kanoh S, Kobayashi H and Motoyoshi K. Exhaled ethane: an in vivo biomarker of lipid peroxidation in interstitial lung diseases. *Chest* 2005;128:2387-2392.
47. Rahman I, Skwarska E, Henry M, Davis M, O'Connor CM, FitzGerald MX, Greening A and MacNee W. Systemic and pulmonary oxidative stress in idiopathic pulmonary fibrosis. *Free Radic Biol Med* 1999;27:60-68.
48. Parkin J, Cohen B. An overview of the immune system. *Lancet* 2001;357:1777-1789.
49. Abbas AK, Lichtman AH. Basic Immunology: functions and disorders of the immune system. Philadelphia 2004; W.B. Saunders Company.

50. Liang Y, Zhou Y, Shen P. NF-kappaB and its regulation on the immune system. *Cell Mol Immunol* 2004;1:343-350
51. Pasare C, Medzhitov R. Toll-like receptors: linking innate and adaptive immunity. *Microbes and Infection* 2004;6:1382-1387
52. Elenkov IJ, Iezoni DG, Daly A, Harris AG, Chrousos GP. Cytokine dysregulation, inflammation and well-being. *Neuroimmunomodulation* 2005;12:255-269
53. Tergaonkar V. NFkappaB pathway: a good signaling paradigm and therapeutic target. *Int J Biochem Cell Biol* 2006;38:1647-1653
54. Lucas PC, McAllister-Lucas LM, Nunez G. NF-kappaB signaling in lymphocytes: a new cast of characters. *J Cell Sci* 2004;117:31-39
55. Rahman I. Oxidative stress, transcription factors and chromatin remodelling in lung inflammation. *Biochem Pharmacol* 2002;64:935-942.
56. Schreck R, Rieber P and Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *Embo J* 1991;10:2247-2258.
57. Rahman I, Gilmour PS, Jimenez LA and MacNee W. Oxidative stress and TNF-alpha induce histone acetylation and NF-kappaB/AP-1 activation in alveolar epithelial cells: potential mechanism in gene transcription in lung inflammation. *Mol Cell Biochem* 2002;234-235: 239-248.
58. Antonicelli F, Parmentier M, Hirani N, Drost E, Rahman I, Donaldson K and MacNee W. LPS stimulation of IL-8 release is inhibited by thiol antioxidant at the transcriptional level in THP-1 macrophage cells. *Am J Respir Crit Care Med* 2000;161:A738.
59. Chandel NS, Trzyna WC, McClintock DS and Schumacker PT. Role of oxidants in NF-kappa B activation and TNF-alpha gene transcription induced by hypoxia and endotoxin. *J Immunol* 2000;165:1013-1021.
60. Droge W, Schulze-Osthoff K, Mihm S, Galter D, Schenk H, Eck HP, Roth S and Gmunder H. Functions of glutathione and glutathione disulfide in immunology and immunopathology. *Faseb J* 1994;8:1131-1138.
61. Schoonbroodt S and Piette J. Oxidative stress interference with the nuclear factor-kappa B activation pathways. *Biochem Pharmacol* 2000;60:1075-1083.
62. Korn SH, Wouters EF, Vos N and Janssen-Heininger YM. Cytokine-induced activation of nuclear factor-kappa B is inhibited by hydrogen peroxide through oxidative inactivation of IkappaB kinase. *J Biol Chem* 2001;276:35693-35700.
63. Lahdenpohja N, Savinainen K and Hurme M. Pre-exposure to oxidative stress decreases the nuclear factor-kappa B-dependent transcription in T lymphocytes. *J Immunol* 1998;160:1354-1358.
64. Flescher E, Tripoli H, Salnikow K and Burns FJ. Oxidative stress suppresses transcription factor activities in stimulated lymphocytes. *Clin Exp Immunol* 1998;112:242-247.
65. Moodie FM, Marwick JA, Anderson CS, Szulakowski P, Biswas SK, Bauter MR, Kilty I and Rahman I. Oxidative stress and cigarette smoke alter chromatin remodeling but differentially regulate NF-kappaB activation and proinflammatory cytokine release in alveolar epithelial cells. *Faseb J* 2004;18:1897-1899.
66. Halliwell B, Gutteridge J. Free radicals in biology and medicine. Oxford 1999; Oxford University Press.
67. Bowie A and O'Neill LA. Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol* 2000;59:13-23.
68. Gloire G, Legrand-Poels S and Piette J. NF-kappaB activation by reactive oxygen species: Fifteen years later. *Biochem Pharmacol* 2006;in press.
69. Kaul N and Forman HJ. Activation of NF kappa B by the respiratory burst of macrophages. *Free Radic Biol Med* 1996;21:401-405.
70. Flohe L, Brigelius-Flohe R, Saliou C, Traber MG and Packer L. Redox regulation of NF-kappa B activation. *Free Radic Biol Med* 1997;22:1115-1126.
71. Ginn-Pease ME and Whisler RL. Redox signals and NF-kappaB activation in T cells. *Free Radic Biol Med* 1998;25:346-361.

72. Kitagawa S, Ohsaka A, Yuo A, Takaku F and Saito M. The respiratory burst of granulocytes: modulation by inflammatory mediators and its mechanism. *Tokai J Exp Clin Med* 1988;13:299-305.
73. Ferrante A. Activation of neutrophils by interleukins-1 and -2 and tumour necrosis factors. *Immunol Ser* 1992;57:417-436.
74. Baggiolini M and Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett* 1992;307:97-101.
75. Sies H. Strategies of antioxidant defense. *Eur J Biochem* 1993;215:213-219.
76. Halliwell B. Antioxidant characterization. Methodology and mechanism. *Biochem Pharmacol* 1995;49:1341-1348.
77. McCord JM and Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* 1969;244:6049-6055.
78. Chaudiere J and Ferrari-Iliou R. Intracellular antioxidants: from chemical to biochemical mechanisms. *Food Chem Toxicol* 1999;37:949-962.
79. McCord JM. Iron- and manganese-containing superoxide dismutases: structure, distribution, and evolutionary relationships. *Adv Exp Med Biol* 1976;74:540-550.
80. Patel RN, Kumar S and Pandeya KB. ESR, visible and SOD studies of imidazolate bridged Cu(2)(II,II), Cu(II)Zn(II) and Cu(II)Ni(II) complexes with pentamethyldiethylenetriamine as capping ligand: a plausible model for superoxide dismutase. *J Inorg Biochem* 2002;89:61-68.
81. Tyler DD. Polarographic assay and intracellular distribution of superoxide dismutase in rat liver. *Biochem J* 1975;147:493-504.
82. Karlsson K, Sandstrom J, Edlund A, Edlund T and Marklund SL. Pharmacokinetics of extracellular-superoxide dismutase in the vascular system. *Free Radic Biol Med* 1993;14:185-190.
83. Steinman HM. Superoxide dismutases: protein chemistry and structure-function relationships. Oberley LW. Superoxide Dismutase, Boca Ration: CRC Press, 1982. 11-68.
84. Zamocky M and Koller F. Understanding the structure and function of catalases: clues from molecular evolution and in vitro mutagenesis. *Prog Biophys Mol Biol* 1999;72:19-66.
85. Nohl H and Jordan W. The metabolic fate of mitochondrial hydrogen peroxide. *Eur J Biochem* 1980;111:203-210.
86. Phung CD, Ezieme JA and Turrens JF. Hydrogen peroxide metabolism in skeletal muscle mitochondria. *Arch Biochem Biophys* 1994;315:479-482.
87. Ursini F, Maiorino M, Brigelius-Flohe R, Aumann KD, Roveri A, Schomburg D and Flohe L. Diversity of glutathione peroxidases. *Methods Enzymol* 1995;252:38-53.
88. Takahashi K and Cohen HJ. Selenium-dependent glutathione peroxidase protein and activity: immunological investigations on cellular and plasma enzymes. *Blood* 1986;68:640-645.
89. Flohe L. Glutathione peroxidase: fact and fiction. *Ciba Found Symp* 1978;95-122.
90. Yamamoto Y and Takahashi K. Glutathione peroxidase isolated from plasma reduces phospholipid hydroperoxides. *Arch Biochem Biophys* 1993;305:541-545.
91. Holben DH and Smith AM. The diverse role of selenium within selenoproteins: a review. *J Am Diet Assoc* 1999;99:836-843.
92. Griffith OW and Meister A. Glutathione: interorgan translocation, turnover, and metabolism. *Proc Natl Acad Sci U S A* 1979;76:5606-5610.
93. Meister A, Griffith OW, Novogrodsky A and Tate SS. New aspects of glutathione metabolism and translocation in mammals. *Ciba Found Symp* 1979;135-161.
94. Van der Vliet A, O'Neill CA, Cross CE, Kooststra JM, Volz WG, Halliwell B and Louie S. Determination of low-molecular-mass antioxidant concentrations in human respiratory tract lining fluids. *Am J Physiol* 1999;276:L289-296.
95. Meister A. On the antioxidant effects of ascorbic acid and glutathione. *Biochem Pharmacol* 1992;44:1905-1915.
96. Van Haaften RI, Haenen GR, Evelo CT and Bast A. Effect of vitamin E on glutathione-dependent enzymes. *Drug Metab Rev* 2003;35:215-253.
97. Meister A. Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy. *Pharmacol Ther* 1991;51:155-194.

98. Filomeni G, Rotilio G and Ciriolo MR. Cell signalling and the glutathione redox system. *Biochem Pharmacol* 2002;64:1057-1064.
99. Chatterjee IB. Evolution and the biosynthesis of ascorbic acid. *Science* 1973;182:1271-1272.
100. Benzie IF. Evolution of antioxidant defence mechanisms. *Eur J Nutr* 2000;39:53-61.
101. Pauling L. Evolution and the need for ascorbic acid. *Proc Natl Acad Sci U S A* 1970;67:1643-1648.
102. Englund S and Seifter S. The biochemical functions of ascorbic acid. *Annu Rev Nutr* 1986;6:365-406.
103. Chatterjee IB, Majumder AK, Nandi BK and Subramanian N. Synthesis and some major functions of vitamin C in animals. *Ann N Y Acad Sci* 1975;258:24-47.
104. Levine M. New concepts in the biology and biochemistry of ascorbic acid. *N Engl J Med* 1986;314:892-902.
105. May JM, Qu ZC and Cobb CE. Human erythrocyte recycling of ascorbic acid: relative contributions from the ascorbate free radical and dehydroascorbic acid. *J Biol Chem* 2004;279:14975-14982.
106. Winkler BS. Unequivocal evidence in support of the nonenzymatic redox coupling between glutathione/glutathione disulfide and ascorbic acid/dehydroascorbic acid. *Biochim Biophys Acta* 1992;1117:287-290.
107. Becker BF. Towards the physiological function of uric acid. *Free Radic Biol Med* 1993;14:615-631.
108. Glantzounis GK, Tsimoyiannis EC, Kappas AM and Galaris DA. Uric acid and oxidative stress. *Curr Pharm Des* 2005;11:4145-4151.
109. Ames BN, Cathcart R, Schwiers E and Hochstein P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proc Natl Acad Sci U S A* 1981;78:6858-6862.
110. Cutler RG. Antioxidants and aging. *Am J Clin Nutr* 1991;53:373S-379S.
111. Kaur H and Halliwell B. Action of biologically-relevant oxidizing species upon uric acid. Identification of uric acid oxidation products. *Chem Biol Interact* 1990;73:235-247.
112. Bast A and Haenen GR. The toxicity of antioxidants and their metabolites. *Environmental Toxicology and Pharmacology* 2002;11:251-258.
113. Haenen GR and Bast A. The use of vitamin supplements in self-medication. *Therapie* 2002;57:119-122.
114. Blomhoff R. Dietary antioxidants and cardiovascular disease. *Curr Opin Lipidol* 2005;16:47-54.
115. Vertuani S, Angusti A and Manfredini S. The antioxidants and pro-antioxidants network: an overview. *Curr Pharm Des* 2004;10:1677-1694.
116. Machlin LJ and Bendich A. Free radical tissue damage: protective role of antioxidant nutrients. *Faseb J* 1987;1:441-445.
117. Halliwell B. The role of oxygen radicals in human disease, with particular reference to the vascular system. *Haemostasis* 1993;23 Suppl 1:118-126.
118. Rothkrantz-Kos S, Drent M, Vuil H, De Boer M, Bast A, Wouters EF, Roos D and van Diejen-Visser MP. Decreased redox state in red blood cells from patients with sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2002;19:114-120.
119. Drent M, van den Berg R, Haenen GR, van den Berg H, Wouters EF and Bast A. NF-kappaB activation in sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2001;18:50-56.
120. Behr J, Maier K, Degenkolb B, Krombach F and Vogelmeier C. Antioxidative and clinical effects of high-dose N-acetylcysteine in fibrosing alveolitis. Adjunctive therapy to maintenance immunosuppression. *Am J Respir Crit Care Med* 1997;156:1897-1901.
121. Demedts M, Behr J, Buhl R, Costabel U, Dekhuijzen R, Jansen HM, MacNee W, Thomeer M, Wallaert B, Laurent F, Nicholson AG, Verbeken EK, Verschakelen J, Flower CD, Capron F, Petruzzelli S, De Vuyst P, van den Bosch JM, Rodriguez-Becerra E, Corvasce G, Lankhorst I, Sardina M and Montanari M. High-dose acetylcysteine in idiopathic pulmonary fibrosis. *N Engl J Med* 2005;353:2229-2242.
122. MacNee W. Pulmonary and systemic oxidant/antioxidant imbalance in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005;2:50-60.

123. Thomeer M, Demedts M., Wuyts W. Epidemiology of sarcoidosis. *Eur Respir Mon* 2005;32: 1-10.
124. Sharma OP. Definition and history of sarcoidosis. *Eur Respir Mon* 2005;1-13.
125. Hunninghake GW, Costabel U, Ando M, Baughman R, Cordier JF, du Bois R, Eklund A, Kitaichi M, Lynch J, Rizzato G, Rose C, Selroos O, Semenzato G and Sharma OP. ATS/ERS/WASOG statement on sarcoidosis. American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders. *Sarcoidosis Vasc Diffuse Lung Dis* 1999;16:149-173.
126. Newman LS, Rose CS and Maier LA. Sarcoidosis. *N Engl J Med* 1997;336:1224-1234.
127. Sharma OP. Sarcoidosis: a worldwide phenomenon. *Sarcoidosis* 1984;1:11-15.
128. Baughman RP, Lower EE and du Bois RM. Sarcoidosis. *Lancet* 2003;361:1111-1118.
129. Semenzato G, Bortoli M, Brunetta E and Agostini C. Immunology and pathophysiology. *Eur Respir Mon* 2005;10:49-63.
130. Agostini C, Meneghin A and Semenzato G. T-lymphocytes and cytokines in sarcoidosis. *Curr Opin Pulm Med* 2002;8:435-440.
131. James DG and Williams WJ. Immunology of sarcoidosis. *Am J Med* 1982;72:5-8.
132. Baughman RP and Iannuzzi M. Tumour necrosis factor in sarcoidosis and its potential for targeted therapy. *BioDrugs* 2003;17:425-431.
133. Moller DR. Treatment of sarcoidosis -- from a basic science point of view. *J Intern Med* 2003;253:31-40.
134. Baughman RP, Strohofer SA, Buchsbaum J and Lower EE. Release of tumor necrosis factor by alveolar macrophages of patients with sarcoidosis. *J Lab Clin Med* 1990;115:36-42.
135. Agostini C, Zambello R, Sancetta R, Cerutti A, Milani A, Tassinari C, Facco M, Cipriani A, Trentin L and Semenzato G. Expression of tumor necrosis factor-receptor superfamily members by lung T lymphocytes in interstitial lung disease. *Am J Respir Crit Care Med* 1996;153:1359-1367.
136. Seitzer U, Swider C, Stuber F, Suchnicki K, Lange A, Richter E, Zabel P, Muller Quernheim J, Flad HD and Gerdes J. Tumour necrosis factor alpha promoter gene polymorphism in sarcoidosis. *Cytokine* 1997;9:787-790.
137. Baughman RP, Judson MA, Teirstein AS, Moller DR and Lower EE. Thalidomide for chronic sarcoidosis. *Chest* 2002;122:227-232.
138. Stridh H, Planck A, Gigliotti D, Eklund A and Grunewald J. Apoptosis resistant bronchoalveolar lavage (BAL) fluid lymphocytes in sarcoidosis. *Thorax* 2002;57:897-901.
139. Moller DR. Cells and cytokines involved in the pathogenesis of sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 1999;16:24-31.
140. Drent M, Bomans PHH, van Suylen RJ, Lamers RJS, Bast A and Wouters EFM. Association of man-made mineral fibre exposure and sarcoidlike granulomas. *Respir Med* 2000;94:815-820.
141. Newman LS. Aetiologies of sarcoidosis. *Eur Respir Mon* 2005;10:23-48.
142. Takashige N, Naruse TK, Matsumori A, Hara M, Nagai S, Morimoto S, Hiramitsu S, Sasayama S and Inoko H. Genetic polymorphisms at the tumour necrosis factor loci (TNFA and TNFB) in cardiac sarcoidosis. *Tissue Antigens* 1999;54:191-193.
143. Grutters JC, Sato H, Pantelidis P, Lagan AL, McGrath DS, Lammers JW, van den Bosch JM, Wells AU, du Bois RM and Welsh KI. Increased frequency of the uncommon tumor necrosis factor -857T allele in British and Dutch patients with sarcoidosis. *Am J Respir Crit Care Med* 2002;165:1119-1124.
144. Poli G, Parola, M. Oxidative damage and fibrogenesis. *Free Radical Biol Med* 1996;22: 287-305.
145. Halliwell B, Gutteridge JM and Cross CE. Free radicals, antioxidants, and human disease: where are we now? *J Lab Clin Med* 1992;119:598-620.
146. Donaldson K. Mechanisms of pneumoconiosis. Banks E. Occupational lung disease, London: Chapman and Hall Medical, 1998. 139-160.
147. Montuschi P, Ciabattoni G, Paredi P, Pantelidis P, du Bois RM, Kharitonov SA and Barnes PJ. 8-Isoprostane as a biomarker of oxidative stress in interstitial lung diseases. *Am J Respir Crit Care Med* 1998;158:1524-1527.

148. Psathakis K, Papatheodorou G, Plataki M, Panagou P, Loukides S, Siafakas NM and Bouros D. 8-Isoprostane, a marker of oxidative stress, is increased in the expired breath condensate of patients with pulmonary sarcoidosis. *Chest* 2004;125:1005-1011.
149. Lenz AG, Costabel U and Maier KL. Oxidized BAL fluid proteins in patients with interstitial lung diseases. *Eur Respir J* 1996;9:307-312.
150. Rottoli P, Magi B, Cianti R, Bargagli E, Vagaggini C, Nikiforakis N, Pallini V and Bini L. Carbonylated proteins in bronchoalveolar lavage of patients with sarcoidosis, pulmonary fibrosis associated with systemic sclerosis and idiopathic pulmonary fibrosis. *Proteomics* 2005;5:2612-2618.
151. Culver DA, Barna BP, Raychaudhuri B, Bonfield TL, Abraham S, Malur A, Farver F, Kavuru MS and Thomassen MJ. Peroxisome proliferator-activated receptor gamma activity is deficient in alveolar macrophages in pulmonary sarcoidosis. *Am J Respir Cell Mol Biol* 2004;30:1-5.
152. Raghu G, Brown KK, Bradford WZ, Starko K, Noble PW, Schwartz DA and King TE, Jr. A placebo-controlled trial of interferon gamma-1b in patients with idiopathic pulmonary fibrosis. *N Engl J Med* 2004;350:125-133.
153. Baughman RP and Lower EE. Therapy for sarcoidosis. *Eur Respir Mon* 2005;32:301-316.
154. Baughman RP, Drent M, Kavuru M, Judson MA, Costabel U, du Bois R, Albera C, Brutsche M, Davis G, Donohue JF, Muller-Quernheim J, Schlenker-Herceg R, Flavin S, Hung Lo K, Oemar B and Barnathan ES. Infliximab therapy in patients with chronic sarcoidosis and pulmonary involvement. *Am J Respir Crit Care Med* 2006;in press.
155. Halliwell B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. *Free Radic Res* 1996;25:57-74.
156. Gaziano JM, Hatta A, Flynn M, Johnson EJ, Krinsky NI, Ridker PM, Hennekens CH and Frei B. Supplementation with beta-carotene in vivo and in vitro does not inhibit low density lipoprotein oxidation. *Atherosclerosis* 1995;112:187-195.
157. Duthie SJ, Jenkinson AM, Crozier A, Mullen W, Pirie L, Kyle J, Yap LS, Christen P and Duthie GG. The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart disease and cancer in healthy human volunteers. *Eur J Nutr* 2006;45:113-122.
158. Meagher EA, Barry OP, Lawson JA, Rokach J and FitzGerald GA. Effects of vitamin E on lipid peroxidation in healthy persons. *Jama* 2001;285:1178-1182.
159. McCall MR and Frei B. Can antioxidant vitamins materially reduce oxidative damage in humans? *Free Radic Biol Med* 1999;26:1034-1053.
160. Moller P, Loft S, Alfthan G and Freese R. Oxidative DNA damage in circulating mononuclear blood cells after ingestion of blackcurrant juice or anthocyanin-rich drink. *Mutat Res* 2004;551:119-126.
161. Lee KW and Lee HJ. Biphasic effects of dietary antioxidants on oxidative stress-mediated carcinogenesis. *Mech Ageing Dev* 2006;127:424-431.
162. Soccio M, Toniato E, Evangelista V, Carluccio M and De Caterina R. Oxidative stress and cardiovascular risk: the role of vascular NAD(P)H oxidase and its genetic variants. *Eur J Clin Invest* 2005;35:305-314.
163. Madamanchi NR, Hakim ZS and Runge MS. Oxidative stress in atherogenesis and arterial thrombosis: the disconnect between cellular studies and clinical outcomes. *J Thromb Haemost* 2005;3:254-267.
164. Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S and Shah AM. NADPH oxidases in cardiovascular health and disease. *Antioxid Redox Signal* 2006;8:691-728.
165. Aslan M and Ozben T. Oxidants in receptor tyrosine kinase signal transduction pathways. *Antioxid Redox Signal* 2003;5:781-788.
166. Neuman I, Nahum H and Ben-Amotz A. Reduction of exercise-induced asthma oxidative stress by lycopene, a natural antioxidant. *Allergy* 2000;55:1184-1189.
167. Dhawan V and Jain S. Garlic supplementation prevents oxidative DNA damage in essential hypertension. *Mol Cell Biochem* 2005;275:85-94.

168. De Backer WA, Amsel B, Jorens PG, Bossaert L, Hiemstra PS, van Noort P and van Overveld FJ. N-acetylcysteine pretreatment of cardiac surgery patients influences plasma neutrophil elastase and neutrophil influx in bronchoalveolar lavage fluid. *Intensive Care Med* 1996;22:900-908.
169. Hofmann MA, Schiekofer S, Isermann B, Kanitz M, Henkels M, Joswig M, Treusch A, Morcos M, Weiss T, Borcea V, Abdel Khalek AK, Amiral J, Tritschler H, Ritz E, Wahl P, Ziegler R, Bierhaus A and Nawroth PP. Peripheral blood mononuclear cells isolated from patients with diabetic nephropathy show increased activation of the oxidative-stress sensitive transcription factor NF-kappaB. *Diabetologia* 1999;42:222-232.
170. Farouque HM, Leung M, Hope SA, Baldi M, Schechter C, Cameron JD and Meredith IT. Acute and chronic effects of flavanol-rich cocoa on vascular function in subjects with coronary artery disease: a randomized double-blind placebo-controlled study. *Clin Sci (Lond)* 2006;111:71-80.
171. Van Overveld FJ, Demkow U, Gorecka D, de Backer WA and Zielinski J. New developments in the treatment of COPD: comparing the effects of inhaled corticosteroids and N-acetylcysteine. *J Physiol Pharmacol* 2005;56 Suppl 4:135-142.
172. Cerda B, Soto C, Albaladejo MD, Martinez P, Sanchez-Gascon F, Tomas-Barberan F and Espin JC. Pomegranate juice supplementation in chronic obstructive pulmonary disease: a 5-week randomized, double-blind, placebo-controlled trial. *Eur J Clin Nutr* 2006;60:245-253.
173. Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Jr., Valanis B, Williams JH, Jr., Barnhart S, Cherniack MG, Brodtkin CA and Hammar S. Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial. *J Natl Cancer Inst* 1996;88:1550-1559.
174. Pryor WA, Stahl W and Rock CL. Beta carotene: from biochemistry to clinical trials. *Nutr Rev* 2000;58:39-53.
175. Albanes D, Heinonen OP, Huttunen JK, Taylor PR, Virtamo J, Edwards BK, Haapakoski J, Rautalahti M, Hartman AM, Palmgren J and et al. Effects of alpha-tocopherol and beta-carotene supplements on cancer incidence in the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study. *Am J Clin Nutr* 1995;62:1427S-1430S.
- 176 Saito K. Possible site of flavonoid synthesis in the photosynthetic apparatus. *Biochem J* 1974;144:431-432.
177. Salunkhe DK, Jadhav SJ, Kadam SS and Chavan JK. Chemical, biochemical, and biological significance of polyphenols in cereals and legumes. *Crit Rev Food Sci Nutr* 1982;17:277-305.
178. Shahidi F and Naczk M. Food phenolics: sources, chemistry, effects, applications. Lancaster 1995; Technomic Publishing Co. Inc.
179. Middleton EJ and Kandaswami C. The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. Harborne JB. The flavonoids: advances in research since 1986, London: Chapman & Hall, 1993. 619-652.
180. Hertog MG, Hollman PC, Katan MB and Kromhout D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr Cancer* 1993;20:21-9.
181. Kuhnau J. The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev Nutr Diet* 1976;24:117-191.
182. Scalbert A and Williamson G. Dietary intake and bioavailability of polyphenols. *J Nutr* 2000;130:2073S-2085S.
183. Hertog MG, Feskens EJ, Hollman PC, Katan MB and Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* 1993;342:1007-1011.
184. De Vries JH, Janssen PL, Hollman PC, van Staveren WA and Katan MB. Consumption of quercetin and kaempferol in free-living subjects eating a variety of diets. *Cancer Lett* 1997;114:141-144.
185. Havsteen B. Flavonoids, a class of natural products of high pharmacological potency. *Biochem Pharmacol* 1983;32:1141-1148.
186. Griffiths LA. Mammalian metabolism of flavonoids. Harborne JB, Mabry, T. The flavonoids: advances in research, London: Chapman & Hall, 1982. 681-718.

187. Hollman PC, de Vries JH, van Leeuwen SD, Mengelers MJ and Katan MB. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am J Clin Nutr* 1995;62:1276-1282.
188. Hollman PC, van Trijp JM, Buysman MN, van der Gaag MS, Mengelers MJ, de Vries JH and Katan MB. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett* 1997;418:152-156.
189. Erlund I, Kosonen T, Alfthan G, Maenpää J, Perttunen K, Kenraali J, Parantainen J and Aro A. Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. *Eur J Clin Pharmacol* 2000;56:545-553.
190. Day AJ, DuPont MS, Ridley S, Rhodes M, Rhodes MJ, Morgan MR and Williamson G. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity. *FEBS Lett* 1998;436:71-75.
191. Nemeth K, Plumb GW, Berrin JG, Juge N, Jacob R, Naim HY, Williamson G, Swallow DM and Kroon PA. Deglycosylation by small intestinal epithelial cell beta-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. *Eur J Nutr* 2003;42:29-42.
192. Gee JM, DuPont MS, Day AJ, Plumb GW, Williamson G and Johnson IT. Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. *J Nutr* 2000;130:2765-2771.
193. Walgren RA, Lin JT, Kinne RK and Walle T. Cellular uptake of dietary flavonoid quercetin 4'-beta-glucoside by sodium-dependent glucose transporter SGLT1. *J Pharmacol Exp Ther* 2000;294:837-843.
194. Day AJ, Canada FJ, Diaz JC, Kroon PA, McLauchlan R, Faulds CB, Plumb GW, Morgan MR and Williamson G. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Lett* 2000;468:166-170.
195. Sesink AL, Arts IC, Faassen-Peters M and Hollman PC. Intestinal uptake of quercetin-3-glucoside in rats involves hydrolysis by lactase phlorizin hydrolase. *J Nutr* 2003;133:773-776.
196. Gee JM, DuPont MS, Rhodes MJ and Johnson IT. Quercetin glucosides interact with the intestinal glucose transport pathway. *Free Radic Biol Med* 1998;25:19-25.
197. Arts IC, Siesink AL and Hollman PC. Quercetin-3-glucoside is transported by the glucose carrier SGLT1 across the brush border membrane of rat small intestine. *J Nutr* 2002;132:2823-2825.
198. Walgren RA, Karnaky KJ, Jr., Lindenmayer GE and Walle T. Efflux of dietary flavonoid quercetin 4'-beta-glucoside across human intestinal Caco-2 cell monolayers by apical multidrug resistance-associated protein-2. *J Pharmacol Exp Ther* 2000;294:830-836.
199. Hollman PC, Katan MB. Absorption, metabolism and bioavailability of flavonoids. Rice-Evans CA, Packer L. Flavonoids in health and disease, New York: Marcel Dekker Inc, 1998. 483-522.
200. Day AJ, Bao Y, Morgan MR and Williamson G. Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radic Biol Med* 2000;29:1234-1243.
201. Hollman PC and Katan MB. Absorption, metabolism and health effects of dietary flavonoids in man. *Biomed Pharmacother* 1997;51:305-310.
202. Hackett AM. The metabolism of flavonoid compounds in mammals. *Prog Clin Biol Res* 1986;213:177-194.
203. Conquer JA, Maiani G, Azzini E, Raguzzini A and Holub BJ. Supplementation with quercetin markedly increases plasma quercetin concentration without effect on selected risk factors for heart disease in healthy subjects. *J Nutr* 1998;128:593-597.
204. Hollman PC, vd Gaag M, Mengelers MJ, van Trijp JM, de Vries JH and Katan MB. Absorption and disposition kinetics of the dietary antioxidant quercetin in man. *Free Radic Biol Med* 1996;21:703-707.
205. De Boer VC, Dihal AA, van der Woude H, Arts IC, Wolffram S, Alink GM, Rietjens IM, Keijer J and Hollman PC. Tissue distribution of quercetin in rats and pigs. *J Nutr* 2005;135:1718-1725.

206. Manach C, Williamson G, Morand C, Scalbert A and Remesy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* 2005;81:230S-242S.
207. Hertog MG, Feskens EJ and Kromhout D. Antioxidant flavonols and coronary heart disease risk. *Lancet* 1997;349:699.
208. Arts IC and Hollman PC. Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr* 2005;81:317S-325S.
209. Hirvonen T, Virtamo J, Korhonen P, Albanes D and Pietinen P. Flavonol and flavone intake and the risk of cancer in male smokers (Finland). *Cancer Causes Control* 2001;12:789-796.
210. Knekt P, Kumpulainen J, Jarvinen R, Rissanen H, Heliovaara M, Reunanen A, Hakulinen T and Aromaa A. Flavonoid intake and risk of chronic diseases. *Am J Clin Nutr* 2002;560-568.
211. Tabak C, Arts IC, Smit HA, Heederik D and Kromhout D. Chronic pulmonary obstructive disease and intake of catechins, flavonols and flavones: the MORGEN study. *Am J Respir Crit Care Med* 1999;164:61-64.
212. Burke V, Milligan RA, Beilin LJ, Dunbar D, Spencer M, Balde E and Gracey MP. Clustering of health-related behaviors among 18-year-old Australians. *Prev Med* 1997;26:724-733.
213. Veenstra J, Schenkel JA, van Erp-Baart AM, Brants HA, Hulshof KF, Kistemaker C, Schaafsma G and Ockhuizen T. Alcohol consumption in relation to food intake and smoking habits in the Dutch National Food Consumption Survey. *Eur J Clin Nutr* 1993;47:482-489.
214. Hulshof KF, Wedel M, Lowik MR, Kok FJ, Kistemaker C, Hermus RJ, Ten Hoor F and Ockhuizen T. Clustering of dietary variables and other lifestyle factors (Dutch Nutritional Surveillance System). *J Epidemiol Community Health* 1992;46:417-424.
215. Tjonneland A, Gronbaek M, Stripp C and Overvad OK. Wine intake and diet in a random sample of 48763 Danish men and women. *Am J Clin Nutr* 1999;69:49-54.
216. Cushnie TP and Lamb AJ. Antimicrobial activity of flavonoids. *Int J Antimicrob Agents* 2005;26:343-356.
217. Hanasaki Y, Ogawa S and Fukui S. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. *Free Radic Biol Med* 1994;16:845-850.
218. Haenen GR and Bast A. Nitric oxide radical scavenging of flavonoids. *Methods Enzymol* 1999;301:490-503.
219. Van Acker SA, Tromp MN, Haenen GR, van der Vijgh WJ and Bast A. Flavonoids as scavengers of nitric oxide radical. *Biochem Biophys Res Commun* 1995;214:755-759.
220. Haenen GR, Paquay JB, Korthouwer RE and Bast A. Peroxynitrite scavenging by flavonoids. *Biochem Biophys Res Commun* 1997;236:591-593.
221. Heijnen CG, Haenen GR, van Acker FA, van der Vijgh WJ and Bast A. Flavonoids as peroxynitrite scavengers: the role of the hydroxyl groups. *Toxicol In Vitro* 2001;15:3-6.
222. Heijnen CG, Haenen GR, Oostveen RM, Stalpers EM and Bast A. Protection of flavonoids against lipid peroxidation: the structure activity relationship revisited. *Free Radic Res* 2002;36:575-581.
223. Arts MJ, Dallinga JS, Voss HP, Haenen GR and Bast A. A new approach to assess the total antioxidant capacity using the TEAC assay. *Food Chem* 2004;88:567-570.
224. Wilms LC, Hollman PC, Boots AW and Kleinjans JC. Protection by quercetin and quercetin-rich fruit juice against induction of oxidative DNA damage and formation of BPDE-DNA adducts in human lymphocytes. *Mutat Res* 2005;582:155-162.
225. Manjeet KR and Ghosh B. Quercetin inhibits LPS-induced nitric oxide and tumor necrosis factor-alpha production in murine macrophages. *Int J Immunopharmacol* 1999;21:435-443.
226. Read MA. Flavonoids: naturally occurring anti-inflammatory agents. *Am J Pathol* 1995;147:235-237.
227. Nair MP, Mahajan S, Reynolds JL, Aalinkeel R, Nair H, Schwartz SA and Kandaswami C. The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF-kappabeta system. *Clin Vaccine Immunol* 2006;13:319-328.
228. Orsolic N, Knezevic AH, Sver L, Terzic S and Basic I. Immunomodulatory and antimetastatic action of propolis and related polyphenolic compounds. *J Ethnopharmacol* 2004;94:307-315.

229. Lee ES, Lee HE, Shin JY, Yoon S and Moon JO. The flavonoid quercetin inhibits dimethylnitrosamine-induced liver damage in rats. *J Pharm Pharmacol* 2003;55:1169-1174.
230. Bucki R, Pastore JJ, Giraud F, Sulpice JC and Janmey PA. Flavonoid inhibition of platelet procoagulant activity and phosphoinositide synthesis. *J Thromb Haemost* 2003;1:1820-1828.
231. Perez-Vizcaino F, Bishop-Bailley D, Lodi F, Duarte J, Cogolludo A, Moreno L, Bosca L, Mitchell JA and Warner TD. The flavonoid quercetin induces apoptosis and inhibits JNK activation in intimal vascular smooth muscle cells. *Biochem Biophys Res Commun* 2006; 919-925.
232. De Whalley CV, Rankin SM, Houlst JR, Jessup W and Leake DS. Flavonoids inhibit the oxidative modification of low density lipoproteins by macrophages. *Biochem Pharmacol* 1990;39:1743-1750.
233. Duarte J, Perez-Palencia R, Vargas F, Ocete MA, Perez-Vizcaino F, Zarzuelo A and Tamargo J. Antihypertensive effects of the flavonoid quercetin in spontaneously hypertensive rats. *Br J Pharmacol* 2001;133:117-124.
234. Kuo SM. Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells. *Cancer Lett* 1996;110:41-48.
235. Gulati N, Laudet N, Zohrabian VM, Murali R and Jhanwar-Uniyal M. The antiproliferative effect of Quercetin in cancer cells is mediated via inhibition of the PI3K-Akt/PKB pathway. *Anticancer Res* 2006;26:1177-1181.
236. Moon YJ, Wang X and Morris ME. Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. *Toxicol In Vitro* 2006;20:187-210.
237. Schwarz D, Kisselev P and Roots I. CYP1A1 genotype-selective inhibition of benzo[a]pyrene activation by quercetin. *Eur J Cancer* 2005;41:151-158.
238. Walle T, Eaton EA and Walle UK. Quercetin, a potent and specific inhibitor of the human P-form phenosulfotransferase. *Biochem Pharmacol* 1995;50:731-734.
239. Pacifici GM. Inhibition of human liver and duodenum sulfotransferases by drugs and dietary chemicals: a review of the literature. *Int J Clin Pharmacol Ther* 2004;42:488-495.
240. Piantelli M, Maggiano N, Ricci R, Larocca LM, Capelli A, Scambia G, Isola G, Natali PG and Ranelletti FO. Tamoxifen and quercetin interact with type II estrogen binding sites and inhibit the growth of human melanoma cells. *J Invest Dermatol* 1995;105:248-253.
241. Caltagirone S, Ranelletti FO, Rinelli A, Maggiano N, Colasante A, Musiani P, Aiello FB and Piantelli M. Interaction with type II estrogen binding sites and antiproliferative activity of tamoxifen and quercetin in human non-small-cell lung cancer. *Am J Respir Cell Mol Biol* 1997;17:51-59.
242. Jorgensen LV, Cornett C, Justesen U, Skibsted LH and Dragsted LO. Two-electron electrochemical oxidation of quercetin and kaempferol changes only the flavonoid C-ring. *Free Radic Res* 1998;29:339-350.
243. Awad HM, Boersma MG, Boeren S, van Bladeren PJ, Vervoort J and Rietjens IM. The regioselectivity of glutathione adduct formation with flavonoid quinone/quinone methides is pH-dependent. *Chem Res Toxicol* 2002;15:343-351.
244. Boots AW, Kubben N, Haenen GR and Bast A. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. *Biochem Biophys Res Commun* 2003;308:560-565.
245. Kalyanaraman B, Premovic PI and Sealy RC. Semiquinone anion radicals from addition of amino acids, peptides, and proteins to quinones derived from oxidation of catechols and catecholamines. An ESR spin stabilization study. *J Biol Chem* 1987;262:11080-11087.
246. Ito S, Kato T and Fujita K. Covalent binding of catechols to proteins through the sulphydryl group. *Biochem Pharmacol* 1988;37:1707-1710.
247. Metodiewa D, Jaiswal AK, Cenas N, Dickanait E and Segura Aguilar J. Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. *Free Radic Biol Med* 1999;26:107-116.
248. Monks TJ, Hanzlik RP, Cohen GM, Ross D and Graham DG. Quinone chemistry and toxicity. *Toxicol Appl Pharmacol* 1992;112:2-16.

249. Galati G, Moridani MY, Chan TS and O'Brien PJ. Peroxidative metabolism of apigenin and naringenin versus luteolin and quercetin: glutathione oxidation and conjugation. *Free Radic Biol Med* 2001;30:370-382.
250. Yen GC, Duh PD, Tsai HL and Huang SL. Pro-oxidative properties of flavonoids in human lymphocytes. *Biosci Biotechnol Biochem* 2003;67:1215-1222.
251. Vrijksen R, Michotte Y and Boeye A. Metabolic activation of quercetin mutagenicity. *Mutat Res* 1990;232:243-248.
252. Silva ID, Gaspar J, da Costa GG, Rodrigues AS, Laires A and Rueff J. Chemical features of flavonols affecting their genotoxicity. Potential implications in their use as therapeutical agents. *Chem Biol Interact* 2000;124:29-51.
253. Rueff J, Gaspar J and Laires A. Structural requirements for mutagenicity of flavonoids upon nitrosation. A structure-activity study. *Mutagenesis* 1995;10:325-328.
254. Jurado J, Alejandro-Duran E, Alonso-Moraga A and Pueyo C. Study on the mutagenic activity of 13 bioflavonoids with the Salmonella Ara test. *Mutagenesis* 1991;6:289-295.
255. Meltz ML and MacGregor JT. Activity of the plant flavanol quercetin in the mouse lymphoma L5178Y TK+/- mutation, DNA single-strand break, and Balb/c 3T3 chemical transformation assays. *Mutat Res* 1981;88:317-324.
256. Jin NZ, Zhu YP, Zhou JW, Mao L, Zhao RC, Fang TH and Wang XR. Preventive Effects of Quercetin against Benzo[a]pyrene-Induced DNA Damages and Pulmonary Precancerous Pathologic Changes in Mice. *Basic Clin Pharmacol Toxicol* 2006;98:593-598.
257. Jalili T, Carlstrom J, Kim S, Freeman D, Jin H, Wu TC, Litwin SE and David Symons J. Quercetin-supplemented diets lower blood pressure and attenuate cardiac hypertrophy in rats with aortic constriction. *J Cardiovasc Pharmacol* 2006;47:531-541.

2

Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants

AW Boots, GRMM Haenen, GJM den Hartog, A Bast

Biochimica et Biophysica Acta 2002;1583:279-284

Abstract

Background

Catechol-containing antioxidants are able to protect against lipid peroxidation by nonenzymatic scavenging of free radicals with their catechol moiety. However, antioxidants become oxidized during their actual scavenging activity and oxidation products such as semiquinone radicals and quinones are formed. These products may display toxic effects. Therefore, the aim of the present study is to evaluate the effect of catechol-containing antioxidants as well as of their oxidation products on free radical damage. 4-Methylcatechol, a catechol-containing antioxidant that gives stable oxidation products, has been used as model compound.

Methods

The formation of the oxidation products of 4-methylcatechol has been evaluated using electron spin resonance (ESR) measurements. The influence of both the antioxidant and its oxidation products on free radical damage has been investigated in liver microsomes by means of various parameters including lipid peroxidation, thiol content and calcium sequestration.

Results

The oxidation products of 4-methylcatechol inactivated both the glutathione (GSH)-dependent protection against lipid peroxidation and the calcium sequestration in liver microsomes.

Conclusion

The found effects of the oxidation products of 4-methylcatechol are probable due to their arylation of free thiol groups of the enzymes responsible for the GSH-dependent protection and calcium sequestration, i.e. the free radical reductase and calcium ATP-ase. Therefore, it is concluded that a catechol-containing antioxidant might shift radical damage from lipid peroxidation to sulfhydryl arylation.

Introduction

Aerobic life-forms are associated with the generation of reactive oxygen species (ROS). ROS can cause oxidative damage to biological macromolecules like DNA, lipids and proteins (1). An important target of free radicals are the poly-unsaturated fatty acids in biomembranes. Their peroxidation, known as lipid peroxidation, may impair membrane functions, increase permeability, reduce the membrane fluidity, inhibit the signal transduction over the membrane and inactivate membrane-bound enzymes and receptors (2). One of the primary targets in toxicity induced by lipid peroxidation is calcium ATP-ase. Microsomal calcium sequestration by calcium ATP-ase is dependent on critically sulfhydryl groups of the enzyme that are affected by lipid peroxidation (3).

Fortunately, cells contain an elaborate defense system to neutralize ROS. Endogenous antioxidants interact in an elaborate network. For example, several groups have stated that glutathione (GSH) protects against microsomal lipid peroxidation in an interplay with vitamin E. During the protection against ROS, vitamin E radicals are formed. It is proposed that this radical is converted back to vitamin E by a free radical reductase at the expense of GSH (4-8).

In some cases, however, an imbalanced radical production, oxidative stress, arises. It is generally accepted that oxidative stress is implicated in several pathophysiological processes such as cardiovascular heart diseases, diabetes and chronic obstructive pulmonary disease (COPD) (9-11).

To prevent oxidative stress-induced pathologies, antioxidant therapy can be used. An important antioxidant pharmacophore is the catechol moiety present in various antioxidants such as catecholamines and numerous flavonoids like quercetin (12). They are able to scavenge highly reactive species such as peroxynitrite and the hydroxyl radical (13,14). It should be noted that during this activity the antioxidant is converted into oxidized products, i.e. semiquinone radicals and quinones. These products may also be toxic (15-17).

The aim of this study is to evaluate the effect of catechol containing antioxidants on free radical damage. To study the effect of the oxidation products of catechol containing antioxidants, we used 4-methylcatechol which gives a stable oxidation product, 4-methyl-ortho-benzoquinone. This strategy has been used previously (18,19). The effect of 4-methylcatechol as well as the effect of its quinone was examined.

Materials and methods

Chemicals

Reduced GSH, Nitro Blue Tetrazolium (NBT) and chromatographically purified xanthine oxidase (grade III) were from Sigma (St Louis, MO). 4-Methylcatechol was obtained from Fluka (Buchs, Switzerland). 4-Methyl-ortho-benzoquinone was synthesized according to Willstätter and Pfannenstiel by the Ag_2O -mediated oxidation of 4-methylcatechol (20).

Methods

Male Wistar rats (Harlan Olec CPB, Zeist, The Netherlands) of 200-250 g were used. Liver microsomes (1mg/ml) were prepared as described previously (4). Lipid peroxidation was measured with the thiobarbituric acid assay (4) and expressed as the absorbance at 535 versus 600 nm ($\Delta A_{535-600}$). Oxidative stress was induced by (a) 50 μM xanthine and 55 mU xanthine oxidase or (b) 1 mM H_2O_2 and 40 nM horseradish peroxidase. The microsomes were suspended in a 150 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl. In the experiment where the calcium sequestration was measured another buffer was used (vide infra).

Pretreatment of the microsomes with 4-methylcatechol and xanthine-xanthine oxidase consisted of an incubation of 10 min at 37 °C with 100 μM catechol, 50 μM xanthine and 55 mU xanthine-oxidase. The incubation was terminated by the addition of an equal volume of ice-cold buffer. The microsomes were immediately centrifuged (40 min, 115,000 x g at 4°C). The pellet was resuspended in the buffer and, in order to induce lipid peroxidation, incubated with 10 μM Fe^{2+} and 0.2 mM ascorbate.

Thiol content of the pretreated microsomes was measured using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). After preincubation, the microsomes were immediately centrifuged (5 min, 115,000 x g at 4°C) and the pellet was resuspended in the buffer. After incubation with 0.6 mM DTNB for 5 minutes, the reduction of DTNB was measured spectrophotometrically at 412 nm.

Electron spin resonance (ESR) spectra were recorded on a Bruker EMX. It was indicated when MgCl_2 , which stabilizes the semi-*ortho*-quinone, was added. Instrumental settings were: modulation amplitude 0.1 G; time constant 30 msec; scan time 30 min; amplifier power 25 mW; receiver gain $2 \cdot 10^5$.

Calcium sequestration was determined by incubating microsomes (1 mg/ml) in 115 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4 , 20 mM Hepes, 4 mM MgCl_2 , pH 7.2 at 37°C in the presence of an ATP regenerating system (10 mM creatine phosphate and 10 U/ml creatine kinase). Calcium sequestration was started by the addition of 2 mM ATP. Changes in the free calcium

concentration were monitored by measuring Quin2 fluorescence (50 μM Quin2) on a Perkin Elmer MPF-2A fluorescence spectrophotometer. An excitation wavelength of 339 nm (10 nm slit) and an emission wavelength of 500 nm (10 nm slit) were used. The concentration of free calcium was within the physiological range (200-350 nM). Calcium sequestered was quantified by releasing the sequestered calcium with ionomycin (2 μM). The calcium concentration was quantified by adding known quantities of calcium into the cuvette.

Protein content was assayed according to Bradford using bovine serum albumin as standard (21).

All experiments were performed at least in triplicate. Unless otherwise mentioned, a typical example is shown.

Results

Oxidative stress induced by xanthine-xanthine oxidase (Figure 2.1A) or H_2O_2 -horseradish peroxidase (Figure 2.1B) resulted in the peroxidation of rat liver microsomes.

Figure 2.1 Protection against microsomal lipid peroxidation by 4-methylcatechol. Lipid peroxidation (expressed as $\Delta A_{535-600}$) was induced by xanthine-xanthine oxidase (panel A) or by H_2O_2 -horseradish peroxidase (panel B). The concentrations of 4-methylcatechol were: 0 μM (closed triangle) 2 μM (closed circle), 4 μM (open circle) or 10 μM (open triangle).

This also resulted in a 20% reduction of the thiol content of the microsomes (Table 2.1). Addition of 4-methylcatechol gave a concentration-dependent protection against lipid peroxidation, irrespective of the method used to induce lipid peroxidation. Both the rate and the final extent of lipid peroxidation were reduced by adding 4-methylcatechol.

Table 2.1 Lipid peroxidation and protein thiol content of liver microsomes after the antioxidant actions of 4-methylcatechol.

H_2O_2 and horse radish peroxidase			Xanthine and xanthine oxidase		
Conc 4-MC (μM)	Lipid peroxidation (%)	Protein thiols (%)	Conc 4-MC (μM)	Lipid peroxidation (%)	Protein thiols (%)
0	100 ± 6	81 ± 2	0	100 ± 9	82 ± 2
100	0	50 ± 1	100	0	49 ± 1

Pre-treatment of the microsomes with H_2O_2 -horseradish peroxidase or xanthine-xanthine oxidase comprised 10 minutes. Lipid peroxidation is expressed relative to a control incubation without H_2O_2 -horseradish peroxidase or xanthine-xanthine oxidase in control membranes $\pm$ SD ($n=3$). One hundred micromolars of 4-methylcatechol (4-MC) itself did not induce lipid peroxidation or reduce the protein thiol content.

The protection by 4-methylcatechol was non-enzymatic, since heating the microsomes had no effect on the inhibition of lipid peroxidation by 4-methylcatechol (data not shown). In contrast to the complete protection by 100 μM 4-methylcatechol against xanthine-xanthine oxidase or H_2O_2 -horseradish peroxidase induced lipid peroxidation, 4-methylcatechol increased the amount of thiols that was consumed from 20% to 50%. 4-Methylcatechol itself had, at a concentration of 100 μM , no effect on the thiol content of the microsomes (Table 2.1).

During the protection by 4-methylcatechol against H_2O_2 -horseradish peroxidase (Figure 2.2) or xanthine-xanthine oxidase (not shown) induced oxidative stress, 4-methylcatechol becomes oxidized and 4-methylbenzoquinone is formed. Using ESR a semi-*ortho*-quinone radical and a secondary radical could be detected in the incubation with H_2O_2 -horseradish peroxidase (Figure 2.3).

Figure 2.2 UV-spectrum of 100 μM 4-methylcatechol (4-MC; trace 1), of 1 mM H_2O_2 and 40 nM horseradish peroxidase (trace 2) and of 100 μM 4-MC with 1 mM H_2O_2 immediately (trace 3) or 10 minutes after (trace 4) the addition of 40 nM horseradish peroxidase. During this reaction 4-MC becomes oxidized and 4-methyl-quinone is formed.

Figure 2.3 ESR spectra of radicals generated from 10 mM 4-methylcatechol by 40 nM horseradish peroxidase and 1 mM H_2O_2 with (panel A) and without 50 mM MgCl_2 (panel B). After stabilization of the semi-*ortho*-quinone with MgCl_2 , a combined spectrum of the semiquinone radical (o) and a secondary radical (x) is obtained. 4-Methylcatechol and H_2O_2 /horse radish peroxidase controls with and without MgCl_2 gave no detectable signals.

The effect of the products of 4-methylcatechol formed during its antioxidant activity on the endogenous protection by the GSH-dependent free radical reductase against microsomal lipid peroxidation was determined. Therefore microsomes subjected to the combination of 100 μM 4-methylcatechol and xanthine-xanthine oxidase were isolated. No lipid peroxidation was observed in these membranes. After 20 minutes the membranes were washed, in order to remove the xanthine-oxidase and the 4-methylcatechol.

Subsequently, in these pretreated membranes the GSH-dependent protection against lipid peroxidation was assessed. To this end, the pretreated liver microsomes were peroxidized by addition of the combination of iron and ascorbate. In control membranes, addition of 1 mM GSH induced a lag time in the occurrence of lipid peroxidation (Figure 2.4A). In microsomes, pretreated with the combination of xanthine-xanthine oxidase and 4-methylcatechol, the GSH-induced lag time was absent (Figure 2.4B).

Figure 2.4 Lipid peroxidation in control microsomes (panel A) and microsomes pretreated with 100 μ M 4-methylcatechol in combination with xanthine-xanthine oxidase (panel B). Lipid peroxidation (expressed as $\Delta A_{535-600}$) was induced by the combination of 10 μ M Fe^{2+} and 0.2 mM ascorbate. Further additions were: none (closed circle) or 1 mM GSH (open circle).

Figure 2.5 Lipid peroxidation in microsomes pretreated with 100 μ M 4-methyl-*ortho*-benzoquinone. Lipid peroxidation (expressed as $\Delta A_{535-600}$) was induced by the combination of 10 μ M Fe^{2+} and 0.2 mM ascorbate. Further additions were: none (closed circle) or 1 mM GSH (open circle).

To investigate whether thiol arylation by 4-methyl-*ortho*-benzoquinone is responsible for the observed inactivation, the effect of addition of GSH was assessed. It was found that the addition of GSH prevented the reduction of the GSH-dependent protection by 4-methyl-*ortho*-benzoquinone (data not shown). This is probably due to the fact that the non-protein thiol GSH, instead of the sulfhydryl group on the free radical reductase, is arylated. Furthermore, preincubation of the microsomes with 4-methyl-*ortho*-benzoquinone resulted in a concentration dependent reduction of the thiol content of the microsomes (Table 2.2).

Table 2.2 Calcium sequestration and protein thiol content of liver microsomes after N-ethylmaleimide or 4-methyl-*ortho*-benzoquinone treatment.

H ₂ O ₂ and horse radish peroxidase			Xanthine and xanthine oxidase		
Conc (μ M)	Ca ²⁺ sequestration (%)	Protein thiols (%)	Conc (μ M)	Ca ²⁺ sequestration (%)	Protein thiols (%)
0	100 $\pm$ 5	100 $\pm$ 2	0	100 $\pm$ 5	100 $\pm$ 2
100	53 $\pm$ 2	59 $\pm$ 2	100	50 $\pm$ 3	49 $\pm$ 1
250	23 $\pm$ 1	33 $\pm$ 1	250	6 $\pm$ 3	31 $\pm$ 2
1000	0	4 $\pm$ 1	1000	n.d.	2 $\pm$ 1

Both calcium sequestration and thiol content of the proteins are expressed relative to the sequestration and the thiol content in control membranes $\pm$ SD (n=3). One hundred micromolars of 4-methylcatechol itself did not inactivate calcium sequestration or reduce the protein thiol content.

Of the other enzymes located on the microsomal membrane, the calcium-ATP-ase was also investigated. The enzyme contains an essential and vulnerable sulfhydryl group, since the synthetic sulfhydryl-alkylating agent N-ethylmaleimide impaired the capacity of the enzyme to catalyse the sequestration of calcium (Table 2.2). It was found that 4-methyl-*ortho*-benzoquinone was equally potent as N-ethylmaleimide in inactivating this enzyme and reducing the thiol content of the microsomes (Table 2.2).

Discussion

4-Methylcatechol is effective in preventing microsomal lipid peroxidation, confirming that the catechol moiety is a potent antioxidant pharmacophore (12,22). This antioxidant pharmacophore is present in numerous flavonoids and other polyphenols. It has previously been described that catechol-containing antioxidants are very potent scavengers of e.g. peroxynitrite (13).

In the protection against lipid peroxidation by the catechols, the catechols are oxidized. Products like semiquinone radicals and quinones are produced during the actual antioxidant activity, i.e. the reaction with radicals. It has been well described that these compounds are potentially toxic, especially due to their ability to arylate protein thiols (16,17,23,24). Indeed, a reference quinone, i.e. 4-methyl-*ortho*-benzoquinone, is able to react with the protein thiols as efficient as the synthetic sulfhydryl-alkylating agent N-ethylmaleimide. This observation suggests that the oxidation

products of catechol-containing antioxidants shift the damage provoked by oxidative stress from lipid peroxidation to sulfhydryl arylation.

One of the consequences of this sulfhydryl arylation is that it may indirectly stimulate lipid peroxidation by inactivating the endogenous defences against lipid peroxidation, i.e. the GSH-dependent free radical reductase. This means that, despite the direct protection offered by catechol-containing antioxidants, lipid peroxidation is stimulated indirectly by the reaction products of these antioxidants formed during this protection.

One of the primary toxic effects of lipid peroxidation is inhibition of calcium ATP-ase. Catechol containing antioxidants inhibit lipid peroxidation, but the reactive products formed during this protection also inhibit calcium ATP-ase. So, despite the apparent protection afforded by catechol-containing antioxidants against lipid peroxidation, the toxic effect on a final target, i.e. calcium ATP-ase, is identical.

Sulfhydryl reactivity of the metabolites of catechol-containing compounds has been observed previously (25,26). For example, the toxicity of the catechol-containing compound α -methyldopa is suggested to be due to the scavenging of superoxide radicals generated by cytochrome-P450, a reaction leading to the production of a reactive semiquinone and quinone (26). Another example of sulfhydryl reactivity is the microsomal glutathione S-transferase (GST) that can be activated by covalent binding of its free thiolgroup (25). By this mechanism 4-methyl-*ortho*-benzoquinone and reactive metabolites of a catecholamine activate the microsomal GST (19).

In conclusion, free radical scavengers offer protection against oxidative stress since they can react with damaging reactive species. Despite this protection, the toxicity of reactive oxidation products generated during the actual antioxidant activity might be substantial, e.g. weakening of the endogenous antioxidant defence and impairment of vital enzymes. This should be considered in the application of antioxidants.

References

1. Diplock AT, Charleux JL, Crozier Willi G, Kok FJ, Rice Evans C, Roberfroid M, Stahl W and Vina Ribes J. Functional food science and defence against reactive oxidative species. *Br J Nutr* 1998;80:1s77-112.
2. Bast A, Haenen GR and Doelman CJ. Oxidants and antioxidants: state of the art. *Am J Med* 1991;91:2s-13s.
3. Thor H, Hartzell P, Svensson SA, Orrenius S, Mirabelli F, Marinoni V and Bellomo G. On the role of thiol groups in the inhibition of liver microsomal Ca^{2+} sequestration by toxic agents. *Biochem Pharmacol* 1985;34:3717-3723.
4. Haenen GR and Bast A. Protection against lipid peroxidation by a microsomal glutathione-dependent labile factor. *FEBS Lett* 1983;159:24-28.
5. Scholz RW, Reddy PV, Wynn MK, Graham KS, Liken AD, Gumprich E and Reddy CC. Glutathione-dependent factors and inhibition of rat liver microsomal lipid peroxidation. *Free Radic Biol Med* 1997;23:815-828.
6. McCay PB, Brueggemann G, Lai EK and Powell SR. Evidence that alpha-tocopherol functions cyclically to quench free radicals in hepatic microsomes. Requirement for glutathione and a heat-labile factor. *Ann N Y Acad Sci* 1989;570:32-45.
7. Wefers H and Sies H. The protection by ascorbate and glutathione against microsomal lipid peroxidation is dependent on vitamin E. *Eur J Biochem* 1988;174:353-357.
8. Burk RF. Glutathione-dependent protection by rat liver microsomal protein against lipid peroxidation. *Biochim Biophys Acta* 1983;757:21-28.
9. Repine JE, Bast A and Lankhorst I. Oxidative stress in chronic obstructive pulmonary disease. Oxidative Stress Study Group. *Am J Respir Crit Care Med* 1997;156:341-357.
10. Parthasarathy S, Khan-Merchant N, Penumetcha M and Santanam N. Oxidative stress in cardiovascular disease. *J Nucl Cardiol* 2001;8:379-389.
11. Rosen P, Nawroth PP, King G, Moller W, Tritschler HJ and Packer L. The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes Metab Res Rev* 2001;17:189-212.
12. Heijnen CG, Haenen GR, Vekemans JA and Bast A. Peroxynitrite scavenging of flavonoids: structure activity relationship. *Environmental Toxicology and Pharmacology* 2001;10:199-206.
13. Heijnen CG, Haenen GR, van Acker FA, van der Vijgh WJ and Bast A. Flavonoids as peroxynitrite scavengers: the role of the hydroxyl groups. *Toxicol In Vitro* 2001;15:3-6.
14. Ohshima H, Yoshie Y, Auriol S and Gilibert I. Antioxidant and pro-oxidant actions of flavonoids: effects on DNA damage induced by nitric oxide, peroxynitrite and nitroxyl anion. *Free Radic Biol Med* 1998;25:1057-1065.
15. Awad HM, Boersma MG, Boeren S, van Bladeren PJ, Vervoort J and Rietjens IM. Structure-activity study on the quinone/quinone methide chemistry of flavonoids. *Chem Res Toxicol* 2001;14:398-408.
16. Metodiewa D, Jaiswal AK, Cenas N, Dickancaite E and Segura Aguilar J. Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. *Free Radic Biol Med* 1999;26:107-116.
17. Ito S, Kato T and Fujita K. Covalent binding of catechols to proteins through the sulphhydryl group. *Biochem Pharmacol* 1988;37:1707-1710.
18. Tse DC, McCreery RL and Adams RN. Potential oxidative pathways of brain catecholamines. *J Med Chem* 1976;19:37-40.
19. Haenen GR, Jansen FP, Vermeulen NP and Bast A. Activation of the microsomal glutathione S-transferase by metabolites of alpha-methyldopa. *Arch Biochem Biophys* 1991;287:48-52.
20. Willstätter R and Pfannenstiel A. Ueber o-chinon. *Chem Ber* 1904;4:4744-4746.
21. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:246-254.

22. van Acker FA, Hulshof JW, Haenen GR, Menge WM, van der Vijgh WJ and Bast A. New synthetic flavonoids as potent protectors against doxorubicin-induced cardiotoxicity. *Free Radic Biol Med* 2001;31:31-37.
23. Kalyanaraman B, Premovic PI and Sealy RC. Semiquinone anion radicals from addition of amino acids, peptides, and proteins to quinones derived from oxidation of catechols and catecholamines. An ESR spin stabilization study. *J Biol Chem* 1987;262:11080-11087.
24. Monks TJ, Hanzlik RP, Cohen GM, Ross D and Graham DG. Contemporary issues in toxicology: quinone chemistry and toxicity. *Toxicol Appl Pharmacol* 1992;112:2-16.
25. Dybing E, Nelson SD, Mitchell JR, Sasame HA and Gillette JR. Oxidation of α -methyldopa and other catechols by CYP 450-generated superoxide anion: possible mechanism of methyldopa hepatitis. *Mol Pharmacol* 1976;12:911-920.
26. Bast A and Haenen GR. The toxicity of antioxidants and their metabolites. *Environmental Toxicology and Pharmacology* 2002;11:251-258.

3

Oxidized quercetin reacts with thiols
rather than with ascorbate:
implication for quercetin
supplementation

AW Boots, N Kubben, GRMM Haenen, A Bast

Biochemical and Biophysical Research Communications 2003;308:560-565

Abstract

Background

When an antioxidant scavenges a reactive species, i.e. when it exerts its antioxidant activity, the antioxidant is converted into potentially harmful oxidation products. In this way the antioxidant quercetin might yield an *ortho*-quinone, denoted as QQ, which has four tautomeric forms, i.e. the *ortho*-quinone and three quinonmethides. We evaluated the interaction of QQ with ascorbate or glutathione (GSH).

Methods

The reaction of QQ with several compounds was monitored spectrophotometrically as well as by high performance liquid chromatography (HPLC).

Results

Ascorbate recycled QQ to the parent compound quercetin, while GSH formed two adducts with QQ, i.e. 6-GSQ and 8-GSQ. When both GSH and ascorbate were present, QQ was converted exclusively into GSQ. In the absence of GSH, protein thiols became arylated by QQ. This protein arylation could not be prevented by ascorbate.

Conclusions

Thiol arylation by quinones and quinonmethides can impair several vital enzymes. This implies that the product formed when quercetin displays its antioxidant scavenging effect is toxic in the absence of GSH. Therefore, an adequate GSH level should be maintained when quercetin is supplemented.

Introduction

Antioxidants have the annotation of being healthy and safe. Many diseases are associated with oxidative stress (1-3) and the use of antioxidant-rich food or antioxidant supplements is often recommended to preserve or regain good health (4,5). Of the dietary antioxidants, flavonoids have gained much interest. One of the most prominent flavonoids is quercetin.

It is not generally recognized that antioxidants may also display adverse effects. Without proper knowledge on side effects, biotransformation or biokinetics relatively high dosages of antioxidants are taken by large populations. Supplements containing up to 500 mg quercetin are being marketed. It should, however, be noted that once an antioxidant such as quercetin exerts its presumed protective effect, i.e. when it neutralizes a reactive species, it is converted into potentially harmful oxidation products (6,7).

Antioxidants do not act in isolation; together they form an intricate antioxidant network. For example, ascorbate can regenerate oxidized vitamin E and glutathione (GSH) can regenerate oxidized ascorbate, as demonstrated by the pioneering work of Hopkins and Morgan (8) and of Slater and coworkers (8,9).

The aim of the present study is to determine how quercetin fits in the antioxidant network. More specifically, we evaluated the interaction of oxidised quercetin with GSH or ascorbate.

Materials and Methods

Materials

Quercetin, reduced GSH, ascorbate, tyrosinase, horseradish peroxidase (HRP) and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma (St. Louis, MO, USA). Hydrogen peroxide was obtained from Merck (Darmstadt, Germany).

Methods

The oxidation of quercetin was performed at 37°C in a 143 mM phosphatebuffer pH 7.4 that contained 50 µM quercetin as well as 25 U/ml tyrosinase, or both 1.6 nM HRP and 33 µM H₂O₂, unless otherwise noted. The reactions were monitored spectrophotometrically as well as by high performance liquid chromatography (HPLC).

Quercetin consumption was determined at 400 nm, ascorbate consumption at 270 nm. Spectra were recorded with a scanspeed of 480 nm/min. Based on HPLC analysis it was concluded that in the incubations without GSH or ascorbate, the spectral changes were only due to the conversion of quercetin into its quinone QQ. Therefore, it is assumed that in these incubations QQ formation equalled quercetin consumption. In the incubations with ascorbate, spectral changes due to ascorbate oxidation could be found. When GSH was present, quercetin was converted only into two glutathionylquercetin adducts (GSQ), based on HPLC analysis. Therefore, it is assumed that in these incubations GSQ formation equalled quercetin consumption.

HPLC of the incubation mixtures was performed using a Supelcosil LC318 column (25 cm x 4.6 mm). Analytical separations were performed according to Awad *et al.* with minor modifications (10). The column was eluted with a mixture of distilled water containing 0.1% (v/v) trifluoroacetic acid and acetonitrile. From 0 to 10 minutes a linear gradient to 5% acetonitrile was applied, followed by a gradient from 10 to 25 minutes to 15% acetonitrile, from 25 to 28 minutes to 35% acetonitrile and from 28 to 40 minutes to 60% acetonitrile. Based on identical elution profiles and UV spectra compared to other studies, it was concluded that the first and lower GSQ peak is 8-GSQ and the second and higher peak is 6-GSQ. QQ was identified using the diode array spectra.

In order to quantify the GSQ adducts, 50 μM quercetin was oxidized by 25 U/ml tyrosinase in the presence of 100 μM GSH. After approximately half of the quercetin was oxidized (the exact degree of quercetin oxidation was determined spectrophotometrically), the solution was acidified in order to stop the reaction. The solution was directly injected on the HPLC system. The peak area of both GSQ adducts (6-GSQ and 8-GSQ) was determined (290 nm) and the concentration of the adducts was calculated assuming that (i) the consumed quercetin was converted quantitatively into GSQ under these conditions and that (ii) the response factor of both GSQ adducts was identical. QQ was identified and quantified using HPLC (290 nm) according to a procedure identical to that of GSQ, albeit that the incubation did not contain GSH. Here only one product was formed. Attempts to synthesize QQ and GSQ failed, probably due to instability of these compounds. Quercetin was quantified using external calibrators (290 nm).

Thiol content of the blood plasma was measured using DTNB. After preincubating 50 μM and 100 μM quercetin with respectively 25 U/ml and 100 U/ml tyrosinase, blood plasma of a healthy 29 year old female volunteer was added in a tenfold dilution with or without 100 μM ascorbate. This reaction mixture was incubated for another 5 min, after which 0.6 mM DTNB

was added. The reduction of DTNB was measured spectrophotometrically at 412 nm.

All experiments were performed, at least, in triplicate. Data are given as mean $\pm$ SD or as a typical example.

Results

Figure 3.1A shows the changes in the UV spectrum during the oxidation of 50 μM quercetin by the enzyme horseradish peroxidase (HRP).

Figure 3.1 UV scans of the incubation mixture containing 1.6 nM HRP, 50 μM quercetin and 33 μM H_2O_2 (A). The reaction was started by the addition of horseradish peroxidase. The same experiment was carried out in the presence of 40 μM ascorbate (B), 100 μM glutathione (C) and both 40 μM ascorbate and 100 μM glutathione (D). The UV scans were started 35 sec (1), 205 sec (2) or 370 sec (3) after the addition of HRP. A typical example is shown.

In the spectrum two isosbestic points are observed at 280 and 360 nm, suggesting the conversion of quercetin into one oxidation product. This is confirmed by HPLC analysis of the incubation mixture, showing the formation

of only one product (data not shown). Based on analogy with other studies (11,12) and on the recorded UV spectrum, it is concluded that the product is the *o*-quinone/quinonmethide of quercetin (QQ). The rate of quercetin oxidation and also of QQ accumulation is 15 ± 2 $\mu\text{M}/\text{min}$.

Addition of 40 μM ascorbate to the incubation mixture containing quercetin and peroxidase prevents the accumulation of QQ. The spectrum of the parent compound, quercetin, is preserved throughout the experiment. At the same time the ascorbate concentration decreases, as determined spectrophotometrically at 270 nm (Figure 3.1B). The rate of ascorbate oxidation is 3.4 ± 1.0 $\mu\text{M}/\text{min}$. In the absence of quercetin the decline in ascorbate concentration is lower (0.35 ± 0.00 $\mu\text{M}/\text{min}$). These data suggest that quercetin is oxidized into QQ and that the QQ formed is immediately regenerated at the expense of ascorbate. The net consumption of ascorbate that is induced by quercetin (3.1 ± 1.0 $\mu\text{M}/\text{min}$) is lower than the formation of QQ in the absence of ascorbate (15 ± 2 $\mu\text{M}/\text{min}$). Assuming that ascorbate and QQ react 1:1 and since no QQ accumulates in the incubation containing ascorbate, the difference in rate without ascorbate (15 ± 2 $\mu\text{M}/\text{min}$) and with ascorbate (3.4 ± 1.0 $\mu\text{M}/\text{min}$) can be attributed to inhibition of HRP by ascorbate. This inhibition depends on the concentration of ascorbate as can be inferred from the data depicted in Figure 3.2. An ascorbate concentration of 25 μM causes 25% inhibition while 40 μM causes 74% inhibition of HRP. Ascorbate concentrations above 40 μM are therefore not tested. It should be noted that ascorbate in a concentration of 25 μM cannot completely prevent a net consumption of quercetin after 60 seconds (Figure 3.2).

Figure 3.2 The decrease in 60 seconds of the concentration of quercetin and ascorbate determined spectrophotometrically. The incubation mixture contained 50 μM quercetin, 1.6 nM HRP and 33 μM H_2O_2 in the presence of 0, 25 and 40 μM ascorbate. The reaction was started by the addition of HRP. Spontaneous oxidation of ascorbate was neglectable, namely 0.35 $\mu\text{M}/\text{min}$ at an ascorbate concentration of 40 μM . All measurements were carried out, at least, in triplicate and expressed as mean $\pm$ SD.

Addition of 100 μM GSH to the incubation mixture containing quercetin and peroxidase gives two isosbestic points at 310 nm and 355 nm in the UV spectrum (Figure 3.1C). Since these isosbestic points differ from the isosbestic points observed during QQ formation, it is concluded that another product is formed in the presence of GSH. HPLC analysis demonstrates the formation of two products which can be identified as 6-glutathionylquercetin (6-GSQ) and 8-GSQ in accordance to Awad *et al.* (10) (Figure 3.3). During the incubation the ratio of the concentration of both adducts remains constant, i.e. 6-GSQ:8-GSQ=1.14:1. This indicates that the rate of formation of 6-GSQ is somewhat faster than the rate of the formation of 8-GSQ. The rate of quercetin consumption is not affected by the presence of 100 μM GSH (rate with GSH 15.0 ± 0.2 $\mu\text{M}/\text{min}$, rate without GSH 15 ± 2 $\mu\text{M}/\text{min}$), indicating that GSH does not inhibit HRP.

Figure 3.3 HPLC-chromatogram at $t=5$ min of the incubation mixture containing 1.6 nM HRP, 33 μM H_2O_2 , 50 μM quercetin and 100 μM GSH. The spectra of the GSQ adducts with a retention time of 7.0 min and 8.3 min are shown. Based on a comparable elution profile it was concluded that the peak at 7.0 min is 8-GSQ and the peak at 8.3 min is 6-GSQ. The reaction was started by the addition of HRP. A typical example is shown.

In vivo both GSH and ascorbate can react with oxidized quercetin. To determine the relative contribution of both reactions, experiments were performed including both GSH and ascorbate.

The UV spectrum of the incubation mixture containing HRP, 50 μM quercetin and both 100 μM GSH and 40 μM ascorbate showed the two isosbestic points at 310 and 355 nm that are indicative for the formation of GSQ (Figure 3.1D). HPLC analysis of the incubation mixture confirms this conclusion since only 6-GSQ and 8-GSQ are detected (data not shown). The rate of this GSQ formation ($7.2 \pm 1.7 \mu\text{M}/\text{min}$) is slower than that of GSQ formation when only GSH is present ($15.0 \pm 0.2 \mu\text{M}/\text{min}$). This demonstrates that the presence of ascorbate inhibits GSQ formation (Figure 3.4A). This inhibition by ascorbate can be achieved either (i) by competition with GSH for QQ or (ii) by inhibition of HRP. As mentioned above, 40 μM ascorbate reduces the enzyme activity to $3.4 \pm 1.0 \mu\text{M}/\text{min}$. The rate of GSQ formation in presence of both GSH and ascorbate ($7.2 \pm 1.7 \mu\text{M}/\text{min}$) is not lower than the enzyme activity with only ascorbate; it is even higher. This suggests that the inhibition of GSQ formation by 40 μM ascorbate proceeds by inhibition of HRP. The lower inhibition of HRP by ascorbate in the presence of GSH suggests that GSH can protect against the inhibition of HRP by ascorbate. Monitoring of ascorbate oxidation in the presence of GSH to quantify ascorbate mediated regeneration is not possible due to the reaction of GSH with oxidized ascorbate to form ascorbate (8). Based on the results it is concluded that 100 μM GSH reacts much faster than 40 μM ascorbate with QQ.

The inhibitory effect of ascorbate on HRP hampers a straightforward interpretation of our results. Moreover, only relatively low ascorbate concentrations compared to the GSH concentration can be used in the experiments. We therefore tested another oxidizing enzyme, i.e. tyrosinase.

As depicted in Figure 3.4B, tyrosinase is also capable of oxidizing quercetin into QQ. Ascorbate in a concentration of 40 μM prevents the apparent quercetin consumption for the greater part, confirming that ascorbate is capable of regenerating quercetin out of QQ. Ascorbate oxidation observed at 270 nm demonstrates that inhibition of tyrosinase by 40 μM ascorbate is much lower than that of HRP (figure not shown).

The presence of GSH during the tyrosinase mediated quercetin oxidation resulted in the formation of 6-GSQ and 8-GSQ in the same ratio as with HRP, based on the two isosbestic points at 310 nm and 355 nm as well as on HPLC analysis. The rate of quercetin consumption in the presence of GSH ($6.6 \pm 0.6 \mu\text{M}/\text{min}$) is equal to that in the absence of GSH ($5.9 \pm 0.4 \mu\text{M}/\text{min}$), suggesting that all the QQ is converted into GSQ and that GSH also does not inhibit tyrosinase.

Figure 3.4 The decrease in 60 seconds of the quercetin (Q) concentration, determined spectrophotometrically. The incubation mixture contained 50 μM quercetin and (A) 1.6 nM HRP and 33 μM H₂O₂ or (B) 25 U/ml tyrosinase in the presence of either 40 μM ascorbate (Asc), 100 μM glutathione (GSH) or both 40 μM ascorbate and 100 μM glutathione. All measurements were carried out in triplicate and expressed as mean ± SD.

Addition of both 40 μM ascorbate and 100 μM GSH together to quercetin and tyrosinase again results in the formation of GSQ. Contrary to the experiments with HRP, no significant difference in the rate of GSQ formation can be seen between the incubation without (6.6±0.6 μM/min) or with (5.4±0.9 μM/min) ascorbate. This again indicates that ascorbate does not affect tyrosinase activity substantially. Therefore, experiments with higher ascorbate concentrations were performed in order to further examine the competition between GSH and ascorbate for QQ.

Ascorbate at a concentration of 100 μM inhibits GSQ formation by 11%. This implies that GSH (the concentration is 100 μM) reacts at least 10 times faster with QQ than ascorbate. Increasing the ascorbate concentration up to 1 mM results in an inhibition of GSQ formation of 30% (Figure 3.5). This suggests that GSH reacts at least 20 times faster with QQ than ascorbate. Since we expect that the major part of the effect of ascorbate is due to inhibition of tyrosinase, the reaction rate of QQ with GSH surpasses that of QQ with ascorbate by far.

In blood plasma GSH is practically absent and ascorbate concentrations are 40 to 60 μM (normal range). Preincubation of blood plasma with 50 μM quercetin and 25 U/ml tyrosinase causes a reduction of the protein thiol content of $25 \pm 6\%$. This reduction is $50 \pm 11\%$ when the blood plasma is preincubated with 100 μM quercetin and 100 U/ml tyrosinase. Thiol consumption after addition of 100 μM ascorbate to the incubation with 50 μM or 100 μM quercetin is $27 \pm 4\%$ or $46 \pm 3\%$ respectively. Apparently, protein thiol consumption is not affected by the presence of ascorbate, implying that ascorbate does not protect protein thiol groups against QQ.

Figure 3.5 Inhibition by ascorbate of GSQ formation. Quercetin at a concentration of 50 μM was oxidised by either 1.6 nM HRP and 33 μM H_2O_2 (A) or by 25 U/ml tyrosinase (B-D). The concentration of ascorbate was 40 μM (A-B), 100 μM (C) or 1 mM (D). The incubation mixture was analysed 5 minutes after the incubation was started by the addition of the enzyme. GSQ formation (the sum of 6-GSQ and 8-GSQ) was quantified using HPLC. The inhibition by ascorbate was calculated by relating GSQ formation in the presence of ascorbate to that in the absence of ascorbate. Experiments were carried out, at least, in triplicate and expressed as mean $\pm$ SD.

Discussion

Free radical scavengers become oxidised when they exert their actual antioxidant effect. This formation of oxidised antioxidants is considered to be an important mechanism for antioxidant toxicity (13). Two electron oxidation of the flavonoid quercetin yields an oxidation product, denoted as QQ, that has four tautomeric forms, i.e. an *ortho*-quinone and three quinonmethides (11,14,15) (Figure 3.6). Oxidised antioxidants can be recycled in an interplay with other antioxidants such as ascorbate and GSH, called antioxidant networking (16,17). In this study we investigated the reaction of QQ with GSH or ascorbate.

The results of this study show that ascorbate recycles QQ to the parent compound quercetin, while GSH forms two adducts with QQ, i.e. 6-GSQ and 8-GSQ (Figure 3.6). In the present study it is shown that GSH reacts much faster than ascorbate with QQ. In other words, when GSH is present QQ will react with GSH.

Figure 3.6 Interplay between quercetin and ascorbate or GSH.

When quercetin becomes oxidized, an *o*-quinone/quinonmethide, referred to as QQ, can be formed. In the figure the 4 tautomeric forms of QQ are given. Ascorbate can recycle QQ to quercetin. During this reaction ascorbate becomes oxidized giving DHA (dehydroascorbate). QQ can also react with GSH, thereby forming 6-GSQ as well as 8-GSQ. QQ reacts much faster with GSH than with ascorbate.

In vivo most cells and body fluids contain relatively high levels of GSH. Under these circumstances GSH will capture QQ resulting in the formation of GSQ. This will be the case in epithelial lining fluid (ELF) with GSH and ascorbate concentrations that are identical to those initially used in the present study, i.e. 100 μM GSH and 40 μM ascorbate (18,19). In most cells the GSH concentration is higher, up to 1 mM in erythrocytes and up to 10 mM in hepatocytes (19,20). Furthermore, the formation of GSQ might be further enhanced by the presence of the glutathione-S-transferases, enzymes that catalyse the reaction of GSH with electrophiles such as QQ (21). This means that in most body compartments GSH will react with the formed QQ, resulting in the formation of GSQ adducts.

In blood plasma GSH is practically absent while the concentration of ascorbate is 40 to 60 μM (22). These conditions suggest that in blood ascorbate might react with QQ, thereby regenerating quercetin so that the flavonoid becomes available again for the antioxidant network. However, in blood plasma ascorbate has to compete with protein thiols. Addition of ascorbate (100 μM) does not alter the reduction of thiol content of blood plasma caused by preincubation with QQ, implying that QQ will react rather with the protein thiols than with ascorbate.

Recently, Van Zanden *et al.* (23) found that QQ arylates cysteine 47 of glutathione-S-transferase-P1-1, thereby inhibiting this isoenzyme. Not only GSH but remarkably, also ascorbate was reported to prevent this inhibition. This protective effect of ascorbate might have been due to the high and non-physiological concentration of ascorbate (1 mM) that was used in this study or to a relatively low reactivity of the thiol group of cysteine 47 towards QQ.

A large body of evidence demonstrates that quercetin can protect against oxidative stress by efficiently scavenging free radicals (24). During this "protection" the antioxidant is converted into reactive products. The present study indicates that in this way oxidative damage is shifted toward thiol arylation. Ascorbate cannot prevent this thiol arylation. We have previously shown that quinones similar to the oxidation product of quercetin can reduce the thiol content of microsomes and inactivate vital enzymes that contain critical thiolgroups (6). Interestingly, Yen *et al.* (25) recently reported that treatment of lymphocytes with quercetin reduced the protein thiol content of the cells. This can also be attributed to oxidation products such as QQ formed during the aerobic incubation of the cells.

In summary, when GSH is present oxidised quercetin reacts with GSH. This reaction however eliminates both the flavonoid and GSH from the antioxidant network. When GSH is absent, protein thiols are arylated by the oxidised antioxidant. Thiol arylation by quinones and quinonmethides can impair several vital enzymes. This implies that the product formed when quercetin

displays its antioxidant scavenging effect is toxic in the absence of GSH. Therefore, an adequate GSH level should be maintained when quercetin is supplemented.

References

1. Repine JE, Bast A and Lankhorst I. Oxidative stress in chronic obstructive pulmonary disease. Oxidative Stress Study Group. *Am J Respir Crit Care Med* 1997;156:341-357.
2. Rosen P, Nawroth PP, King G, Moller W, Tritschler HJ and Packer L. The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes Metab Res Rev* 2001;17:189-212.
3. Parthasarathy S, Khan Merchant N, Penumetcha M and Santanam N. Oxidative stress in cardiovascular disease. *J Nucl Cardiol* 2001;8:379-389.
4. Block G, Patterson B and Subar A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr Cancer* 1992;18:1-29.
5. Diplock AT. Antioxidants and disease prevention. *Mol Aspects Med* 1994;15:293-376.
6. Boots AW, Haenen GR, den Hartog GJ and Bast A. Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants. *Biochim Biophys Acta* 2002;1583:279-284.
7. Ito S, Kato T and Fujita K. Covalent binding of catechols to proteins through the sulphhydryl group. *Biochem Pharmacol* 1988;37:1707-1710.
8. Hopkins FG and Morgan JM. Some relations between ascorbic acid and glutathione. *Biochem J* 1936;30:1446-1462.
9. Packer JE, Slater TF and Willson RL. Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* 1979;278:737-738.
10. Awad HM, Boersma MG, Boeren S, van Bladeren PJ, Vervoort J and Rietjens IM. Structure-activity study on the quinone/quinone methide chemistry of flavonoids. *Chem Res Toxicol* 2001;14:398-408.
11. Awad HM, Boersma MG, Vervoort J and Rietjens IM. Peroxidase-catalyzed formation of quercetin quinone methide-glutathione adducts. *Arch Biochem Biophys* 2000;378:224-233.
12. Metodiewa D, Jaiswal AK, Cenas N, Dickanait E and Segura Aguilar J. Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. *Free Radical Biol Med* 1999;26:107-116.
13. Bast A and Haenen GR. The toxicity of antioxidants and their metabolites. *Environmental Toxicology and Pharmacology* 2002;11:251-258.
14. Jorgensen LV, Cornett C, Justesen U, Skibsted LH and Dragsted LO. Two-electron electrochemical oxidation of quercetin and kaempferol changes only the flavonoid C-ring. *Free Radic Res* 1998;29:339-350.
15. Penning TM, Burczynski ME, Hung CF, McCoull KD, Palackal NT and Tsuruda LS. Dihydrodiol dehydrogenases and polycyclic aromatic hydrocarbon activation: generation of reactive and redox active o-quinones. *Chem Res Toxicol* 1999;12:1-18.
16. Bast A, Haenen GR and Doelman CJ. Oxidants and antioxidants: state of the art. *Am J Med* 1991;91:2s-13s.
17. Meister A. Glutathione-ascorbic acid antioxidant system in animals. *J Biol Chem* 1994;269:9397-9400.
18. Bast A. Oxidants and antioxidants in the lung. In *COPD: diagnosis and treatment*. Eds. van Herwaarden C.L.A., Repine J.E., Vermeire P., van Weel C. Excerpta Medica Amsterdam 1996;33-39.
19. Kelly FJ, Cotgrove M and Mudway IS. Respiratory tract lining fluid antioxidants: the first line of defence against gaseous pollutants. *Cent Eur J Public Health* 1996;4 Suppl:11-14.
20. Ponsoda X, Jover R, Gomez Lechon MJ, Fabra R, Trullenque R and Castell JV. Intracellular glutathione in human hepatocytes incubated with S-adenosyl-L-methionine and GSH-depleting drugs. *Toxicology* 1991;70:293-302.
21. Mannervik B and Danielson UH. Glutathione transferases--structure and catalytic activity. *CRC Crit Rev Biochem* 1988;23:283-337.
22. Brubacher D, Moser U and Jordan P. Vitamin C concentrations in plasma as a function of intake: a meta-analysis. *Int J Vitam Nutr Res* 2000;70:226-237.

23. van Zanden JJ, Ben Hamman O, van Iersel ML, Boeren S, Cnubben NH, Lo Bello M, Vervoort J, van Bladeren PJ and Rietjens IM. Inhibition of human glutathione S-transferase P1-1 by the flavonoid quercetin. *Chem Biol Interact* 2003;145:139-148.
24. Heijnen CG, Haenen GR, Vekemans JA and Bast A. Peroxynitrite scavenging of flavonoids: structure activity relationship. *Environmental Toxicology and Pharmacology* 2001;10:199-206.
25. Yen GC, Duh PD, Tsai HL and Huang SL. Pro-oxidative properties of flavonoids in human lymphocytes. *Biosci Biotechnol Biochem* 2003;67:1215-1222.

A large, light gray, stylized letter 'Q' that serves as a background for the slide content.

4

No role of DT-diaphorase (NQO1) in
the protection against oxidized
quercetin

AW Boots, A Bast, GRMM Haenen

FEBS letters 2005;579:677-682

Abstract

Background

Quercetin is one of the most studied alimentary antioxidants. During its antioxidant activity quercetin becomes oxidized into its ortho-quinone/quinone methide, denoted as QQ. QQ is toxic since it is highly reactive towards thiols. DT-diaphorase (NQO1) might protect against QQ toxicity by reducing QQ to quercetin. However, conflicting data have been reported. The aim of the present study is to elucidate the role of DT-diaphorase in the protection against QQ-mediated thiol reactivity.

Methods

Several competition experiments have been performed with isolated compounds as well as with liver cytosol. The thiol reactivity of QQ, and the influence of various reactants, has been assessed using blood plasma. All experiments were monitored spectrophotometrically as well as by high performance liquid chromatography (HPLC).

Results

It was found that QQ is indeed a substrate for DT-diaphorase. However, compared to its reaction with the enzyme, QQ reacted much faster with either glutathione or protein thiols. Moreover, even ascorbate, known to react much slower with QQ than glutathione (GSH), reacted faster with the quinine than DT-diaphorase did.

Conclusions

The results of the present study indicate that QQ will primarily react with thiols, i.e. GSH or when GSH is depleted with protein thiols. Therefore, it is concluded that DT-diaphorase has no role in the protection against QQ.

Introduction

One of the most prominent dietary antioxidants is quercetin, a flavonoid that is nearly ubiquitous in foods, including vegetables, tea, fruit and wine (1,2) as well as in countless food supplements. Quercetin can scavenge highly reactive species such as peroxynitrite and the hydroxyl radical (3,4) and this activity is involved in the beneficial health effect of quercetin. During this antioxidant activity, quercetin becomes oxidized to its ortho-quinone/quinone methide, denoted as QQ (5,6). QQ can exert toxic effects since it is highly reactive towards thiols.

Protection against quinone-mediated toxicity might be provided by DT-diaphorase [NAD(P):H-(quinone acceptor) oxidoreductase NQO1, EC 1.6.99.2] (7). This is a cytosolic enzyme that acts as a two-electron reductase. It has a flavin-adenine dinucleotide (FAD) prosthetic group in the active site that directly donates two electrons to reduce a quinone to its corresponding hydroquinone (8). A tyrosine residue in the active site is involved in the reduction of para-quinones, the primary substrates of DT-diaphorase (9). The reduction by DT-diaphorase is often regarded as the first line of defence against quinone-mediated toxicity (7,10). Data on the role of DT-diaphorase in the protection against the ortho-quinone/quinone methide QQ that is generated when quercetin scavenges highly reactive pro-oxidants are conflicting, since DT-diaphorase is reported to enhance (11) or protect against (12) this toxicity.

The aim of the present study is to elucidate the role of DT-diaphorase in the regeneration of QQ. To this end the reaction rate of QQ with DT-diaphorase is compared to the reaction rate of QQ with ascorbate and glutathione (GSH), endogenous compounds known to react readily with QQ. Experiments are performed with isolated compounds as well as with human liver cytosol.

Materials and methods

Materials

Quercetin, reduced glutathione (GSH), ascorbate, tyrosinase, menadione and DT-diaphorase (E.C. 1.6.99.2, also called [NAD(P):H-(quinone acceptor) oxidoreductase or NQO1] were purchased from Sigma (St. Louis, MO, USA). Reduced β -Nicotinamide adenine dinucleotide disodium salt (NADH) and 5',5'-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from ICN Biomedicals (Ohio, USA). Human liver was obtained from liver tissue that was removed by surgical excision of a liver tumour from 40 to 60 years old

patients. A “healthy” part of the excised material that did not contain any tumour cells was used. The livers were removed according to the standard surgical procedure and the use of the material is in accordance with the medical and ethical guidelines of the academic hospital of Maastricht. Liver cytosol was prepared as described previously (13). Blood plasma was obtained from two healthy volunteers (age 27 and 30) again in accordance with the medical and ethical guidelines of the Academic Hospital of Maastricht.

Methods

All incubations were performed at 37°C in a 143 mM phosphatebuffer pH 257.4 and were monitored spectrophotometrically as well as by high performance liquid chromatography (HPLC).

QQ was formed by oxidizing 100 μ M quercetin with 25 U/ml tyrosinase. The oxidation product that accumulated during 5 minutes oxidation of quercetin by tyrosinase appeared not to be a substrate for DT-diaphorase (data not shown). The product also did not react with GSH, indicating that the product formed is not QQ, since GSH is known to readily react with QQ (14,15). HPLC analysis revealed that two products accumulated after the oxidation of quercetin with tyrosinase (6-QOH and 8-QOH), denoted as QOH. It should be noted that in some studies (11) it was stated that preformed QQ is used, which actually is QOH.

Due to this relative instability of QQ, QQ had to be generated in situ for each experiment in the presence of the reactants studied. DT-diaphorase activity was studied at an enzyme concentration of 10 U/ml plus 0.2 mM NADH and quantified by determining the NADH decrease at 240 nm. Quercetin consumption was determined spectrophotometrically at 400 nm and ascorbate consumption at 270 nm. Spectra were recorded with a scanspeed of 480 nm/min.

HPLC of the incubation mixtures was performed using a Supelcosil LC318 column (25 cm x 4.6 mm). The column was eluted isocratically with water containing 0.1% (v/v) trifluoroacetic acid and 5% acetonitrile during 5 minutes, followed by a linear gradient to 20% acetonitrile from 5 to 10 minutes and to 30% acetonitrile from 10 to 16 minutes.

Quercetin was quantified using external calibrators (detection at 290 nm). To quantify the glutathionyl adducts, 50 μ M quercetin was oxidized by 25 U/ml tyrosinase in the presence of 100 μ M GSH. After approximately half of the quercetin was oxidized based on spectrophotometrical analysis (the exact degree of quercetin oxidation was determined using HPLC), the solution was directly injected on the HPLC system. The peak area of both glutathionyl adducts (6-GSQ and 8-GSQ) was determined (detection at 290 nm) and the concentration of the adducts was calculated assuming that

(i) all quercetin consumed is converted quantitatively into GSQ under these conditions and (ii) the response factor of both glutathionyl adducts is identical. Comparable to the strategy used for the glutathionyl adducts, the decomposition products of QQ (QOH) were quantified using HPLC (detection at 290 nm). Attempts to synthesize QOH and GSQ failed, probably due to the relative instability of these compounds. QQ formation was quantified by trapping QQ with GSH and determination of GSQ.

In the experiments performed with liver cytosol and blood plasma, the DT-diaphorase activity was determined by adding 0.2 mM NADH and 50 μ M of the substrate menadione to the cytosol and monitoring spectrophotometrically the NADH consumption due to DT-diaphorase activity at 340 nm. As reference the same incubation without menadione was used. The GSH concentration of the liver cytosol was measured by adding 0.3 mM DTNB and determining the absorption of the incubation mixture at 412 nm. In the reference no DTNB was added.

NADH consumption due to DT-diaphorase reduction of oxidized quercetin was quantified by monitoring spectrophotometrically the decrease at 340 nm in the incubation mixture containing liver cytosol, 0.2 mM NADH, 50 μ M quercetin and 25 U/ml tyrosinase. In the reference quercetin was omitted. GSH consumption was measured by adding 0.3 mM DTNB to 1 ml of this incubation mixture after 1 minute incubation and the absorption at 412 nm was immediately determined. In the blanc DTNB was replaced by buffer and the results were compared to an incubation that did not contain quercetin. Quercetin and GSH consumption as well as QOH and GSQ formation were also determined using HPLC as described above. To this end, all incubation mixtures were deproteinated by adding an equal volume of 10% TCA to the sample before vortexing and centrifuging it for 1 minute at 14.000 rpm. The supernatant was injected on the HPLC system.

Protein thiol arylation by QQ was assessed in blood plasma. The plasma was incubated in a 10-fold dilution with 50 μ M quercetin with or without 25 U/ml tyrosinase in either in the presence or the absence of 10 U/ml DT-diaphorase plus 0.2 mM NADH. After 5 minutes incubation 0.6 mM DTNB was added to this reaction mixture. The reduction of DTNB was measured spectrophotometrically at 412 nm. In the blanc DTNB was replaced by buffer.

Formation of glutathionyl adducts and NADH or ascorbate consumption showed that addition of ascorbate or DT-diaphorase plus NADH results in a small inhibition of the formation of quercetin by tyrosinase as also reported previously for ascorbate (14). DT-diaphorase mediated NADH reduction is constant in time in the experiments. This indicates that there is no relevant inhibition by the compounds under our experimental conditions.

All experiments were performed, at least, in triplicate. Data are given as mean or as a typical example. The SD of all results was usually less than 5%

and always less than 10%. Statistical analysis was performed using Student's t-test.

Results

The ability of DT-diaphorase to reduce the quinone/quinone methide of quercetin (QQ) was investigated. QQ was generated by the oxidation of 50 μM quercetin with 25 U/ml tyrosinase.

Due to the reactivity of QQ, QQ was generated in situ in the presence of the reactants studied. It appeared that QQ is a substrate for DT-diaphorase (10 U/ml) based on the NADH consumption (Figure 4.1C). Menadione, a para-quinone and one of the most studied substrates for DT-diaphorase, was used as a control (Figure 4.1A). Ascorbate (100 μM) had no effect on the reaction of DT-diaphorase (10 U/ml) with menadione (50 μM) (Figure 4.1B). Ascorbate almost completely prevented the DT-diaphorase catalysed consumption of NADH by QQ, indicating that ascorbate reacts faster with QQ than DT-diaphorase does (Figure 4.1D).

GSH (100 μM) reacts with QQ forming glutathionyl adducts, as is seen in the spectral changes that occur in the incubation mixture containing quercetin, tyrosinase and GSH (Figure 4.2C). The increase of the absorption at 340 nm and the isosbestic points at 310 nm and 355 nm can be attributed to the formation of the glutathionyl adducts (14). When DT-diaphorase is also present, again the spectral changes indicative for the formation of glutathionyl adducts occur (Figure 4.2D). The formation of GSQ was confirmed using HPLC analysis, which also shows that DT-diaphorase could not compete with GSH for QQ (Figure 4.3).

Addition of GSH to the incubation mixture containing menadione, DT-diaphorase and NADH had no effect on the DT-diaphorase mediated reduction of menadione (data not shown). These results made us conclude that the reaction of QQ with GSH exceeds that of QQ with DT-diaphorase by far (Figure 4.2).

Figure 4.1 Repetitive UV scans of the incubation mixture containing 0.2 mM NADH, 10 U/ml DT-diaphorase and 50 μ M menadione in the absence (A) or presence (B) of 100 μ M ascorbate. The same experiment was also performed with 50 μ M quercetin, instead of menadione, in the presence of 25 U/ml tyrosinase and in the absence (C) or presence (D) of 100 μ M ascorbate. The reactions were started by the addition of DT-diaphorase (A and B) or a mixture of DT-diaphorase and tyrosinase (C and D). The UV scans were measured 0 (black), 3 (light grey) or 5 minutes (dark grey) after the addition of the enzyme(s). Scanspeed was 480 nm/min. A typical example is shown.

To assess the relative contribution of the reaction of QQ with each reactant studied, competition experiments were performed (Figure 4.3). In these experiments, QQ formation by tyrosinase was quantified by trapping QQ with GSH and determination of the amount of glutathionyl adducts formed.

Figure 4.2 Repetitive UV scans of the incubation mixture containing 50 μM quercetin and 25 U/ml tyrosinase (A). Further additions were 10 U/ml DT-diaphorase plus 0.2 mM NADH (B), 100 μM glutathione (C) and both 10 U/ml DT-diaphorase plus 0.2 mM NADH and 100 μM GSH (D). All reactions were started by the addition of a mixture of DT-diaphorase and tyrosinase. The UV scans were measured 0 (black), 3 (light grey) or 5 minutes (dark grey) after the addition of the enzyme mixture. Scanspeed was 480 nm/min. A typical example is shown.

When DT-diaphorase and NADH were added to the incubations (i.e. panel B and D), inserts are added to show the time dependent decrease of NADH (rate).

In the control experiment containing none of the reactants, all the QQ generated was converted into the decomposition products, QOH. Under the conditions used, addition of DT-diaphorase resulted in approximately 60% regeneration of quercetin out of QQ. With ascorbate, this regeneration was approximately 90%. By the combination of DT-diaphorase and ascorbate, all QQ was converted back into quercetin. In the presence of GSH, all the QQ was transformed into the glutathionyl adducts, irrespective of the presence of DT-diaphorase or ascorbate (Figure 4.3).

Figure 4.3 Influence of DT-diaphorase, ascorbate and glutathione on the products formed during the oxidation of quercetin by tyrosinase. Quercetin at a concentration of $50\ \mu\text{M}$ was oxidized by $25\ \text{U/ml}$ tyrosinase in the absence (upper row) or presence (lower row) of $100\ \mu\text{M}$ glutathione. Further additions were $10\ \text{U/ml}$ DT-diaphorase plus $0.2\ \text{mM}$ NADH (+ DTD), $100\ \mu\text{M}$ ascorbate (+ Asc) or the combination of $10\ \text{U/ml}$ DT-diaphorase plus $0.2\ \text{mM}$ NADH and $100\ \mu\text{M}$ ascorbate (+ DTD and Asc). In all incubations, the fraction of quercetin that either did not react (Q black) or after oxidation was regenerated (Q grey) was determined, as well as the formation of decomposition products (QOH) and of the glutathionyl adducts (GSQ). The incubation mixture was analysed 1 min after the incubation was started by the addition of tyrosinase. Quantification was performed using HPLC. Experiments were carried out, at least, in triplicate and expressed as means. The SD for all values is less than $3\ \mu\text{M}$ (not depicted). The extent of regeneration of all incubations was significantly different ($P < 0.05$).

In human liver cytosol, DT-diaphorase activity is $9.4 \pm 1.1\ \text{U/ml}$ and the GSH concentration is $4.7 \pm 0.5\ \text{mM}$. The spectral changes observed in the incubation mixture containing quercetin, tyrosinase and cytosol in the absence (Figure 4.4A) or presence of NADH (Figure 4.4B) are indicative for the formation of glutathionyl adducts. During these incubations, the quercetin consumed ($30 \pm 1\ \mu\text{M/min}$ in the absence and $32 \pm 2\ \mu\text{M/min}$ in the presence of NADH) equalled the GSH consumption ($33 \pm 2\ \mu\text{M/min}$ in the absence and $31 \pm 1\ \mu\text{M}$ in the presence of NADH) while the NADH consumption was negligible ($1 \pm 0.1\ \mu\text{M/min}$) (Figure 4.4, insert). This indicates that GSH is the primary target of QQ in the liver and that the role of DT-diaphorase in the reaction with oxidized quercetin is negligible.

In human blood plasma GSH is practically absent and DT-diaphorase activity was below $0.1\ \text{U/ml}$. Incubation of blood plasma with QQ resulted in a $61 \pm 8\%$ reduction of the protein thiol content (Figure 4.5). This thiol consumption did not change significantly when extra DT-diaphorase ($10\ \text{U/ml}$)

and NADH (0.2 mM) were added to the incubation and was $58\pm5\%$. Apparently, protein thiol consumption is not prevented by the addition of DT-diaphorase, implying that DT-diaphorase cannot protect protein thiols against QQ.

Figure 4.4 UV-scans of the reaction of QQ in human liver cytosol. The cytosol was diluted to give a final GSH concentration of 100 μM . QQ was generated in situ by adding 50 μM quercetin and 40 U/ml tyrosinase. In panel B, 0.2 mM NADH was added to promote DT-diaphorase activity. The reactions were started by the addition of tyrosinase. The UV scans were measured 0 (black), 3 (light grey) or 5 minutes (dark grey) after the addition of the enzyme. Scanspeed was 480 nm/min. A typical example is shown. The inserts show the quercetin (Q), GSH and NADH consumption during these incubations in $\mu\text{M}/\text{min}$. No substantial NADH consumption (measured at 340 nm) is observed, indicating that, in the liver, DT-diaphorase cannot compete with GSH for oxidized quercetin (QQ).

Figure 4.5 Thiol content of blood plasma after the following incubations: 10 U/ml DT-diaphorase plus 0.2 mM NADH (DTD), 50 μM quercetin plus 25 U/ml tyrosinase (QQ) or the combination of 10 U/ml DT-diaphorase plus 0.2 mM NADH and 50 μM quercetin plus 25 U/ml tyrosinase (QQ + DTD). Thiol content is expressed as percentage and relative to that of the control incubation. * $P<0.01$ compared to the control; # $P<0.01$ compared to the control and not significantly different from QQ.

Discussion

Quercetin is an alimentary antioxidant with an alleged positive health effect. Quercetin is able to scavenge all sorts of reactive species. This results in protection against the damage by these reactive species, but the price that is being paid is the accumulation of oxidized quercetin, e.g. QQ. Although the general reactivity of QQ, compared to e.g. $\text{OH}^\bullet$ or ONOOH , is relatively low, QQ displays a selective toxicity since it is highly reactive towards thiols, including GSH and protein thiols. So by quercetin administration, the general damage inflicted by oxidative stress tends to become focussed on cellular thiols (6).

DT-diaphorase can protect against quinone toxicity by converting a quinone into the corresponding hydroquinone. For QQ this would mean that DT-diaphorase converts it into quercetin, thereby not only preventing QQ toxicity but also regenerating the consumed antioxidant and thus boosting the antioxidant effect.

In the present study it was found that 100 μM ascorbate reacted faster with QQ than 10 U/ml DT-diaphorase. GSH (100 μM) reacts at least 1000 times as fast as ascorbate (100 μM) with QQ (14). Combining both findings made us conclude that 100 μM GSH reacts at least 1000 times faster with QQ than 10 U/ml DT-diaphorase does. This indicates that DT-diaphorase cannot compete with GSH in the reaction with QQ.

The activity of DT-diaphorase used in the present *in vitro* study (10 U/ml, i.e. an activity of 10.000 μM substrate/min) is in the same range as the enzyme activity we measured in human liver, i.e. 9.4 U/ml, and corresponds nicely with the enzyme activity reported in literature, i.e. 4-6 U/ml or 4.000-6.000 $\mu\text{M}/\text{min}$ in liver and lung (10,16). The GSH level is in most cells much higher than the 100 μM used in this *in vitro* study, e.g. the GSH concentration in the lung is ~ 1 mM (17) and in liver cytosol even 4.7 mM (this study). This means that in the liver GSH reacts at least 47.000 times as fast as DT-diaphorase with QQ. This strengthens the conclusion that the role of DT-diaphorase in the protection against QQ is negligible. Indeed, no DT-diaphorase activity was observed when QQ was formed in human liver cytosol (Figure 4.4). In blood plasma, where practically no GSH is present (18) and no DT-diaphorase activity could be detected, addition of DT-diaphorase (10 U/ml) did also not protect against protein thiol loss in blood plasma caused by QQ (Figure 4.5). This indicates that DT-diaphorase also cannot compete with protein thiols for QQ.

Gliszczynska-Swiglo et al. found that quercetin gave a similar reduction in cell proliferation ($\sim 20\%$) in wild type chinese hamster ovary (CHO) cells (DT-diaphorase activity 11 $\mu\text{M}\cdot\text{min}^{-1}$) as in CHO cells over-expressing DT-diaphorase (244 $\mu\text{M}\cdot\text{min}^{-1}$) (12). They observed that addition of

dicoumerol, an inhibitor of DT-diaphorase, increased the quercetin toxicity in both cell types (cell proliferation was reduced by ~ 70%). The observed lack of difference in quercetin toxicity between wild type cells and cells overexpressing DT-diaphorase made the authors conclude that even in wild type cells DT-diaphorase activity is sufficient for optimal protection against QQ toxicity. An alternative explanation, not considered by Gliszczynska-Swiglo et al., is that DT-diaphorase plays no role in quercetin toxicity. Opposite to the results of Gliszczynska-Swiglo et al., Metodiewa and co-workers observed a significant lower toxicity of quercetin in wild type CHO cells compared to CHO cells over-expressing DT-diaphorase (11). Also opposite to Gliszczynska-Swiglo et al., they found that dicoumerol reduced quercetin-induced toxicity. These conflicting results once again indicate that experiments in cells using “specific” inhibitors are not conclusive and fraught with artefacts.

Our results show that DT-diaphorase efficiently reacts with menadione and that both GSH and ascorbate do not compete with DT-diaphorase for menadione. Similarly, Gliszczynska-Swiglo et al. have shown that menadione toxicity was less in DT-diaphorase enriched cells compared to wild type cells (12). Dicoumerol abolished the protection observed in DT-diaphorase enriched cells against menadione toxicity. This indicates that for menadione toxicity DT-diaphorase might play a role.

The present study demonstrates that GSH reacts much faster than DT-diaphorase with QQ. Moreover, in most body compartments GSH levels are relatively high and DT-diaphorase activities are in the same order of magnitude as the levels used in our experiments. In blood plasma, where GSH is practically absent, protein thiols are the primary reactants of QQ and DT-diaphorase (10 U/ml added) could not protect against the arylation of these protein thiols by QQ (Figure 4.5). Apparently, DT-diaphorase reacts sluggishly with QQ compared to the fast reaction of QQ with protein thiols. Besides DT-diaphorase, also ascorbate cannot protect against protein thiol arylation by QQ [12].

It has been reported that treatment of lymphocytes with quercetin reduces the protein thiol content of the cells (19). Moreover, Walle et al. (2003) has shown that quercetin covalently binds to cellular DNA and proteins in human cell lines (20). As also proposed by these authors (20), conversion of quercetin into QQ is the primary step involved in the covalent binding to proteins (20).

Based on literature and the data of the present study the scheme depicted in Figure 4.6 has been constructed. During its antioxidant activity quercetin becomes oxidized into QQ, which will react with thiols, i.e. with GSH or, when GSH is depleted, with protein thiols. The results of the present study show that DT-diaphorase has no role in the protection against QQ.

Figure 4.6 Reactions of the oxidation product of quercetin. When quercetin is oxidized, an *o*-quinone/quinonmethide (referred to as QQ) is formed. In the figure only one of the four tautomeric forms of QQ is given. Quercetin can be regenerated from QQ by ascorbate, resulting in the formation of dehydroascorbate (DHA), or by DT-diaphorase (DTD). The latter regenerates quercetin using NADH as cofactor. QQ can also react with glutathione (GSH), thereby forming 6-glutathionylquercetin (6-GSQ) and 8-glutathionylquercetin (8-GSQ).

References

1. Hertog MG, Hollman PC, Katan MB and Kromhout D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr Cancer* 1993;20:21-29.
2. Beecher GR, Warden BA and Merken H. Analysis of tea polyphenols. *Proc Soc Exp Biol Med* 1999;220:267-270.
3. Bors W, Michel C and Saran M. Flavonoid antioxidants: rate constants for reactions with oxygen radicals. *Methods Enzymol* 1994;234:420-429.
4. Heijnen CG, Haenen GR, van Acker FA, van der Vijgh WJ and Bast A. Flavonoids as peroxynitrite scavengers: the role of the hydroxyl groups. *Toxicol In Vitro* 2001;15:3-6.
5. Awad HM, Boersma MG, Boeren S, van Bladeren PJ, Vervoort J and Rietjens IM. Structure-activity study on the quinone/quinone methide chemistry of flavonoids. *Chem Res Toxicol* 2001;14:398-408.
6. Boots AW, Haenen GR, den Hartog GJ and Bast A. Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants. *Biochim Biophys Acta* 2002;1583:279-284.
7. Cadenas E. Antioxidant and prooxidant functions of DT-diaphorase in quinone metabolism. *Biochem Pharmacol* 1995;49:127-140.
8. Skelly JV, Sanderson MR, Suter DA, Baumann U, Read MA, Gregory DS, Bennett M, Hobbs SM and Neidle S. Crystal structure of human DT-diaphorase: a model for interaction with the cytotoxic prodrug 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954). *J Med Chem* 1999;42:4325-4330.
9. Chen S, Wu K, Zhang D, Sherman M, Knox R and Yang CS. Molecular characterization of binding of substrates and inhibitors to DT-diaphorase: combined approach involving site-directed mutagenesis, inhibitor-binding analysis, and computer modeling. *Mol Pharmacol* 1999;56:272-278.
10. Malkinson AM, Siegel D, Forrest GL, Gazdar AF, Oie HK, Chan DC, Bunn PA, Mabry M, Dykes DJ, Harrison SD et al. Elevated DT-diaphorase activity and messenger RNA content in human non-small cell lung carcinoma: relationship to the response of lung tumor xenografts to mitomycin C. *Cancer Res* 1992;52:4752-4757.
11. Metodiewa D, Jaiswal AK, Cenas N, Dickanaité E and Segura-Aguilar J. Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. *Free Radical Biol Med* 1999;26:107-116.
12. Gliszczynska Swiglo A, van der Woude H, de Haan L, Tyrakowska B, Aarts JM and Rietjens IM. The role of quinone reductase (NQO1) and quinone chemistry in quercetin cytotoxicity. *Toxicol In Vitro* 2003;17:423-431.
13. Haenen GR and Bast A. Protection against lipid peroxidation by a microsomal glutathione-dependent labile factor. *FEBS Lett* 1983;159:24-28.
14. Boots AW, Kubben N, Haenen GR and Bast A. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. *Biochem Biophys Res Commun* 2003;308:560-565.
15. Awad HM, Boersma MG, Boeren S, van Bladeren PJ, Vervoort J and Rietjens IM. The regioselectivity of glutathione adduct formation with flavonoid quinone/quinone methides is pH-dependent. *Chem Res Toxicol* 2002;15:343-351.
16. Schlager JJ and Powis G. Cytosolic NAD(P)H:(quinone-acceptor)oxidoreductase in human normal and tumor tissue: effects of cigarette smoking and alcohol. *Int J Cancer* 1990;45:403-409.
17. Rowell NP, McCready VR, Cronin B, Pepper J, Higley B, Burke JF and Tyrrell DA. ⁹⁹Tcm-labelled meso-HMPAO and glutathione content of human lung tumours. *Nucl Med Commun* 1989;10:503-508.
18. Jones DP, Carlson JL, Samiec PS, Sternberg P, Jr, Mody VC, Jr, Reed RL and Brown LA. Glutathione measurement in human plasma. Evaluation of sample collection, storage and derivatization conditions for analysis of dansyl derivatives by HPLC. *Clin Chim Acta* 1998;275:175-184.

19. Yen GC, Duh PD, Tsai HL and Haung SL. Pro-oxidative properties of flavonoids in human lymphocytes. *Biosci Biotechnol Biochem* 2003;67:1215-1222.
20. Walle T, Vincent TS and Walle UK. Evidence of covalent binding of the dietary flavonoid quercetin to DNA and protein in human intestinal and hepatic cells. *Biochem Pharmacol* 2003;65:1603-1610.

5

The reversibility of the glutathionyl-
quercetin adduct spreads oxidized
quercetin-induced toxicity

AW Boots, JM Balk, A Bast, GRMM Haenen

Biochemical and Biophysical Research Communications 2005;338:923-929

Abstract

Background

Quercetin is one of the most prominent dietary antioxidants. During its antioxidant activity, quercetin becomes oxidized into its *o*-quinone/quinone methide QQ. QQ is toxic since it instantaneously reacts with thiols of e.g. proteins. In cells, QQ will initially form an adduct with glutathione (GSH), giving glutathionyl-quercetin adducts, denoted as GSQ. Little is known about the fate of GSQ once formed in a biological system. Therefore, the aim of the present study is to investigate the stability of GSQ and to examine the exchange of GSH in GSQ for other thiols.

Methods

All incubations were monitored spectrophotometrically as well as by high performance liquid chromatography (HPLC).

Results

We have found that GSQ is not stable; it dissociated continuously into GSH and QQ with a half life of 2 minutes. Surprisingly, GSQ incubated with 2-mercapto-ethanol (MSH), a far less reactive thiol, resulted in the conversion of GSQ into the MSH-adduct MSQ. A similar conversion of GSQ into relatively stable protein thiol-quercetin adducts is expected. With the dithiol dihydrolipoic acid ($L(SH)_2$), quercetin was formed out of GSQ.

Conclusions

The results of the present study indicate that GSQ might act as transport and storage of QQ. In that way, the initially highly focussed toxicity of QQ is dispersed by the formation of GSQ that finally spreads QQ-induced toxicity, probably even over cells.

Introduction

Quercetin is the most studied flavonoid and is ubiquitous in our diet, especially in vegetables, tea, fruit and wine (1,2). Moreover, quercetin is a major constituent of countless food supplements and other nutraceuticals. Quercetin has gained special attention since it is an antioxidant which very efficiently scavenges highly reactive biological species such as peroxynitrite (3,4) and the hydroxyl radical (5,6).

Oxidative stress is defined as an imbalance between the formation of highly reactive species and the protection against these species by antioxidants. This condition is implicated in the etiology of various diseases including chronic obstructive pulmonary disease (COPD) (7,8), sarcoidosis (9), fibrosis (10), diabetes (11,12) and cancer (13,14). An increased intake of dietary antioxidants is expected to reduce oxidative stress. Quercetin seems to be an excellent candidate to empower the endogenous antioxidant defence system.

During the scavenging of highly reactive species, an antioxidant is converted into an oxidation product. The oxidation product often is relatively stable but it usually retains a part of the reactivity of the species it has scavenged (15). Oxidation of quercetin results in the formation of an *o*-quinone/quinone methide, denoted as QQ (16,17). QQ is highly reactive towards thiols groups and is therefore toxic (18). Glutathione (GSH) is the primary target of QQ in cells (17). GSH rapidly reacts with QQ, resulting in the formation of two glutathionyl-quercetin adducts, i.e. 6-glutathionyl-quercetin (6-GSQ) and 8-glutathionyl-quercetin (8-GSQ) (16,17,19), together denoted as GSQ. Little is known about the fate of these adducts once formed in a biological system. This prompted us to study the stability of GSQ and to examine the exchange of GSH in GSQ for other thiols.

Materials and methods

Materials

Quercetin, reduced GSH, tyrosinase, 2-mercapto-ethanol (MSH), dihydroic lipoic acid (L(SH)₂) and 1-chloro-2,4-dinitrobenzene (CDNB) were all purchased from Sigma (St. Louis, MO, USA). Sep-Pak C18 cartridges were obtained from Waters (Ireland).

Methods

All incubations were performed at 37°C in a 143 mM phosphatebuffer pH 7.4 and were monitored spectrophotometrically as well as by high performance liquid chromatography (HPLC).

Formation of QQ

Due to the relative instability of QQ, it had to be generated *in situ* for each experiment. The oxidation of quercetin leading to the formation of its *o*-quinone methide (QQ) was performed by adding 100 U/ml tyrosinase to 100 μ M quercetin. After 30 seconds, the solution was filtered using a Sep-Pak C18 cartridge in order to stop the reaction by removing the enzyme. The concentration of QQ was calculated as previously described (17). QQ generated in this way contained not oxidized quercetin. In the reactions of QQ studied, quercetin did not interfere.

Synthesis of GSQ, MSQ and QOH

GSQ, MSQ and QOH also had to be synthesized *in situ* due to limited stability. Synthesis of GSQ was performed by adding 10 U/ml tyrosinase to 100 μ M quercetin in the presence of 120 μ M GSH. After approximately 80% of the quercetin was oxidized, based on spectrophotometrical analysis (the exact degree of quercetin oxidation was determined using HPLC), the solution was directly filtered using a Sep-Pak C18 cartridge in order to stop the reaction and purify the GSQ formed during this reaction. The GSQ concentration was quantified as previously described (17). The formed GSQ contained a substantial amount of quercetin and the excess of GSH. The GSH present in GSQ might have an effect on the rate constant obtained with relatively low concentrations of MSH and $L(SH)_2$. To reduce possible interference, these experiments were performed with concentrations of MSH and $L(SH)_2$ that were much higher than the concentrations of GSH present in GSQ.

As model compound for protein thiols 2-mercapto-ethanol (MSH) was used. The adduct of MSH with quercetin (MSQ) is easier to detect than adducts of proteins such as albumin with quercetin. The synthesis of MSQ was performed similar to the strategy used for the synthesis of GSQ. 10 U/ml tyrosinase was added to 100 μ M quercetin in the presence of 120 μ M MSH. Purification and calibration are comparable to that described for GSQ.

QOH was synthesized according to Boots *et al.* (20).

HPLC analysis

For qualitative and quantitative HPLC analysis of the three synthesized compounds, a reversed phase Supelcosil LC318 column (25 cm x 4.6 mm) was used. For GSQ, the column was eluted with water containing 0.1% (v/v) trifluoroacetic acid and 5% acetonitrile for 5 minutes, followed by a gradient to 20% acetonitrile from 5 to 10 minutes and to 30% acetonitrile from 10 to 16 minutes. For MSQ, the column was eluted with water containing 0.1% (v/v) trifluoroacetic acid and 7.5% acetonitrile for 5 minutes, followed by a gradient to 15% acetonitrile from 5 to 22 minutes and to 30% acetonitrile from 22 to 38 minutes. For QOH, the column was eluted with 20% acetonitrile and 80% 0.1% (v/v) trifluoroacetic acid. Typical chromatograms of GSQ, MSQ and QOH are shown in Figure 5.1.

HPLC analysis of the incubation mixtures, unless otherwise noted, was performed using a Supelcosil LC318 column (25 cm x 4.6 mm). The column was eluted isocratically with water containing 0.1% (v/v) trifluoroacetic acid and 5% acetonitrile during 5 minutes, followed by a linear gradient to 20% acetonitrile from 5 to 10 minutes and to 30% acetonitrile from 10 to 16 minutes.

Figure 5.1 The product formed by the reaction of QQ with either GSH (trace A), H₂O (trace B) or MSH (trace C).

The HPLC analysis shows the different isomers of the adducts formed, i.e. 6-GSQ and 8-GSQ with GSH, 6-QOH and 8-QOH with H₂O and 6-MSQ and 8-MSQ with MSH. A typical example is shown.

Thiol reactivity

The reactivity of GSH, MSH and L(SH)₂ with CDNB was determined by mixing 1 mM thiol with 1 mM CDNB. This reaction was monitored for 10 minutes. The increase in absorption at 340 nm was used to calculate the second order rate constant k_{CDNB} for each thiol.

Statistics

All experiments were performed, at least, in triplicate. Data are given as mean $\pm$ SEM or as a typical example.

Results

Incubation of GSQ with mercapto-ethanol (MSH) results in the formation of a MSH-QQ adduct, i.e. MSQ. In an excess of MSH, the reaction follows the kinetics of a (pseudo) first order reaction (Figure 2A and 2B).

Figure 5.2 Reaction kinetics of the incubation of GSQ with MSH. Panel A shows the formation of MSQ (open squares) out of GSQ (closed dots) in time during the incubation of GSQ (initial concentration of 50 μM) with MSH (100 μM). In panel B the natural logarithm of the GSQ concentration is plotted in time. In panel C k_{MSH} is plotted as function of the MSH concentration. Experiments are performed at least in triplicate and data are shown as mean $\pm$ SEM.

The (apparent) first order reaction constant (k_{MSH}) increases upon increasing the concentration of MSH, but surprisingly the rate does not linearly depend on the concentration of MSH (Figure 5.2B). At high concentrations of MSH, the rate constant k_{MSH} approaches 0.4 min^{-1} (Figure 5.2C).

Incubation of GSQ with the dithiol lipoic acid ($\text{L}(\text{SH})_2$) results in the formation of quercetin. Again, in an excess of $\text{L}(\text{SH})_2$ the reaction follows the kinetics of a (pseudo) first order reaction (Figure 5.3A and 5.3B). Similarly to MSH, the rate with $\text{L}(\text{SH})_2$ does not linearly depend on the concentration of the thiol and the rate constant $k_{\text{L}(\text{SH})_2}$ approaches 0.4 min^{-1} at high concentrations of $\text{L}(\text{SH})_2$ (Figure 5.3C).

Figure 5.3 Reaction kinetics of the incubation of GSQ with $\text{L}(\text{SH})_2$. Panel A shows the formation of quercetin (open squares) out of GSQ (closed dots) in time during the incubation of GSQ (initial concentration of 50 μM) with $\text{L}(\text{SH})_2$ (100 μM). In panel B the natural logarithm of the GSQ concentration is plotted in time. In panel C $k_{\text{L}(\text{SH})_2}$ is plotted as function of the $\text{L}(\text{SH})_2$ concentration. Experiments are performed at least in triplicate and data are shown as mean $\pm$ SEM.

When QQ is formed in the presence of both GSH and MSH, initially GSQ is formed (Figure 5.4). After a longer time of incubation, GSQ is gradually converted into MSQ. When QQ is formed in the presence of both GSH and $L(SH)_2$ again initially GSQ is formed. After a longer time of incubation, GSQ gradually disappears and quercetin reappears (data not shown).

The (apparent) first order reaction constant (k_{MSH}) increases upon increasing the concentration of MSH, but surprisingly the rate does not linearly depend on the concentration of MSH (Fig 2B). At high concentrations of MSH, the rate constant k_{MSH} approaches 0.4 min^{-1} (Figure 5.2C).

Incubation of GSQ with the dithiol lipoic acid ($L(SH)_2$) results in the formation of quercetin. Again, in an excess of $L(SH)_2$ the reaction follows the kinetics of a (pseudo) first order reaction (Figure 5.3A and 5.3B). Similarly to MSH, the rate with $L(SH)_2$ does not linearly depend on the concentration of the thiol and the rate constant $k_{L(SH)_2}$ approaches 0.4 min^{-1} at high concentrations of $L(SH)_2$ (Figure 5.3C).

When QQ is formed in the presence of both GSH and MSH, initially GSQ is formed (Figure 5.4). After a longer time of incubation, GSQ is gradually converted into MSQ. When QQ is formed in the presence of both GSH and $L(SH)_2$ again initially GSQ is formed. After a longer time of incubation, GSQ gradually disappears and quercetin reappears (data not shown).

Figure 5.4 The fate of QQ in the presence of equimolar concentrations of both GSH and MSH. The initial concentration of GSH and MSH is $500 \mu\text{M}$. QQ is formed *in situ* by adding 100 U/ml tyrosinase (tyr) to $100 \mu\text{M}$ quercetin (open dots, dotted line). After 30 seconds, this reaction is stopped by filtering the solution, thereby removing the enzyme and stopping the QQ formation. Initially, GSQ (closed dots) is formed. In time GSQ is readily converted into MSQ (open squares). All experiments are performed at least in triplicate and data are expressed as mean $\pm$ SEM.

The reactivity of the thiols used in the present study was determined by monitoring the reaction of the thiols with CDNB. The rate constant of the

second order reaction with GSH ($0.020 \text{ mM}^{-1}\cdot\text{min}^{-1}$) was twice that with MSH ($0.0073 \text{ mM}^{-1}\cdot\text{min}^{-1}$) and $\text{L}(\text{SH})_2$ ($0.011 \text{ mM}^{-1}\cdot\text{min}^{-1}$) (Figure 5.5).

Figure 5.5 Second order rate constant of the reaction of GSH, MSH and $\text{L}(\text{SH})_2$ with CDNB. Data are expressed as mean $\pm$ SEM ($n=3$).

Discussion

QQ formed during the oxidation of quercetin is highly reactive towards thiol groups (17). *In vivo*, the most abundant thiol is glutathione (GSH). Furthermore, GSH is a better nucleophile than most other cellular thiols and its nucleophilicity is often further enhanced by the catalytic activity of various GSH-S-transferases. Therefore it is expected that initially QQ will react with GSH to form GSQ. The formation of the glutathionyl-adducts 6-GSQ and 8-GSQ from QQ under various conditions has been reported (16,17,19).

In the present study the fate of GSQ is examined. Our results show that GSQ is not stable; in both glutathionyl adducts of quercetin GSH is readily exchanged for another thiol such as MSH or $\text{L}(\text{SH})_2$.

The reaction of MSH with GSQ results in the formation of an MSH-QQ adduct, i.e. MSQ. Similar to GSQ there are two mercapto-ethanyl-adducts of quercetin, tentatively identified as 6-MSQ and 8-MSQ. Both forms of MSQ are formed in approximately equimolar concentrations and in all experiments the ratio of both adducts was constant. For sake of clarity MSQ is used to denote the combination of 6-MSQ and 8-MSQ. At a high concentration of MSH, the rate of MSQ formation is independent of the MSH concentration. This suggests a reaction scheme for the MSQ formation as depicted in Figure 5.6A. The binding of GSH to QQ is reversible and at high concentrations of MSH, the dissociation of GSQ is the rate limiting step in the MSQ formation. The first order rate constant of the GSQ dissociation (k_{GSQ}) is 0.4 min^{-1} at pH 7.4 and 37°C . Under these conditions the half life of GSQ is approximately 2 minutes.

Figure 5.6 Proposed schemes for the reaction of GSQ with MSH (scheme A) or $\text{L}(\text{SH})_2$ (panel B). As depicted in both schemes, GSQ will continuously dissociate into GSH and QQ ($k_2 = k_{\text{GSQ}}$). When the GSH concentration is high enough, QQ will again react with GSH to form GSQ at a relatively high rate (k_1). However, when the GSH concentration is relatively low, the dissociated QQ will react with other thiols, although k_3 is usually lower than k_1 . When QQ reacts with MSH (scheme A) the quercetin-thiol-adduct MSQ is formed. MSQ dissociates at a low rate (k_4) and may therefore accumulate.

The conversion of GSQ into MSQ is in line with the previously reported conversion of isolated pure 6-GSQ into an equimolar mixture of 6-GSQ and 8-GSQ (16). It was suggested that this conversion was due to an intramolecular isomerization. The results of the present study indicate that this is rather due to the dissociation of GSQ into QQ and GSH, followed by the recombination of GSH with QQ either at the 6 or 8 position. The half life of either isomer, deduced from the data of Boersma *et al.*, is approximately 2 minutes, identical to the half life of GSQ found in this study.

With $\text{L}(\text{SH})_2$ a comparable scheme as for MSH can be constructed (Figure 6B). Here, again the dissociation of GSQ is the rate limiting step ($k_{\text{GSQ}} = 0.4 \text{ min}^{-1}$) at high concentrations of the thiol. The difference between $\text{L}(\text{SH})_2$ and MSH is that with MSH a thiol-adduct of QQ is formed while with $\text{L}(\text{SH})_2$ quercetin is formed. It is proposed that with $\text{L}(\text{SH})_2$ at first also a thiol-QQ adduct is formed, i.e. the $\text{L}(\text{SH})\text{SQ}$ adduct. For the $\text{L}(\text{SH})\text{SQ}$ adduct four isomers are probably formed, namely 6(6S-lipoic acid) quercetin, 6(8S-lipoic acid) quercetin, 8(6S-lipoic acid) quercetin and 8(8S-lipoic acid) quercetin.

When QQ reacts with L(SH)_2 (scheme B), quercetin is formed. It is suggested that the formation of quercetin is preceded by the formation of a quercetin-thiol-adduct, i.e. L(SH)SQ -adduct. This adduct has 4 isomers, but for sake of clarity only two isomers are presented in the scheme. Due to the presence of a second intramolecular thiol group, the L(SH)SQ -adducts are rapidly converted into quercetin and lipoic acid (LS_2) (k_7).

The reaction constant rates k_{MSH} (k_3) and $k_{\text{L(SH)}_2}$ (k_5) approach k_2 at high concentrations of respectively MSH and L(SH)_2 .

Due to the presence of an intramolecular thiol group, these adducts are readily converted into quercetin and lipoic acid (LS_2). With monothiols, the reaction of a second thiol with the thiol-QQ adduct to form quercetin and a disulfide proceeds very slowly. Incubation of GSQ with high concentrations of either MSH or GSH for 15 minutes did indeed not result in the formation of detectable amounts of quercetin (data not shown). A similar superiority of L(SH)_2 compared to monothiols with regard to the reduction of thiol adducts has previously been described (21).

The reactivity of the thiol group of GSH at a pH of 7.4, determined with CDNB, is twice as high as that of MSH and L(SH)_2 . This is in line with the high initial formation of GSQ versus MSQ when QQ is generated in the presence of equimolar concentrations of both thiols. After a longer time of incubation, GSQ is converted into MSQ, despite the higher reactivity of GSH.

This can be explained by the fact that GS^- is a better leaving group compared to MS^- . The leaving group quality is correlated with the pK_a of the protonated form of the leaving group; the lower the pK_a , the better the leaving group quality. The pK_a of GSH is 8.6 whereas the pK_a of MSH is 10.6

(22), indicating that GS^- is indeed the better leaving group and QQ is trapped as MSQ in the end, despite the higher reactivity of GSH.

The reactivity of the thiol group in nucleophilic reactions also depends on the pK_a . In most reactions, including the Michael addition studied in the present study, the thiolate anion is the most reactive form of a thiol. The thiolate of MSH, i.e. MS^- , has a higher nucleophilicity and will thus be more reactive than the thiolate of GSH, i.e. GS^- , deduced from the higher pK_a of MSH. Since the pH of 7.4 is well below the pK_a of both compounds, only a small fraction of the thiols will be deprotonated. It can be calculated that for GSH the deprotonated fraction is higher (6%) than for MSH (0.1%). Figure 5.5 shows that at a pH of 7.4 GSH is more reactive than MSH with CDNB. This implicates that the difference in fraction of the thiol that is deprotonated has a higher contribution to the overall reactivity of the thiol than the intrinsic reactivity of the thiolate. In glutathione-S transferases the pK_a of GSH is reduced, partially explaining the catalytic mechanism of these enzymes.

Based on these results it can be concluded that at first QQ will form an adduct with thiols such as GSH, i.e. thiols with a relatively low pK_a and high reactivity. In the end this adduct will be converted into an adduct of QQ with thiols that are in first instance less reactive, for example protein thiols. The latter QQ-adduct is far more stable than GSQ. Incubation of MSQ with an excess of GSH or $\text{L}(\text{SH})_2$ did not show any detectable conversion of MSQ into GSQ or quercetin (data not shown). This indicates a high stability of MSQ and protein-quercetin adducts with half lives of over one hour.

Previously, we have shown that H_2O can also react with QQ to form QOH, i.e. 6-hydroxy-quercetin and 8-hydroxy-quercetin (20). The pK_a of H_2O is 14, indicating the relatively poor leaving group quality of the OH group in QOH. It is therefore expected that QOH is even more stable than MSQ. Based on the high stability of QOH, it could be argued that QQ would finally end up as QOH. QOH does not react with thiols (20). However, QOH is not formed out of QQ in aqueous solutions when thiols are present. Apparently, the nucleophilicity of H_2O is too low to form QOH in the presence of thiols. This suggests that there is an optimum in nucleophilicity of a compound in forming an adduct with QQ. What kind of QQ-adduct will be present depends on (i) the reactivity of the nucleophiles present (ii) the quality of the leaving group of the adducts (iii) the relative concentrations of the various nucleophiles and (iv) the time span of the incubation period. The reactivity of the nucleophile and the leaving group quality of the nucleophile of a thiol at physiological pH are respectively negatively and positively correlated with the pK_a value, as explained above.

QQ is extremely reactive, e.g. it reacts with thiols instantaneously. It will react at the site it is formed. As stated above, in cells GSQ is expected to

be the initial product of QQ. GSQ is far less reactive than QQ as indicated by their half lives of respectively 2 minutes and a fraction of a second. The half life of 2 minutes indicates that GSQ will diffuse over a relatively great distance.

The relatively long life time of GSQ indicates that this adduct can also be transported by an active metabolically dependent process, the ATP-dependent glutathione S-conjugate export pump (23-25). In this way QQ induced toxicity is even further spread *in vivo* by transporting QQ to sites different from those of its generation. The glutathione-S-transferases, that reduce the pK_a value and thus increase the leaving group quality of GSH, may promote QQ induced toxicity by catalysing the dissociation of GSQ into GS^- and QQ as previously suggested for other GSH adducts (26). Therefore, the glutathione-S-transferases might play a dual role by enhancing the formation as well as the dissociation of GSQ.

The non-enzymatic dissociation of GSQ into GSH and QQ will occur continuously. As long as the GSH concentration is high enough, QQ will react with GSH to form GSQ again. In this way GSH will still protect against QQ. However, when the GSH concentration is low, the dissociated QQ will react with other thiol groups, such as protein thiols (Figure 5.7). Protein thiols usually have a higher pK_a value than GSH. Therefore, the formed quercetin-protein-adducts are expected to be far more stable than GSQ. This will result for example in the non-competitive blocking of essential enzymes with critical thiol groups, a major mechanism for cell toxicity (18). It is therefore concluded that the "protection" of GSH against thiol arylation by QQ is ambiguous. Initially, GSH protects against QQ by scavenging QQ at the site of formation, whereas on the long run GSH will spread QQ toxicity.

Efficient protection against QQ toxicity is offered by dithiols that easily form intramolecular disulfides such as $L(SH)_2$. These dithiols convert QQ into quercetin. The synergistic interplay between $L(SH)_2$ and quercetin is expected to form an efficient antioxidant network, since regeneration of quercetin by $L(SH)_2$ makes the flavonoid available to the antioxidant network again. In this way the positive effect of quercetin during elevated oxidative stress might be boosted.

In conclusion it can be stated that GSH protects against QQ by formation of GSQ. However, GSQ is not stable, it dissociates into GS^- and QQ with a dissociation rate constant of 0.4 min^{-1} . The dissociated QQ may react with for example protein thiols, resulting in a much more stable quercetin-protein adduct. On the long run these more stable adducts might accumulate. Therefore, GSQ formation will not offer complete protection against QQ, it may also serve as transport or storage of QQ. This demonstrates that glutathione conjugation, generally regarded as a detoxification mechanism, may also spread toxicity.

Figure 5.7 Possible reactions of the oxidation product of quercetin (adapted from Boots *et al.*, 2003).

When quercetin is oxidized, an *o*-quinone/quinonmethide (referred to as QQ) is formed. QQ is highly reactive, it will react at the site it is formed. The most likely primary target of QQ is glutathione (GSH). At low GSH concentrations, QQ reacts with protein-thiols (P-SH) to form protein-quercetin adducts. The reaction of QQ with H₂O is too slow to occur *in vivo*. Regeneration of QQ by either ascorbate or DT-diaphorase (DTD) is also negligible compared to the thiol-adduct formation.

DHA= dehydroascorbate; NAD⁺= nicotinamide adenine dinucleotide; NADH= reduced NAD⁺.

References

1. Bravo L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr Rev* 1998;56:317-333.
2. Beecher GR, Warden BA and Merken H. Analysis of tea polyphenols. *Proc Soc Exp Biol Med* 1999;220:267-270.
3. Haenen GR, Paquay JB, Korthouwer RE and Bast A. Peroxynitrite scavenging by flavonoids. *Biochem Biophys Res Commun* 1997;236:591-593.
4. Heijnen CG, Haenen GR, van Acker FA, van der Vijgh WJ and Bast A. Flavonoids as peroxynitrite scavengers: the role of the hydroxyl groups. *Toxicol In Vitro* 2001;15:3-6.
5. Haenen GR, Jansen FP and Bast A. The antioxidant properties of five O-(β -hydroxyethyl)-rutosides of the flavonoid mixture Venoruton. *Phlebology* 1993;Suppl 1:10-17.
6. Diplock AT, Charleux JL, Crozier Willi G, Kok FJ, Rice Evans C, Roberfroid M, Stahl W and Vina Ribes J. Functional food science and defence against reactive oxidative species. *Br J Nutr* 1998;80:1s77-112.
7. Rahman I. The role of oxidative stress in the pathogenesis of COPD: implications for therapy. *Treat Respir Med* 2005;4:175-200.
8. Boots AW, Haenen GR and Bast A. Oxidant metabolism in chronic obstructive pulmonary disease. *Eur Respir J* 2003;22:14s-27s.
9. Psathakis K, Papatheodorou G, Plataki M, Panagou P, Loukides S, Siafakas NM and Bouros D. 8-Isoprostane, a marker of oxidative stress, is increased in the expired breath condensate of patients with pulmonary sarcoidosis. *Chest* 2004;125:1005-1011.
10. Kinnula VL, Fattman CL, Tan RJ and Oury TD. Oxidative stress in pulmonary fibrosis: a possible role for redox modulatory therapy. *Am J Respir Crit Care Med* 2005;172:417-422.
11. Penckofer S, Schwartz D and Florczak K. Oxidative stress and cardiovascular disease in type 2 diabetes: the role of antioxidants and pro-oxidants. *J Cardiovasc Nurs* 2002;16:68-85.
12. Kuyvenhoven JP and Meinders AE. Oxidative stress and diabetes mellitus: Pathogenesis of long-term complications. *Eur J Int Med* 1999;10:9-19.
13. Engel RH and Evens AM. Oxidative stress and apoptosis: a new treatment paradigm in cancer. *Front Biosci* 2006;11:300-312.
14. Gromadzinska J, Wasowicz W, Rydzynski K and Szeszenia Dabrowska N. Oxidative-stress markers in blood of lung cancer patients occupationally exposed to carcinogens. *Biol Trace Elem Res* 2003;91:203-215.
15. Bast A and Haenen GR. The toxicity of antioxidants and their metabolites. *Environmental Toxicology and Pharmacology* 2002; 11:251-258
16. Boersma MG, Vervoort J, Szymusiak H, Lemanska K, Tyrakowska B, Cenas N, Segura Aguilar J and Rietjens IM. Regioselectivity and reversibility of the glutathione conjugation of quercetin quinone methide. *Chem Res Toxicol* 2000;13:185-191.
17. Boots AW, Kubben N, Haenen GR and Bast A. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. *Biochem Biophys Res Commun* 2003;308:560-565.
18. Orrenius S. Biochemical mechanisms of cytotoxicity. *Trends in Pharmacol Sci* 1985;6: S15-S20.
19. Galati G, Moridani MY, Chan TS and O'Brien PJ. Peroxidative metabolism of apigenin and naringenin versus luteolin and quercetin: glutathione oxidation and conjugation. *Free Radic Biol Med* 2001;30:370-382.
20. Boots AW, Bast A and Haenen GR. No role of DT-diaphorase (NQO1) in the protection against oxidized quercetin. *FEBS Lett* 2005;579:677-682.
21. Haenen GR, De Rooij BM, Vermeulen NP and Bast A. Mechanism of the reaction of ebselen with endogenous thiols: dihydrolipoate is a better cofactor than glutathione in the peroxidase activity of ebselen. *Mol Pharmacol* 1990;37:412-422.
22. Jocelyn PC. Biochemistry of the SH group: the occurrence, chemical properties, metabolism and biological function of thiols and disulphides, London. Academic Press, 1972.

23. Kondo T, Yoshida K, Urata Y, Goto S, Gasa S and Taniguchi N. gamma-Glutamylcysteine synthetase and active transport of glutathione S-conjugate are responsive to heat shock in K562 erythroid cells. *J Biol Chem* 1993;268:20366-20372.
24. Ishikawa T. The ATP-dependent glutathione S-conjugate export pump. *Trends Biochem Sci* 1992;17:463-468.
25. Olive C and Board P. Glutathione S-conjugate transport by cultured human cells. *BBA* 1994;1224:264-268.
26. Chen J and Armstrong RN. Stereoselective catalysis of a retro-michael reaction by class mu glutathione transferases. Consequences for the internal distribution of products in the active site. *Chem Res Toxicol* 1994;8:580-585.

A large, light gray, stylized letter 'Q' that serves as a background for the page. It has a thick stroke and a short, slightly curved tail.

6

The quercetin paradox

AW Boots, H Li, RPF Schins, R Duffin, JWM Heemskerk, A Bast,
GRMM Haenen

Submitted

Abstract

Background

Free radical scavenging antioxidants, such as quercetin, are chemically converted into oxidation products when they protect against free radicals. The oxidation product of quercetin, however, displays a high reactivity towards thiols, which can lead to the loss of protein function. The quercetin paradox is that in the process of offering protection, quercetin is converted into a potential toxic product. In the present study this paradox is evaluated using rat lung epithelial (RLE) cells.

Methods

Markers of cellular toxicity (i.e. DNA damage, glutathione (GSH) consumption and LDH leakage) and cellular function loss (i.e. cytosolic free calcium concentration) have been measured as parameters of both the protective and the possible toxic effects of quercetin. All experiments have been performed in cultured lung cells using hydrogen peroxide (H_2O_2) as stressor.

Results

It was found that quercetin efficiently protected against H_2O_2 -induced DNA damage in RLE cells, but this damage was swapped for a reduction in GSH level, an increase in LDH leakage as well as an increase of the cytosolic free calcium concentration. This so-called quercetin paradox, i.e. the exchange of damage caused by quercetin and its metabolites, became more pronounced following depletion of GSH in the cells.

Conclusions

To our knowledge, this is the first study that indicates that the quercetin paradox also occurs in intact lung cells. This paradox implies that the antioxidant directs oxidative damage selectively to thiol arylation. The more pronounced paradox after GSH depletion confirms that the formation of thiol reactive quercetin metabolites is involved. Apparently, the potential toxicity of metabolites formed during the actual antioxidant activity of free radical scavengers should be considered in antioxidant supplementation.

Introduction

Quercetin is one of the most frequently studied dietary flavonoids and is ubiquitously present in various vegetables, fruits, seeds, nuts, tea and red wine (1-3). Quercetin is an excellent free radical scavenging antioxidant (4). A diet rich in flavonoids reduces the risk for oxidative-stress related chronic diseases such as diabetes, coronary heart disease and stroke (5,6). This has been associated with the antioxidant activity of flavonoids such as quercetin.

Free radical scavenging antioxidants form products that usually have taken over some of the reactivity of the radical that has been scavenged (7). In isolated membranes and blood plasma, these products of catechol-containing antioxidants such as quercetin react with thiols and impair several enzymes (8,9). The potential toxicity of quercetin metabolites, formed during the protection offered by quercetin, in relation to the protective effect of the flavonoid itself has not been evaluated in intact cells before.

In the present study the overall effect of quercetin on oxidative stress-induced damage is determined for the first time in an integrated system, i.e. cultured lung cells. The role of glutathione (GSH) in this interaction is also considered.

Lung cells have been chosen since the oxygen tension is relatively high in the lung and because after supplementation in rats and pigs the highest quercetin concentration is found in the lung (10). Furthermore, oxidative stress plays a prominent role in the etiology of lung diseases such as emphysema (11), sarcoidosis (12) and chronic obstructive pulmonary disease (13). H_2O_2 is used as stressor because H_2O_2 levels are known to be elevated during lung pathology (14,15). Furthermore, H_2O_2 released from inflammatory cells during chronic pulmonary inflammation following inhalation of toxic air pollution particles has been implicated in lung epithelial DNA damage (16). As parameters of both the protective as well as the possible toxic effects of quercetin, markers of cellular toxicity (DNA damage, GSH consumption and LDH leakage) and cellular function (cytosolic free calcium concentration) are measured.

Materials and methods

Materials

Quercetin, hydrogen peroxide (H_2O_2), trypan blue, buthionine sulfoximine (BSO), bovine serum albumin (BSA), potassium dihydrogen orthophosphate (KH_2PO_4), di-potassium hydrogen orthophosphate (K_2HPO_4), ethylenediamine-tetra acetic acid (EDTA), sulfosalicylic acid (SSA), triton

X-100, 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB), reduced β -nicotinamide adenine dinucleotide phosphate (β -NADPH), Dulbecco's phosphate buffered saline (PBS), trypsin, agarose, low melting point (LMP) agarose, sodiumpyruvate, sodiumchlorid, sodiumhydroxid solution, dimethylsulfoxide (DMSO), sodium lauroyl sarcosinate, ethidium bromide, glutathione reductase and reduced glutathione (GSH) were purchased from Sigma (St. Louis, MO, USA). Reduced β -Nicotinamide adenine dinucleotide disodium salt (NADH) was obtained from ICN Biomedicals (Ohio, USA). Ethanol was acquired from Biosolve (Valkenswaard, the Netherlands). Hanks' Balanced Salt Solution (HBSS, cat. no. 21-022) and cell culture medium were purchased from Life Technologies (Breda, the Netherlands). 1 AM Fura-2/AM was obtained from Molecular Probes (Leiden, the Netherlands). 4-Methylcatechol quinone was synthesized according to Willstätter and Pfannenstiel by the Ag_2O -mediated oxidation of 4-methylcatechol (17).

Methods

Incubation of Rat Lung Epithelial (RLE) cells

The rat lung epithelial cell line (RLE cells) was kindly provided by K. Driscoll (Proctor and Gamble, Cincinnati, USA). The RLE cells were seeded in 24-well culture dishes and cultivated in HAM's F12 medium (5% FCS, 1% penicillin, 1% streptomycin, 1% glutamine) under a 95% oxygen and 5% CO_2 atmosphere until 90% confluency was reached. In designated wells, RLE cells were treated for 24 hours with 0.1% buthionine sulfoximine (BSO) to deplete cells of GSH. The monolayers of RLE cells were treated for 30 minutes with 100 μM quercetin or HBSS as control. Subsequently 20 μM H_2O_2 , or HBSS as control, was added for a further 30 minutes. Quercetin was relatively stable in HBSS over the 1 hour incubation period (data not shown). The presence of H_2O_2 did not influence the stability of quercetin and quercetin did not directly react with H_2O_2 within the 1 hour incubation period (data not shown).

Addition of quercetin, that was dissolved in ethanol, resulted in an ethanol concentration in the incubation mixture of 0.5%. Control incubations with only 0.5% ethanol were performed and showed that ethanol had no effect.

Cell viability during all treatments was measured using the trypan blue dye exclusion.

Single Cell Gel Electrophoresis

DNA strand break formation in the RLE cells was determined using the comet assay (18) as described by Schins *et al.* (19) with minor modifications. RLE cells were seeded in designated wells of 24 well tissue culture dishes and

grown further for 48 hours. After incubation, the cells were rinsed twice with PBS, treated with 500 μ l trypsin-EDTA for 2 minutes and immediately after detachment of the cells 2 ml complete culture medium was added. Cells were centrifuged for 5 min at 800g and resuspended in 70 μ l PBS. Slides previously covered with a layer of 0.65% agarose were covered with a second layer consisting of 80 μ l low melting point (LMP) agarose and 30 μ l cell suspension. After storage for 30 minutes at 4°C to allow solidification of the second layer, the slides were covered with another layer of LMP agarose and stored again for at least 30 minutes at 4°C to allow solidification. Subsequently, slides were immersed in lysis buffer (2.5 mM NaCl, 100 mM EDTA, 10 mM Tris-base, 1% sodium lauryl sarcosinate, 10% DMSO and 1% Triton X-100) and stored overnight at 4°C.

The following day, slides were rinsed with distilled water and for 20 minutes placed in an electrophoresis tank filled with ice-cold electrophoresis solution containing 300 mM NaOH and 1 mM EDTA. Electrophoresis was conducted at 300 mA and 25 V for 15 minutes. Following three times neutralisation of the slides for 10 minutes using neutralization buffer (0.4 mM Tris, pH 7.5), slides were stained with 20 μ g/ml ethidium bromide in H₂O.

All steps described above were performed in the dark or under dimmed red light to prevent additional DNA damage. Analysis of comet appearance was performed on an Olympus BX60 fluorescence microscope at 200x magnification. For each individual experiment, at least 2 times 50 cells were analysed randomly per treatment, using a comet image analysis software program (Comet Assay II, Perceptive Instruments, Haverhill, UK). DNA damage was scored by determination of Olive tail moments, defined as the product of tail length and the fraction of total DNA in the comet tail.

Measurement of total cellular glutathione (GSH + GSSG)

After incubation, the cells were washed with PBS, harvested by gentle scraping and washed again in PBS. Total intracellular glutathione was determined according to the method of Tietze (20) with DTNB and glutathione reductase in a 96-well plate (21). The total cellular concentration of glutathione in the samples was determined using linear regression to calculate the values obtained from a standard curve and expressed as nmol per mg of protein.

Measurement of LDH leakage

After incubation of the RLE cells, the incubation media were collected and stored at -80°C. The cells were washed with PBS, harvested by gentle scraping and washed again in PBS. To lyse the cells 50 μ l 1% Triton X-100 in 143 mM potassium phosphate buffer pH 7.4 was added. Samples were then vortexed and stored at -80°C.

Before LDH analysis, all samples, i.e. media as well as lysed cells, were thawed and thoroughly vortexed. After diluting the lysed cells with 600 μl 143 mM potassium phosphate buffer pH 7.4, 50 μl of 1% Triton X-100 was added to 700 μl of each sample, followed by vortexing and centrifuging (3' at 14.000 g). Subsequently 700 μl of the obtained solutions was added to 200 μl 143 mM potassium phosphate buffer pH 7.4 containing 1.125 mg/ml natriumpyruvate and 0.9 mg/ml NADH. After mixing the samples, the decrease in NADH was measured spectrophotometrically at 340 nm for 3 minutes. Total LDH activity was calculated as the sum of LDH activity in the medium and in the cell lysate. The percentage of LDH leakage was calculated by relating the LDH activity present in the medium expressed to the total LDH activity, i.e. percentage LDH leakage=(LDH in medium/total LDH)*100%.

Measurement of cytosolic free calcium concentration ($[\text{Ca}^{2+}]_c$)

Cytosolic free concentrations of calcium were measured as described previously with minor modifications (22). Briefly, confluent monolayers of RLEs were cultured on gelatine-coated cover slips and mounted in a thermostatic (37°C) open chamber placed on a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan). Subsequently, the cells on the cover slips were loaded with 1 μM Fura-2/AM in DMSO for 30 minutes. Quercetin or 4-methyl-ortho-benzoquinone (positive control) were added as indicated. After washing the cover slips, hydrogen peroxide or fresh medium (positive control) was added and the measurement started ($t=0$ min). Changes in Fura-2 fluorescence were monitored in individual cells for 30 minutes by ratio fluorometry, using a high-sensitive, camera-based Quanticell video imaging system (Visitech, Sunderland, UK). Calibration of single cell measurements to values of $[\text{Ca}^{2+}]_c$ was performed by following earlier described procedures (23).

Statistics

All experiments were performed at least in triplicate and data are presented as mean $\pm$ SEM. The SEM values corresponding to Figure 6.1 and 6.4 were low and omitted from these figures for sake of clarity, but given in the results section. Statistical analysis was carried out using the non-parametric Wilcoxon's signed rank test; the Mann Whitney U test was applied to evaluate the BSO effect. Differences were considered to be significant if $P \leq 0.05$.

Results

The effect of quercetin on control rat lung epithelial cells (RLE cells) and BSO pretreated RLE cells that are exposed to hydrogen peroxide (H_2O_2) was investigated. DNA damage, GSH consumption, LDH leakage were determined

as markers of cell toxicity, whereas cytosolic free calcium concentration was measured as marker of function loss.

DNA damage in RLE cells treated with 20 μM H_2O_2 was measured using the Comet assay. In this assay DNA damage becomes visible as a “tail” of DNA fragments behind the cell and was scored by determination of these tail moments, defined as the product of tail length and the fraction of total DNA in the comet tail. The higher the tail moment, the more DNA damage has occurred in the cell. DNA damage induced by H_2O_2 (13.0 ± 2.0) was far greater than that occurring in control cells (0.8 ± 0.4) as depicted in Figure 6.1. Quercetin (100 μM) did not induce DNA damage in control cells (0.8 ± 0.2). Pre-treatment with quercetin offered full protection against H_2O_2 -induced DNA damage (2.1 ± 1.5) ($P < 0.01$).

H_2O_2 or quercetin induced a decrease in the GSH content of the cells of $18\% \pm 7$ or $19\% \pm 5$, respectively. This decrease was more substantial, i.e. $50\% \pm 5$, when RLE cells were treated with the combination of quercetin and H_2O_2 (Figure 6.1).

Figure 6.1 Effect of quercetin on H_2O_2 -induced oxidative stress in RLE cells. The cells were pre-treated for 30 minutes with or without 100 μM quercetin (Q). Subsequently, 20 μM H_2O_2 was added for another 30 minutes. In Q control incubations no H_2O_2 was added. DNA damage, LDH leakage and GSH consumption were measured as markers of genotoxicity and/or cell toxicity. Values are expressed as mean. DNA damage is presented as Olive tail moment and both GSH consumption and LDH leakage as percentage of respectively the whole GSH or LDH content of the cells in the control incubation. * : $P < 0.05$ compared to the incubation without quercetin and without H_2O_2 ; # : $P < 0.01$ compared to the incubation without quercetin but with H_2O_2 .

H_2O_2 did not induce detectable LDH leakage in control cells, as shown in Figure 6.1. Treatment with quercetin alone resulted in a cell viability of $97 \pm 1\%$ and a small LDH leakage of $3\% \pm 2$. The combination of H_2O_2 and quercetin lead to a cell viability of $89 \pm 1\%$ and drastically increased LDH leakage to $10\% \pm 2$ (Figure 6.1).

Treatment of the RLE cells with H_2O_2 resulted in the occurrence of both stress fibres and membrane bleb formation (Figure 2). These morphologic changes were slightly more pronounced after treatment with both H_2O_2 and quercetin (Figure 6.2).

Figure 6.2 The effect of H_2O_2 and of H_2O_2 plus quercetin on RLE cells in time. (A) the morphologic changes in the cells treated with H_2O_2 (H, upper panels) or with H_2O_2 and quercetin (HQ, lower panels). (B) the fluorescent images of cells treated with H_2O_2 (H, upper panels) or with H_2O_2 and quercetin (HQ, lower panels). Images were taken before ($t=0$ min), half-way ($t=15$ min) and after H_2O_2 treatment ($t=30$ min).

No changes in free cytosolic calcium concentration, a marker of cell function loss, after 30 min were found following treatment with quercetin (Figure 6.3).

Hydrogen peroxide treatment of the RLE cells induced repetitive spiking, i.e. oscillation, of the cytosolic free calcium-concentration of individual cells (Figure 6.2). Combining the reactions of all individual cells, these spikes result in a significant net increase of $[Ca^{2+}]_i$ in all cells after 30 minutes (36 ± 0.1 nM vs 43 ± 0.9 nM).

Figure 6.3 The increase in cytosolic free calcium concentration ($\Delta[Ca^{2+}]_i$) of either all RLE cells treated (upper figure) or representative single cells (lower figures) after 30 min treatment with 4-methylquinone (4-MCQ, 20 μ M), H_2O_2 (20 μ M), quercetin (Q, 100 μ M) or both H_2O_2 (20 μ M) and quercetin (100 μ M).

H_2O_2 treatment induced repetitive spiking of the cytosolic free calcium concentration of individual cells rather than an increase in the total net cytosolic free calcium concentration. Data are expressed as mean $\pm$ SEM. * : $P < 0.05$ compared to the incubation at $t = 0$ min.

Treatment of the cells with both quercetin and H_2O_2 did not cause any calcium-spikes, but did significantly increase the cytosolic free calcium concentration of the cells, i.e. from 36 ± 0.1 nM to 74 ± 2.4 nM after 30 minutes

(Figure 6.2). This increase was more than twice that caused by 20 μM of the positive control, i.e. the thiol-reactive 4-methylortho-benzoquinone (from 35 ± 0.1 nM to 50 ± 1.7 nM in 30 min) (Figure 6.3).

Pre-treatment of RLE cells with the GSH synthase inhibitor BSO (0.1 mM for 24 hours) caused a $80 \pm 5\%$ reduction in the GSH content of the cells (Figure 6.4). Treatment of the cells with H_2O_2 , quercetin or both compounds together did not further reduce the GSH content of BSO-pretreated cells.

Figure 6.4 Effect of quercetin on H_2O_2 -induced oxidative stress in BSO-pretreated RLE cells. The cells were pre-treated for 30 minutes with or without 100 μM quercetin (Q). Subsequently, 20 μM H_2O_2 was added for another 30 minutes. In Q control incubations no H_2O_2 was added. DNA damage, LDH leakage and GSH consumption were measured as markers of genotoxicity and cell toxicity. Values are expressed as mean. DNA damage is presented as tailmoment and both GSH consumption and LDH leakage as percentage of respectively the whole GSH or LDH content of the cells in the control incubation. * : $P < 0.05$ compared to the incubation without quercetin and without H_2O_2 ; ‡ : $P < 0.05$ compared to the corresponding incubations without BSO treatment depicted in Figure 6.1; # : $P < 0.01$ compared to the incubation without quercetin but with H_2O_2 .

BSO pre-treatment resulted, for all conditions tested, in a significantly increased DNA damage ($P < 0.01$) compared to that in control cells (Figure 6.4). H_2O_2 -induced DNA damage was slightly more pronounced after pre-treatment with BSO (13.0 ± 2.0 for control cells vs 15.5 ± 1.5 for cells pre-treated with BSO). Quercetin was still able to fully protect against this toxicity (3.2 ± 2.5) (Figure 6.4).

H₂O₂ did not induce LDH leakage in BSO pre-treated cells, as shown in Figure 6.5. LDH leakage induced by quercetin in BSO pre-treated cells was 8%±1, whereas cell viability during this treatment was 92±1%. The combination of H₂O₂ and quercetin resulted in a cell viability of 83±2% and further enhanced LDH leakage to 15±2%.

Discussion

As demonstrated in this study, H₂O₂ induces DNA damage in RLE cells and quercetin completely reverses this toxicity. Similar protective effects of quercetin against H₂O₂-induced DNA damage have been obtained previously in numerous other studies (24-26). In general, the protection observed is attributed to the antioxidant activity of quercetin. H₂O₂ has been implicated in the induction of DNA damage by acting as a precursor of free radicals, including the highly reactive hydroxyl radical (OH[•]), that can non-selectively cause oxidative damage to virtually any molecule including DNA bases (27). Quercetin is an efficient scavenger of these radicals (4).

The paradox of quercetin is that, although a highly reactive species is being neutralized, during the same process a thiol-reactive quercetin metabolite is being formed. In this way, the aselective toxicity of the highly reactive radical is swapped for a more selective toxicity of the quercetin metabolite. This thiol reactivity manifests itself by the preferential reaction with GSH (9). The binding of oxidized quercetin with GSH is reversible (28). At low GSH concentrations, oxidized quercetin will react with protein thiols, leading to the formation of a relatively stable protein-oxidized quercetin adduct (28). Binding of oxidized quercetin to protein thiols has already been shown in isolated membranes and lymphocytes as well as in blood plasma (8, 9,29) and can result in function loss like the impairment of enzymes such as the sarco- and endoplasmatic reticulum calcium ATPase (SERCA) that is responsible for calcium sequestration (8).

The present study demonstrates for the first time that the quercetin paradox also occurs in cultured cells; through quercetin the H₂O₂-induced DNA damage in RLE cells is swapped for a reduction in GSH level and an increase in both cytosolic free calcium concentration and LDH leakage. Again, the protection offered and the toxicity caused by quercetin can be attributed to the scavenging of radicals by quercetin and the concurrent formation of a thiol-reactive oxidation product of quercetin (8,9,29). The oxidation product of quercetin is not stable and therefore the relatively stable 4-methyl-ortho-benzoquinone has been used as a reference compound that displays a similar thiol-reactivity as oxidized quercetin (28). 4-Methyl-ortho-benzoquinone induces a comparable increase in the intracellular calcium concentration as

the combined treatment with quercetin and H_2O_2 . This effect of 4-methyl-ortho-benzoquinone indicates that quercetin-induced toxicity is indeed caused by the formation of thiol-reactive oxidation products of quercetin.

Since it is known that GSH efficiently protects against arylation and subsequent function loss of proteins (8), it is expected that reduction of the GSH levels will increase quercetin-induced toxicity. BSO pre-treatment of the RLE cells reduced GSH levels by 80% and indeed drastically enhanced the LDH leakage induced by the combined treatment with both quercetin and H_2O_2 . This is in line with the previous finding that a loss of GSH, caused by oxidation of this thiol, is involved in peroxide-induced cytoskeletal reorganisation and bleb formation in endothelial cells (22,30).

Under normal conditions, the cytosolic free calcium concentration is kept very low by a delicate control mechanism consisting of cellular channels, transporters and large calcium stores in e.g. the endoplasmatic reticulum (31). Reduction of the calcium sequestration in these stores, for example by impairing SERCA, results in an increase in the cytosolic free calcium concentration. One of the most important targets of thiol reactive compounds is the critical sulfhydryl group of this calcium-ATPase (32). In isolated membranes, it has already been shown that catechol-containing antioxidants such as quercetin impair this enzyme (8). In the present study, H_2O_2 alone evokes sudden increases in cytosolic free calcium concentration that are followed by sharp decreases of this concentration, thus reflecting high calcium-ATPase activity. The combined treatment with quercetin and H_2O_2 , however, induces a low but consistent increase in cytosolic free calcium concentration without a subsequently lowering of the calcium concentration, indicating that oxidized quercetin can also impair calcium-ATPase.

Elevated cytosolic free calcium can cause cytotoxicity or trigger apoptosis (33). Calcium can induce cell death by for example (i) mediating cytochrome c release from mitochondria via increasing the mitochondrial permeability (33), (ii) activating caspases (31,33), (iii) causing the expression of ligands that bind to and activate death receptors (31), (iv) activating hydrolytic enzymes (34) or by (v) initiating cytoskeletal degradation (34). First signs of apoptosis are the appearance of so-called stress fibres and membrane blebs at the plasma membrane, caused by a reassembly of the actin cytoskeleton (22).

In our study, the H_2O_2 -induced net increase in the cytosolic free calcium concentration was indeed accompanied by concurrent signs of the formation of stress fibres and blebbing. The increase in cytosolic free calcium, and thus the resulting stress fibres and blebbing, are slightly more pronounced after combined treatment with both quercetin and H_2O_2 than with the latter alone.

Moreover, after BSO-pretreatment, the combined treatment caused a reduced cell viability. These findings suggest that, despite the direct protection against H_2O_2 -induced DNA damage offered by quercetin, toxic changes that may result in LDH leakage, blebbing, decreased cell viability and eventually even apoptosis, are indirectly induced by the oxidation product of quercetin formed during this protection. In that way, the apparent protection offered by quercetin is actually a swap of one form of toxicity, i.e. DNA-damage, for another, i.e. impaired calcium sequestration.

Figure 6.5 Schematic overview of the quercetin paradox. When no quercetin is present, H_2O_2 damages DNA by the formation of free radicals. When quercetin is present, it efficiently scavenges radicals and protects the cells against H_2O_2 -induced DNA damage. During this protection, quercetin is converted into oxidized quercetin. Oxidized quercetin is thiol reactive and will arylate GSH as well as protein thiol groups, leading to GSH consumption, an increase in cytosolic free calcium concentration ($[\text{Ca}^{2+}]_c$) and LDH leakage.

Although the low oral bioavailability and the relatively high concentration of quercetin used in the present study hamper a straightforward extrapolation to *in vivo* conditions, our results point at some practical implications of the supplementation with quercetin. In subjects with elevated oxidative stress, i.e. subjects expected to benefit from antioxidant therapy, the formation of oxidized quercetin will be relatively high. Additionally, in these subjects GSH levels might be low, making them even more susceptible to damage by quercetin oxidation metabolites. Studies conducted with healthy volunteers on the efficacy or safety are, therefore, not representative for the actual application of quercetin. When clinical

studies with quercetin are executed, toxicity might be monitored by determining the effect of quercetin on the endogenous GSH-level.

The quercetin paradox is that, as a result of its protection, quercetin becomes toxic (depicted in Figure 6.5). Such a paradox might also apply for other free radical scavenging antioxidants. The toxicity of the metabolites that are formed during the actual antioxidant activity of free radical scavengers, might shade or even eclipse the direct positive effects of antioxidant supplementation.

References

1. Formica JV and Regelson W. Review of the biology of Quercetin and related bioflavonoids. *Food Chem Toxicol* 1995;33:1061-1080.
2. Beecher GR, Warden BA and Merken H. Analysis of tea polyphenols. *Proc Soc Exp Biol Med* 1999;220:267-270.
3. Hertog MG, Hollman PC, Katan MB and Kromhout D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr Cancer* 1993;20:21-29.
4. Bors W, Michel C and Saran M. Flavonoid antioxidants: rate constants for reactions with oxygen radicals. *Methods Enzymol* 1994;234:420-429.
5. Skibola CF and Smith MT. Potential health impacts of excessive flavonoid intake. *Free Radic Biol Med* 2000;29:375-383.
6. Hollman PC and Katan MB. Dietary flavonoids: intake, health effects and bioavailability. *Food Chem Toxicol* 1999;37:937-942.
7. Bast A and Haenen GR. The toxicity of antioxidants and their metabolites. *Environ Tox Pharm* 2002;11:251-258.
8. Boots AW, Haenen GR, den Hartog GJ and Bast A. Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants. *Biochim Biophys Acta* 2002;1583:279-284.
9. Boots AW, Kubben N, Haenen GR and Bast A. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. *Biochem Biophys Res Commun* 2003;308:560-565.
10. De Boer VC, Dihal AA, van der Woude H, Arts IC, Wolffram S, Alink GM, Rietjens IM, Keijer J and Hollman PC. Tissue distribution of quercetin in rats and pigs. *J Nutr* 2005;135:1718-1725.
11. Tuder RM, Zhen L, Cho CY, Taraseviciene Stewart L, Kasahara Y, Salvemini D, Voelkel NF and Flores SC. Oxidative stress and apoptosis interact and cause emphysema due to vascular endothelial growth factor receptor blockade. *Am J Respir Cell Mol Biol* 2003;29:88-97.
12. Psathakis K, Papatheodorou G, Platakis M, Panagou P, Loukides S, Siafakas NM and Bouros D. 8-Isoprostane, a marker of oxidative stress, is increased in the expired breath condensate of patients with pulmonary sarcoidosis. *Chest* 2004;125:1005-1011.
13. Boots AW, Haenen GR and Bast A. Oxidant metabolism in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 2003;46:14s-27s.
14. Dekhuijzen PN, Aben KK, Dekker I, Aarts LP, Wielders PL, van Herwaarden CL and Bast A. Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1996;154:813-816.
15. Kostikas K, Papatheodorou G, Psathakis K, Panagou P and Loukides S. Oxidative stress in expired breath condensate of patients with COPD. *Chest* 2003;124:1373-1380.
16. Knaapen AM, Borm PJ, Albrecht C and Schins RP. Inhaled particles and lung cancer. Part A: Mechanisms. *Int J Cancer* 2004;109:799-809.
17. Willstätter R and Pfannenstiel, A. Ueber o-chinon. *Chem. Berichte* 1904;4:4744-4746.
18. Singh NP, McCoy MT, Tice RR and Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 1988;175:184-191.
19. Schins RP, Duffin R, Hohn D, Knaapen AM, Shi T, Weishaupt C, Stone V, Donaldson K and Borm PJ. Surface modification of quartz inhibits toxicity, particle uptake, and oxidative DNA damage in human lung epithelial cells. *Chem Res Toxicol* 2002;15:1166-1173.
20. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969;27:502-522.
21. Jardine H, MacNee W, Donaldson K and Rahman I. Molecular mechanism of transforming growth factor (TGF)-beta1-induced glutathione depletion in alveolar epithelial cells. Involvement of AP-1/ARE and Fra-1. *J Biol Chem* 2002;277:21158-21166.

22. Van Gorp RM, Heeneman S, Broers JL, Bronnenberg NM, van Dam-Mieras MC and Heemskerk JW. Glutathione oxidation in calcium- and p38 MAPK-dependent membrane blebbing of endothelial cells. *Biochim Biophys Acta* 2002;1591:129-138.
23. Heemskerk JW, Vuist WM, Feijge MA, Reutelingsperger CP and Lindhout T. Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca^{2+} responses. *Blood* 1997;90:2615-2625.
24. Peng IW and Kuo SM. Flavonoid structure affects the inhibition of lipid peroxidation in Caco-2 intestinal cells at physiological concentrations. *J Nutr* 2003;133:2184-2187.
25. Musonda CA and Chipman JK. Quercetin inhibits hydrogen peroxide (H_2O_2)-induced NF-kappaB DNA binding activity and DNA damage in HepG2 cells. *Carcinogenesis* 1998;19:1583-1589.
26. Blasiak J, Arabski M, Pertynski T, Malecka Panas E, Wozniak K and Drzewoski J. DNA damage in human colonic mucosa cells evoked by nickel and protective action of quercetin - involvement of free radicals? *Cell Biol Toxicol* 2002;18:279-288.
27. Spencer JP, Jenner A, Chimel K, Aruoma OI, Cross CE, Wu R and Halliwell B. DNA strand breakage and base modification induced by hydrogen peroxide treatment of human respiratory tract epithelial cells. *FEBS Lett* 1995;374:233-236.
28. Boots AW, Balk JM, Bast A and Haenen GR. The reversibility of the glutathionyl-quercetin adduct spreads oxidized quercetin-induced toxicity. *Biochem Biophys Res Commun* 2005;338:923-929.
29. Yen GC, Duh PD, Tsai HL and Huang SL. Pro-oxidative properties of flavonoids in human lymphocytes. *Biosci Biotechnol Biochem* 2003;67:1215-1222.
30. Van Gorp RM, Broers JL, Reutelingsperger CP, Bronnenberg NM, Hornstra G, van Dam-Mieras MC and Heemskerk JW. Peroxide-induced membrane blebbing in endothelial cells associated with glutathione oxidation but not apoptosis. *Am J Physiol* 1999;277:C20-28.
31. Kass GE and Orrenius S. Calcium signaling and cytotoxicity. *Environ Health Perspect* 1999;107 Suppl 1:25-35.
32. Thor H, Hartzell P, Svensson SA, Orrenius S, Mirabelli F, Marinoni V and Bellomo G. On the role of thiol groups in the inhibition of liver microsomal Ca^{2+} sequestration by toxic agents. *Biochem Pharmacol* 1985;34:3717-3723.
33. Orrenius S, Zhivotovsky B and Nicotera P. Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol* 2003;4:552-565.
34. Nicotera P and Orrenius S. The role of calcium in apoptosis. *Cell Calcium* 1998;23:173-180.

7

The anti-inflammatory activity of quercetin

AW Boots, LC Wilms, ELR Swennen, JCS Kleinjans, A Bast,
GRMM Haenen

Submitted

Abstract

Background

Quercetin, the most commonly occurring flavonoid, is an excellent antioxidant that is also suggested to possess other beneficial activities. The present study has been designed to investigate the possible anti-inflammatory effects of physiologically attainable quercetin concentrations.

Methods

Quercetin was added to blood in the test tube, or blood taken after four weeks quercetin supplementation. Primary pro-inflammatory cytokine measured in this study was tumour necrosis factor alpha (TNF α), of which the production has been evoked using lipopolysaccharide (LPS).

Results

It was found that quercetin dose-dependently inhibited *in vitro* LPS-induced TNF α production in the blood of healthy volunteers. A concentration of 1 μ M of the flavonoid already reduced the cytokine production to 77%. The *in vitro* LPS-induced IL-10 production remained unaffected by quercetin. Four weeks quercetin supplementation resulted in a significant increase in plasma quercetin concentration. The supplementation also increased the total plasma antioxidant status, but did not affect the glutathione, vitamin C and uric acid plasma concentrations. Basal as well as *ex vivo* LPS-induced TNF α levels were also not altered by the supplementation.

Conclusions

The present study shows that quercetin increases the antioxidant capacity *in vivo* and displays anti-inflammatory effects *in vitro*, but not *in vivo* or *ex vivo*, in the blood of healthy volunteers. The lack of effect in the healthy volunteers is probably due to their low cytokine and high antioxidant levels at baseline, indicating that neither inflammation nor oxidative stress is present. Only in people with increased levels of inflammation and oxidative stress, e.g. patients suffering from a disease of which the pathology is associated with these two processes, antioxidant supplementation is expected to be fruitful.

Introduction

Flavonoids are a class of naturally occurring polyphenolic compounds that are ubiquitously present in fruits, vegetables, nuts, plant-derived beverages such as tea and wine as well as in some traditional herbal-containing medicines (1). The total amount of flavonoids consumed in the Netherlands is estimated at several hundreds of mg per day (2). The Dutch intake of flavones and flavonols, two important subgroups of the flavonoids, is determined as 23-24 mg per day and 70% of this amount is quercetin (3). Higher estimates, reporting an average daily flavonoid intake of 1 gram including about 50 mg of quercetin, have been made in other Western countries (4).

Much attention has been given to the potential health-promoting properties of flavonoids in general and of quercetin in particular. Several epidemiological studies have reported an inverse relation between flavonoid intake and both the risk for cardiovascular diseases and the incidence of lung and colorectal cancer (5). These beneficial effects have been attributed to the anti-oxidative capacities of flavonoids which have already been determined both *in vitro* (6,7) and *in vivo* (8,9). However, these findings are not conclusive since various other studies have failed to demonstrate such health-promoting effects of flavonoids (5).

Recently, some studies have also reported that, *in vitro*, quercetin can inhibit various cytokines, including tumour necrosis factor α (TNF α) (10,11). Extrapolation of these findings to a physiological relevant effect of *in vivo* quercetin supplementation is difficult, since most studies have been performed in immortalized cancer cell lines with relatively high concentrations of the flavonoid. This prompted us to investigate the anti-inflammatory effects of physiologically attainable quercetin concentrations in whole blood from healthy subjects, a model more closely resembling the *in vivo* situation. The effects of quercetin are tested both *in vitro*, i.e. added to blood in the test tube, and *ex vivo*, i.e. in blood taken after the administration of quercetin in a supplementation study. Moreover, the direct *in vivo* effect of the quercetin supplementation on basal cytokine levels is also assessed. The primary cytokine measured in this study is TNF α since this cytokine is an important mediator of inflammation and reported to be elevated in various chronic diseases such as sarcoidosis and idiopathic pulmonary fibrosis (12). Lipopolysaccharide (LPS), a patho-physiological relevant stimulator of monocytes, neutrophils and B lymphocytes, is used to evoke TNF α production *ex vivo* (13-16).

Materials and Methods

Chemicals

Quercetin, reduced glutathione (GSH), oxidized glutathione (GSSG), GSSG reductase, sulfosalicylic acid (SSA), 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB), O-phenylenediamine (OPDA), ascorbic acid and lipopolysaccharide (LPS, *E. coli* 0.26:B6) were purchased from Sigma Chemical Co. (St. Louis, USA). RPMI 1640 medium containing L-glutamine was obtained from Gibco (Paisley, UK). Ascorbate oxidase spatula were purchased by Roche Diagnostics (Basel, Switzerland). Human TNF α (7300 pg/ml) and human IL-10 (4000 pg/ml) were acquired from CLB/Sanquin (Amsterdam, The Netherlands). All other chemicals were of analytical grade. The quercetin-rich blueberry-apple juice mixture was produced specifically for this study by Riedel Drinks (Riedel, Ede, The Netherlands).

Methods

Participants

The *in vitro* experiments, of which the data are shown in Figure 7.1 and 7.2, are performed with the blood of 3 healthy volunteers (2 male, 1 female) between the age of 27 and 45 years.

All other experiments are performed with the blood of 7 healthy volunteers (3 male, 4 female) between the age of 20 and 40 years. In a comparable study, it has been shown that for a biomarker of oxidative stress, i.e. the total plasma antioxidant capacity (TEAC), a 10% difference between treatments ($\alpha=0.05$; two-sided and a power of 80%) can be demonstrated with such a small number of volunteers (17).

All healthy volunteers were recruited through advertisements in local newspapers. Volunteers were included if they were non-smoking and did not use any medication or vitamin supplementation at the moment of the intervention. All participants filled in a questionnaire regarding their dietary habits and self-experienced health status and were, based on that information, considered healthy. No large differences in dietary habits were found. Mean quercetin intake of all volunteers was approximately 15 mg per day.

The Medical Ethical Committee of Maastricht University and the Academic Hospital Maastricht had approved the protocol before the beginning of the study. All participants were fully informed about the aim and details of the study and have given their written informed consent.

Supplementation study

Prior to the actual supplementation period, volunteers were subjected to a five-day during “washout”-period. During this period they were not allowed to consume food ingredients rich in flavonoids in general and in quercetin in particular. These food ingredients included onions, apples, red wine, tea, biological and freshly pressed fruit juices, berries (e.g. blueberries and elderberries), grapes, cherries, raisins, parsley, broccoli, cabbage, beans and tomatoes (8). Subjects also had to minimise the use of spices and herbs during this period.

The paired design of this supplementation study comprises that each subject acts as his or her own control. Based on the results from an earlier pilot study (8), it was concluded that best results would be obtained after 4 weeks of supplementation. The five-day flavonoid washout period was therefore followed by a four-week supplementation period with a large increase in quercetin intake, established by consuming a blueberry-apple juice mixture. This juice contains about 97 mg of quercetin per litre, most of which is bound to a glucoside or a galactoside at the 3-position, making it well biologically available (18). After the washout-period as well as after the supplementation study venous blood samples were drawn into EDTA-containing vacutainer tubes (Vacutainer, Becton-Dickinson) and kept on ice prior to processing which occurred within 1 hour after blood collection.

The design of this supplementation as well as the efficacy of the washout period is based on a pilot study, described earlier (8).

Preparation of the blood samples

Blood was aliquoted into eppendorfs for both the ascorbic acid and the GSH/GSSG analysis: for the former 10% TCA was added to the whole blood, whereas 1.3% SSA in 10 mM HCl was used to preserve the samples for the latter. Another aliquot of blood was used for the incubations required for the blood-based cytokine production assay as described in that section below. The remaining blood was centrifuged (3000 rpm, 5' at 4°C) to obtain plasma. Deproteinization of an aliquot of this plasma, using 10% TCA (1:1) followed by centrifugation (13,000 rpm, 5' at 4°C), was carried for the trolox equivalent antioxidant capacity measurement. All samples were stored at -80°C prior to analysis.

Determination of total plasma quercetin concentration

Total quercetin concentration was analysed in plasma by means of high performance liquid chromatography (HPLC) with coulometric array-detection after enzymatic hydrolysis as described previously (19).

Trolox antioxidant capacity

The trolox equivalent antioxidant capacity (TEAC value) is a measurement for the total antioxidant status, relating the free radical scavenging properties of a solution or a compound to that of the synthetic antioxidant trolox. The assay is performed as previously described (20). The relative contribution of uric acid, vitamin C and quercetin to the total TEAC value is calculated using the TEAC value described for each individual antioxidant, i.e. 1, 1 and 6.24 respectively (21).

Ascorbic acid measurement

Ascorbic acid has been included in the present study since it is known to be an important contributor to the TEAC. Calibrators were prepared freshly, containing the same amount of TCA as the samples. Samples and calibrators were processed identically as described previously (22).

Uric acid measurement

Uric acid has been included in the present study since it is known to be an important contributor to the TEAC. Uric acid was measured in the plasma of all samples as described previously (23).

GSH, GSSG and haemoglobin measurement

Both GSH and GSSG calibrators were prepared freshly and contained the same concentrations of SSA as the samples. Samples and calibrators were treated identically and GSH and GSSG levels measured were related to the haemoglobin content as described previously (24).

Blood-based cytokine production assay

Within one hour after blood collection, the blood-based cytokine production assay was performed as described previously (14). Care was taken that handling of the blood prior to LPS-stimulation did not influence the cytokine release.

The advantage of the current *ex vivo* blood assay is that this model, unlike models using isolated cells or cell lines grown in medium, represents a more physiological and well reproducible model to measure cytokine production *ex vivo*. The natural cell-to-cell interactions are preserved and all blood components are present in *in vivo* ratios with non-cellular components, resulting in a system that reflects the *in vivo* condition well (14).

Enzyme linked immune sorbent assay (ELISA) measurement

Both TNF α and IL-10 were quantified using PeliKine Compact human ELISA kits (CLB/Sanquin, The Netherlands) based on appropriate and validated sets of monoclonal antibodies. Assays were performed as described in the manufacturer's instructions. Cytokine production was related to that

of the control incubation without quercetin. The ethanol (0.5%) used to dissolve quercetin did not show any influence on the *ex vivo* LPS-induced cytokine production (data not shown).

Statistics

The *in vitro* data as well as the before versus the after data were compared using the Wilcoxon's signed rank test. A one-tailed probability value (P-value) of less than 0.05 was considered to be statistically significant.

Results

To determine the possible anti-inflammatory effects of the antioxidant quercetin, inflammation has been evoked *ex vivo*, using LPS, in the blood of healthy volunteers. To optimize this assay, the $\text{TNF}\alpha$ -inducing ability of various concentrations of LPS, as well as the effect of 30 μM quercetin on this production, have been measured. Figure 7.1 shows that LPS, starting at the very low concentration of 0.005 ng/ml, dose-dependently induced $\text{TNF}\alpha$ -production. Quercetin (30 μM) inhibited this LPS-induced $\text{TNF}\alpha$ -production, but the percentage of inhibition caused by the flavonoid depended on the amount of $\text{TNF}\alpha$ present. The most pronounced inhibitory effect of quercetin ($61 \pm 4\%$) was found when 0.1 ng/ml LPS was used to induce $\text{TNF}\alpha$ -production. This relatively low LPS-concentration has been used to further evaluate the anti-inflammatory effect of quercetin.

Figure 7.1 LPS-induced $\text{TNF}\alpha$ production *in vitro* after 24 hours, without or with (insert) 30 μM quercetin (30 min pre-incubation) in the blood of healthy volunteers. In the insert, results are expressed as % inhibition of the LPS-induced $\text{TNF}\alpha$ release. Data are expressed as mean $\pm$ SEM ($n=3$).

Quercetin dose-dependently inhibited the LPS-induced $\text{TNF}\alpha$ -production as is depicted in Figure 7.2.

Figure 7.2 The inhibitory effect of quercetin on *ex vivo* LPS-induced $\text{TNF}\alpha$ production in the blood of healthy volunteers. Blood was incubated with increasing quercetin concentrations for 30 minutes and subsequently stimulated with 0.1 ng/ml LPS for 24 hours. Results are expressed in percentage with 100% representing $\text{TNF}\alpha$ under stimulation by LPS in the absence of quercetin. Data are expressed as mean $\pm$ SEM (n=7). * = $P < 0.01$ compared to the control incubation without quercetin

At a concentration of 1 μM , the flavonoid already reduced the cytokine production to $77 \pm 7\%$. The LPS-induced IL-10 production remained unaffected by the flavonoid. As a result, quercetin was also capable of significantly reducing the ratio of pro- versus anti-inflammatory marker $\text{TNF}\alpha/\text{IL-10}$, a frequently used diagnostic parameter of inflammation. It was found that this *in vitro* effect of quercetin greatly depends on the level of $\text{TNF}\alpha$ induced by LPS (Figure 7.3).

Four weeks quercetin supplementation resulted in a significant increase in both the plasma quercetin concentration and the total plasma antioxidant capacity, i.e. the total sum of all plasma antioxidants that is expressed as Trolox equivalent, in the blood of the healthy volunteers (Figure 7.4). The glutathione (GSH) levels and basal $\text{TNF}\alpha$ levels were unaffected by this quercetin supplementation (Figure 7.5). No significant changes were found between the uric acid and vitamin C levels before and after the supplementation (data not shown).

Figure 7.3 The relation between the amount of $\text{TNF}\alpha$ present and the effect of quercetin on these $\text{TNF}\alpha$ levels *in vitro*. Blood was pre-treated with $30 \mu\text{M}$ quercetin prior to 24-hours incubation with various concentrations of LPS (37°C , $5\% \text{CO}_2$). Afterwards, the $\text{TNF}\alpha$ released into the supernatants was analyzed by ELISA and expressed on the x-axis. The decrease in $\text{TNF}\alpha$ production caused by quercetin is expressed, as percentage, on the y-axis. Data are expressed as mean $\pm$ SEM ($n=3$).

Figure 7.4. The effect of four weeks quercetin-supplementation in healthy volunteers regarding the plasma quercetin concentration (panel A), the total plasma antioxidant capacity (expressed as Trolox equivalents, panel B), the GSH concentration (panel C) and the basal $\text{TNF}\alpha$ level (panel D). Blood was drawn before and after the quercetin-intervention and all data are individually expressed ($n=7$); the light grey bars represent the mean.

*= $P<0.05$ compared to the “before” measurement prior to the intervention

The relative contribution of endogenous antioxidants uric acid and vitamin C, as well as that of the exogenous quercetin, to the total plasma antioxidant status is depicted in Figure 7.5. This reveals that the relative contribution of quercetin to the total plasma antioxidant capacity was rather small (0.04% before vs 0.06% after). A major part of this capacity was due to plasma anti-oxidants other than uric acid and vitamin C, such as low molecular protein thiols (Figure 7.5). This residual plasma antioxidant capacity was significantly increased after the four weeks of quercetin supplementation.

Figure 7.5 Total antioxidant capacity (expressed as Trolox equivalents) and the relative contribution of uric acid, vitamin C and quercetin in healthy volunteers before and after four weeks quercetin intervention. Subtraction of these three contributions results in a residual plasma antioxidant capacity (TEACres) that is significant higher after the intervention compared to the control values before the intervention ($563 \pm 14 \mu\text{M}$ vs $590 \pm 19 \mu\text{M}$). Data are expressed as mean $\pm$ SEM ($n=7$).
* = $P < 0.05$ compared to the “before” measurement prior to the intervention

Ex vivo LPS-induced $\text{TNF}\alpha$ production did not show a significant decrease after four weeks quercetin supplementation ($P \leq 0.1$, Figure 7.6). Also no clear correlation was found between the changes in the *ex vivo* LPS-induced $\text{TNF}\alpha$ production of the individual volunteers after the four weeks of quercetin supplementation and the increase in either the quercetin plasma concentration (Figure 7.7) or the total plasma antioxidant capacity (data not shown).

Figure 7.6 The effect of four weeks quercetin-intervention in healthy volunteers on *ex vivo* LPS-induced $\text{TNF}\alpha$ production. Data are individually expressed ($n=7$); the light grey bars represent the mean.

Figure 7.7 The relation between the increase in quercetin concentration ($\Delta[\text{quercetin}]_{\text{plasma}}$) and the decrease in the *ex vivo* LPS-induced $\text{TNF}\alpha$ production ($\Delta[\text{TNF}\alpha]$) in healthy volunteers after four weeks quercetin intervention. Each point represents the individual values of one healthy volunteer ($n=7$).

Discussion

The present study shows that quercetin dose-dependently reduces $\text{TNF}\alpha$ production *in vitro* in the blood of healthy volunteers by LPS.

LPS is a pro-inflammatory glycolipid component of the gram-negative bacterial cell wall. LPS acts as a polyclonal mitogen for B lymphocytes (16) and as an activator of macrophages and neutrophils via the LPS-binding protein (LBP)/CD14/Toll like receptor (TLR)-4-dependent pathway, resulting in the production of specific cytokines (25). Short-term exposure to LPS induces an inflammatory reaction in the lung mediated primarily by human

blood monocytes and alveolar macrophages, which release an array of inflammatory cytokines including $\text{TNF}\alpha$ (26).

Under normal circumstances, ambient air contains low amounts of LPS (approximately 0.36 ng/m^3) that can easily be eliminated by the human endogenous immune defense system (27). However, LPS is present in larger amounts in airborne particles in polluted air (28) and cigarette smoke (29). Moreover, a variety of infections may provide a pathological burden of mediators like LPS (30). Various studies have shown a relation between LPS exposure and airway inflammation or obstruction (31-33). Moreover, long-term exposure to LPS in mice results in inflammatory and pathological changes that mimic changes observed in human subjects suffering from chronic lung diseases such as chronic obstructive pulmonary disease (COPD) (25). Altogether, this suggests that long-term exposure to LPS might play a large role in the pathogenesis of chronic lung diseases.

The *ex vivo* model applied in the present study uses a relatively low LPS concentration, i.e. 0.1 ng/ml , that could well be achieved *in vivo* when taken into consideration that smoking 1 cigarette delivers a local LPS dose of 120 ng (29). Moreover, the $\text{TNF}\alpha$ production evoked by the employed low LPS concentration shows a significant higher sensitivity towards quercetin than that induced by increasing amounts of LPS. This suggests that especially during exposure to relatively low LPS doses, antioxidants such as quercetin will have a relatively more pronounced effect on cytokine production.

LPS induces the reactive oxygen species (ROS)-producing enzymes inducible nitric oxide synthase and NADPH-oxidase in monocytes and macrophages, leading to the extensive production of $\text{NO}^\cdot$, $\text{O}_2^{\cdot-}$, peroxynitrite and other reactive oxygen (ROS) or nitrogen species (RNS) (34,35). It is known that ROS are capable of promoting inflammation by activating transcription factors like nuclear factor kappa-B (NF- κ B) and activator protein-1 that induce not only more ROS but also pro-inflammatory cytokines like $\text{TNF}\alpha$ (36,37). Since $\text{TNF}\alpha$ can also activate NF- κ B (36,38), a feed-forward mechanism, resulting in increased production of both cytokines and ROS, will be set in motion upon LPS exposure.

Quercetin is an excellent scavenger of both ROS and RNS. Consequently, the flavonoid might be used to reduce both oxidative stress, i.e. an imbalance between the production of and the protection against reactive species, and inflammation. Moreover, quercetin can also inhibit NF- κ B activation, thereby directly reducing the cytokine production via this transcription factor (10). Both these capacities of the flavonoid may contribute to the counteracting effect of quercetin on the LPS-induced $\text{TNF}\alpha$ production, as we observed *in vitro* in the present study. This is in line with the inhibiting effect of the antioxidant β -carotene found in alveolar macrophages on LPS-induced NF- κ B activation and ROS generation (39).

The total antioxidant status shows a small, but significant, increase after the quercetin supplementation. Interestingly, this increase significantly surpasses the increase of the plasma quercetin concentration. Vitamin C and uric acid levels, important determinants of the total antioxidant capacity, are not affected by the supplementation. This indicates that various metabolites of quercetin, such as small phenolic compounds, might have a substantial contribution to the rise in total antioxidant capacity. The residual antioxidant capacity, also corrected for the contribution of quercetin in the plasma, is still significantly enlarged after four weeks quercetin supplementation. Apparently, the supplementation also has a small, but persistent, effect on the residual antioxidant capacity.

The *in vitro* experiments with quercetin and LPS added to the blood show that the inhibitory effect of quercetin was more potent at higher $\text{TNF}\alpha$ levels. This means that quercetin displays a more pronounced anti- $\text{TNF}\alpha$ effect when the production of this cytokine is elevated. Consequently, especially in people suffering from a disease of which the pathology is associated with elevated levels of inflammation, quercetin supplementation might exert a beneficial effect. The same probably applies for oxidative stress; only in people suffering from oxidative stress, strengthening the antioxidant defence through quercetin supplementation might be expected to be beneficial. This indicates that health-benefit studies regarding antioxidant supplementation should not be conducted in healthy volunteers that are expected not to display inflammation or oxidative stress during the intervention period.

In the *in vivo* experiment, the basal $\text{TNF}\alpha$ levels are not affected by the quercetin supplementation. As indicated by the *in vitro* experiment, this absence of an *in vivo* effect might be due to the baseline $\text{TNF}\alpha$ levels in the healthy subjects that were already very low and could therefore not further be reduced.

The effect of the quercetin supplementation has also been evaluated *ex vivo*. To the blood of the volunteers obtained both before and after supplementation, LPS was added to induce an inflammatory response. Still, no significant decrease of *ex vivo*-induced $\text{TNF}\alpha$ production could be observed after supplementation. Moreover, no correlation could be found between the increase in plasma quercetin concentration and the *ex vivo*-induced $\text{TNF}\alpha$ -levels. This absence of an effect of quercetin supplementation on the LPS-induced $\text{TNF}\alpha$ production *ex vivo* might be due to the small effect of the supplementation on the total plasma antioxidant capacity. Although the increase caused by the supplementation is statistically significant, it is relatively low compared to the fairly high total plasma antioxidant capacity already present at baseline in healthy volunteers. This is an extra argument that health benefit studies regarding antioxidants should not be conducted in

healthy volunteers not suffering from oxidative stress. When the intake of antioxidants, e.g. by a healthy diet, is adequate and there is no oxidative stress, there is no need to supply extra antioxidants. When the antioxidant capacity has declined, as for instance in diseases associated with increased production of ROS, empowering the weakened endogenous antioxidant shield is expected to be helpful. The rationale for antioxidant supplementation in healthy subjects is present when it is foreseen that a situation resulting in oxidative stress is likely to arise. The supplementation might then be used as a protective measure to prevent damage.

In the protection against free radicals, quercetin becomes converted into highly thiol-reactive and potential toxic oxidation products (40,41). In the present study, the level of glutathione (GSH), the most abundant and reactive endogenous thiol, is unaltered by the given quercetin supplementation. This indicates that the possible formation of reactive oxidation products has no major consequences in the applied dosing regime. Since this study concerns healthy volunteers with hardly any oxidative stress, the absence of quercetin-induced toxicity might be due to the limited formation of oxidized quercetin compared to the high levels of GSH. The expected limited formation of oxidized quercetin makes the information obtained with supplementation studies in healthy volunteers of limited value for predicting the toxicity of quercetin in patients with oxidative stress. In these patients, the formation of oxidized quercetin is higher and levels of GSH might be lower. This indicates that in patients with oxidative stress, the formation of reactive oxidation products of quercetin and the toxicity induced by these reactive metabolites might be more pronounced.

In conclusion, the results of the present study show anti-inflammatory effects of quercetin *in vitro* but not *ex vivo* or *in vivo* in healthy volunteers. The fact that no indication has been found for an anti-inflammatory effect in healthy volunteers after increasing the dietary intake of an antioxidant, is probably due to (i) the relatively small increase of the antioxidant capacity of healthy volunteers that is already high at baseline and (ii) the low inflammatory status of these subjects. In retrospect, the design of the present study is thus not optimal for demonstrating potential health effects of quercetin. Antioxidant supplementation in healthy volunteers that are not likely to have inflammation or oxidative stress is superfluous and, therefore, will not result in substantial beneficial health effects in these subjects. A healthy and diverse diet normally supplies sufficient antioxidants, provided that no enhanced ROS production is expected. There is only a rationale for any supplementation when on forehand a beneficial effect is foreseen. Actually, this has insufficiently been realised in the design of a great many antioxidant studies and may explain why various major trials on the preventive effect of antioxidant supplementation in healthy subjects had a

disappointing outcome. Particularly in people with increased levels of inflammation and oxidative stress, antioxidant supplementation is expected to be fruitful.

References

1. Diplock AT, Charleux JL, Crozier-Willi G, Kok FJ, Rice-Evans C, Roberfroid M, Stahl W and Vifia-Ribes J. Functional food science and defence against reactive oxidative species. *Brit. J. Nutr.* 1988;80:S77-S112.
2. Hertog MG, Feskens EJ, Hollman PC, Katan MB and Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* 1993;342:1007-1011.
3. De Vries JH, Janssen PL, Hollman PC, van Staveren WA and Katan MB. Consumption of quercetin and kaempferol in free-living subjects eating a variety of diets. *Cancer Lett* 1997;114:141-144.
4. Kuehnau A. The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev Nutr Diet* 1976;24:117-191.
5. Arts IC and Hollman PC. Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr* 2005;81:317S-325S.
6. Haenen GR, Paquay JB, Korthouwer RE and Bast A. Peroxynitrite scavenging by flavonoids. *Biochem Biophys Res Commun* 1997;236:591-593.
7. Haenen GR and Bast A. Nitric oxide radical scavenging of flavonoids. *Methods Enzymol* 1999;301:490-503.
8. Wilms LC, Hollman PC, Boots AW and Kleinjans JC. Protection by quercetin and quercetin-rich fruit juice against induction of oxidative DNA damage and formation of BPDE-DNA adducts in human lymphocytes. *Mutat Res* 2005;582:155-162.
9. Zhang Y, Li H, Zhao Y and Gao Z. Dietary supplementation of baicalin and quercetin attenuates iron overload induced mouse liver injury. *Eur J Pharmacol* 2006;535:263-269.
10. Nair MP, Mahajan S, Reynolds JL, Aalinkeel R, Nair H, Schwartz SA and Kandaswami C. The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF-kappabeta system. *Clin Vaccine Immunol* 2006;13:319-328.
11. Manjeet KR and Ghosh B. Quercetin inhibits LPS-induced nitric oxide and tumor necrosis factor-alpha production in murine macrophages. *Int J Immunopharmacol* 1999;21:435-443.
12. Ziegenhagen MW, Schrum S, Zissel G, Zipfel PF, Schlaak M and Muller-Quernheim J. Increased expression of proinflammatory chemokines in bronchoalveolar lavage cells of patients with progressing idiopathic pulmonary fibrosis and sarcoidosis. *J Investig Med* 1998;46:223-231.
13. Thorn J. The inflammatory response in humans after inhalation of bacterial endotoxin: a review. *Inflamm Res* 2001;50:254-261.
14. Swennen EL, Bast A and Dagnelie PC. Immunoregulatory effects of adenosine 5'-triphosphate on cytokine release from stimulated whole blood. *Eur J Immunol* 2005;35:852-858.
15. Fischer KD, Tedford K and Wirth T. New roles for Bcl10 in B-cell development and LPS response. *Trends Immunol* 2004;25:113-116.
16. De Groote D, Zangerle PF, Gevaert Y, Fassotte MF, Beguin Y, Noizat-Pirenne F, Pirenne J, Gathy R, Lopez M, Dehart I et al. Direct stimulation of cytokines (IL-1 beta, TNF-alpha, IL-6, IL-2, IFN-gamma and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation. *Cytokine* 1992;4:239-248.
17. Van den Berg R, van Vliet T, Broekmans WM, Cnubben NH, Vaes WH, Roza L, Haenen GR, Bast A and van den Berg H. A vegetable/fruit concentrate with high antioxidant capacity has no effect on biomarkers of antioxidant status in male smokers. *J Nutr* 2001;131:1714-1722.
18. Olthof MR, Hollman PC, Vree TB and Katan MB. Bioavailabilities of quercetin-3-glucoside and quercetin-4'-glucoside do not differ in humans. *J Nutr* 2000;130:1200-1203.
19. Arts IC, Venema DP and Hollman PC. Quantitative determination of flavonoids in plant foods and biological fluids. Santos-Buelga C. *Methods in polyphenols analysis*, Cambridge: The Royal Society of Chemistry, 2003.

20. Fischer MA, Gransier TJ, Beckers LM, Bekers O, Bast A and Haenen GR. Determination of the antioxidant capacity in blood. *Clin Chem Lab Med* 2005;43:735-740.
21. Arts MJ, Dallinga JS, Voss HP, Haenen GR and Bast A. A new approach to assess the total antioxidant capacity using the TEAC assay. *Food Chem* 2004;88:567-570.
22. Speek AJ, Schrijver J and Schreurs WH. Fluoremetric determination of total vitamin C in whole blood by high performance liquid chromatography with pre-column derivatization. *J Chrom Biomed Appl* 1984;305:53-60.
23. Benzie IF, Chung W and Tomlinson B. Simultaneous measurement of allantoin and urate in plasma: analytical evaluation and potential clinical application in oxidant:antioxidant balance studies. *Clin Chem* 1999;45:901-904.
24. Griffith OW. Determination of GSH and GSSG using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 1980;106:207-212.
25. Vernooy JH, Dentener MA, van Suylen RJ, Buurman WA and Wouters EF. Long-term intratracheal lipopolysaccharide exposure in mice results in chronic lung inflammation and persistent pathology. *Am J Respir Cell Mol Biol* 2002;26:152-159.
26. Chen LY, Zuraw BL, Zhao M, Liu FT, Huang S and Pan ZK. Involvement of protein tyrosine kinase in Toll-like receptor 4-mediated NF-kappa B activation in human peripheral blood monocytes. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L607-613.
27. Hartung J and Seedorf J. Orienting endotoxin measurement in the atmosphere. *Dtsch Tierarztl Wochenschr* 1999;106:522-525.
28. Kline JN, Cowden JD, Hunninghake GW, Schutte BC, Watt JL, Wohlford-Lenane CL, Powers LS, Jones MP and Schwartz DA. Variable airway responsiveness to inhaled lipopolysaccharide. *Am J Respir Crit Care Med* 1999;160:297-303.
29. Hasday JD, Bascom R, Costa JJ, Fitzgerald T and Dubin W. Bacterial endotoxin is an active component of cigarette smoke. *Chest* 1999;115:829-835.
30. Paquay JB, Haenen GR, Stender G, Wiseman SA, Tjburg LB and Bast A. Protection against nitric oxide toxicity by tea. *J Agric Food Chem* 2000;48:5768-5772.
31. Michel O, Kips J, Duchateau J, Vertongen F, Robert L, Collet H, Pauwels R and Sergysels R. Severity of asthma is related to endotoxin in house dust. *Am J Respir Crit Care Med* 1996;154:1641-1646.
32. Schwartz DA, Thorne PS, Yagla SJ, Burmeister LF, Olenchock SA, Watt JL and Quinn TJ. The role of endotoxin in grain dust-induced lung disease. *Am J Respir Crit Care Med* 1995;152:603-608.
33. Smid T, Heederik D, Houba R and Quanjer PH. Dust- and endotoxin-related acute lung function changes and work-related symptoms in workers in the animal feed industry. *Am J Ind Med* 1994;25:877-888.
34. Srisook K, Han SS, Choi HS, Li MH, Ueda H, Kim C and Cha YN. CO from enhanced HO activity or from CORM-2 inhibits both O₂⁻ and NO production and downregulates HO-1 expression in LPS-stimulated macrophages. *Biochem Pharmacol* 2006;71:307-318.
35. Khanduja KL, Sohi KK, Pathak CM and Kaushik G. Nimesulide inhibits lipopolysaccharide-induced production of superoxide anions and nitric oxide and iNOS expression in alveolar macrophages. *Life Sci* 2006;78:1662-1669.
36. Rahman I. Oxidative stress, transcription factors and chromatin remodelling in lung inflammation. *Biochem Pharmacol* 2002;64:935-942.
37. MacNee W. Oxidative stress and lung inflammation in airways disease. *Eur J Pharmacol* 2001;429:195-207.
38. Rahman I and MacNee W. Role of transcription factors in inflammatory lung diseases. *Thorax* 1998;53:601-612.
39. Bai SK, Lee SJ, Na HJ, Ha KS, Han JA, Lee H, Kwon YG, Chung CK and Kim YM. beta-Carotene inhibits inflammatory gene expression in lipopolysaccharide-stimulated macrophages by suppressing redox-based NF-kappaB activation. *Exp Mol Med* 2005;37:323-334.
40. Galati G, Moridani MY, Chan TS and O'Brien PJ. Peroxidative metabolism of apigenin and naringenin versus luteolin and quercetin: glutathione oxidation and conjugation. *Free Radic Biol Med* 2001;30:370-382.

41. Boots AW, Kubben N, Haenen GR and Bast A. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. *Biochem Biophys Res Commun* 2003;308:560-565.

8

Antioxidant status associated with inflammation in sarcoidosis: a potential role for antioxidants

AW Boots, M Drent, ELR Swennen, HJJ Moonen, A Bast, GRMM Haenen
Submitted

Abstract

Background

Enhanced production of reactive oxygen species (ROS) is suggested to play a pivotal role in sarcoidosis. The high production of ROS is expected to reduce antioxidant levels. Since ROS can enhance inflammation, antioxidant supplementation might, also mitigate elevated inflammation. The aim of the present study is to determine both the antioxidant and inflammatory status in sarcoidosis. Furthermore, the potential of the antioxidant quercetin to mitigate inflammation will be assessed.

Methods

Non-smoking patients with sarcoidosis were enrolled as well as matched controls. Measurements included assessment of total plasma antioxidant capacity, vitamin C, uric acid, glutathione, basal and lipopolysaccharide (LPS)-induced levels of tumour necrosis factor alpha (TNF α), interleukin (IL)-8 and IL-10 and the effect of quercetin on these levels.

Results

Sarcoidosis patients displayed a significantly lower total plasma antioxidant capacity and decreased levels of the antioxidants vitamin C, uric acid and glutathione compared to their controls. Basal TNF α and IL-8 levels were significantly increased in the sarcoidosis group, whereas basal IL-10 levels were unaffected. Quercetin significantly decreased *ex vivo* LPS-induced TNF α - and IL-8 production in a concentration-dependent manner, but did not affect LPS-induced IL-10 production, in both the patient and control group. Interestingly, this quercetin effect was more pronounced in sarcoidosis patients.

Conclusions

The endogenous antioxidant defence is significantly reduced in sarcoidosis, suggesting that increased production of ROS is associated with the pathology of this disease. Moreover, the levels of the pro-inflammatory cytokines TNF α and IL-8 are significantly enhanced in sarcoidosis. The antioxidant quercetin significantly reduces the *ex vivo* TNF α and IL-8 production and this effect is more pronounced in sarcoidosis patients. This implies that these patients might benefit from antioxidant supplementation not only by empowering the relatively low protection against ROS but also by reducing inflammation.

Introduction

Sarcoidosis is an interstitial lung disease which incidence varies among different countries over the world. In Scandinavian countries the incidence is higher compared to more southern countries (1). In the Netherlands and Germany it is estimated that the incidence is approximately 20-25 per 100.000 inhabitants (1).

Sarcoidosis is an antigen-driven, multisystem, granulomatous disorder of which the exact aetiology is unknown (2,3). Current evidence supports the concept that the pathogenesis of sarcoidosis involves a highly polarized T-helper 1 Th1 immune response to pathogenic tissue antigens or specific environmental factors (1). Granuloma formation is regulated by a complex interaction between Th lymphocytes and macrophages, in which cytokines such as tumour necrosis factor alpha (TNF α) play an important role (2).

Enhanced formation of reactive oxygen species (ROS) is suggested to play a pivotal role in the aetiology of sarcoidosis (4,5). However, little is known about the endogenous defence levels present in sarcoidosis, i.e. the antioxidant levels that can offer protection against ROS-mediated damage. This prompted us to determine the endogenous antioxidant levels present in sarcoidosis patients. It is expected that the high production of ROS that occurs in sarcoidosis will consume antioxidants, thereby reducing their levels. As a result, antioxidant supplementation might be beneficial in sarcoidosis treatment.

ROS are also capable of initiating and mediating inflammation in the lung (6,7). Besides enhanced ROS formation, inflammation plays a key-role in the occurrence and progression of sarcoidosis too (8,9). Conventional treatment is focused on attenuating granuloma formation with drugs that inhibit antigen presentation or with nonspecific anti-inflammatory agents such as glucocorticosteroids, methotrexate, or azathioprine (2). However, all these therapies fail to be completely efficacious (10,11). Strengthening the endogenous antioxidant defence with antioxidant supplementation, and thereby decreasing the levels of ROS production and ROS-mediated damage, might mitigate the elevated inflammation present.

The past few years, much attention has been given to the potential health-beneficial properties of flavonoids, natural occurring polyphenolic compounds, and to quercetin, the most commonly occurring flavonoid, in particular. Quercetin is an extremely powerful antioxidant (12) and therefore it is tempting to speculate that quercetin can exert positive effects in sarcoidosis.

The aim of the present study is to determine both the anti-oxidant and inflammatory status in sarcoidosis. As markers of the antioxidant status, the endogenous antioxidants glutathione (GSH), vitamin C and uric acid are

measured as well as the total antioxidant capacity. As markers of the inflammatory status, the pro-inflammatory cytokines $\text{TNF}\alpha$ and interleukin (IL)-8 and the anti-inflammatory cytokine IL-10 are measured. Furthermore, the potential anti-inflammatory effects of antioxidants, exemplified with the flavonoid quercetin, will be assessed in sarcoidosis. To this extent, cytokine production will be evoked *ex vivo* by lipopolysaccharide (LPS), a pathophysiological relevant stimulator of monocytes, neutrophils and B lymphocytes (13-16).

Material and Methods

Chemicals

Quercetin and lipopolysaccharide (LPS, *E. coli* 0.26:B6) were purchased from Sigma Chemical Co. (St. Louis, USA). RPMI 1640 medium containing L-glutamine was obtained from *Gibco (UK)*. Human $\text{TNF}\alpha$ (7300 pg/ml), human IL-10 (4000 pg/ml) and human IL-8 (10 ng/ml) were acquired from CLB/Sanquin (Amsterdam, The Netherlands). All other chemicals were of analytical grade.

Methods

Participants

General information

All participating patients were recruited from the patients visiting the out-patient clinic of the university hospital Maastricht, a tertiary referral for the Netherlands. The experiment took place at the day they had to come in for a regular check-up. Based on food questionnaires, all patients had comparable dietary habits with an average daily intake of quercetin of approximately 15 mg (data not shown). None of the participants used any vitamin or food supplementation.

The Medical Ethical Committee of Maastricht University and the University Hospital Maastricht had approved the protocol before the beginning of the study. All participants were fully informed about the aim and details of the study and have given their written informed consent.

Sarcoidosis patients

Twenty non-smoking patients with symptomatic sarcoidosis (age 44 ± 1) were enrolled in this study. Patients had been diagnosed with sarcoidosis based on clinical features, together with bronchoalveolar lavage (BAL) fluid analysis results (data not shown) (17). Moreover, 17 out of the 20 sarcoidosis

patients had a biopsy confirmation of the disease. The clinical symptoms of all patients included respiratory symptoms, i.e. dyspnea, coughing and chest pain. The characteristics of the studies population are summarized in Table 8.1.

Table 8.1 Characteristics of the participants.

	Sarcoidosis	Controls
number (m/f)	20 (16/4)	11 (7/4)
age	31-60 (44 ± 1)	40-58 (48 ± 3)
length	158-194 (177 ± 2)	162-188 (175 ± 3)
weight	52-107 (80 ± 4)	59-95 (77 ± 3)
body mass index	19-31 (25 ± 1)	19-30 (26 ± 1)
time since diagnosis	0-30 (5 ± 2)	-
biopsy taken	yes: 17 no: 3	-
DLCO	39-107 (80 ± 3)	-
FEV ₁	42-135 (84 ± 6)	-
FVC	49-152 (92 ± 6)	-
chest radiograph stage 0/I/II/III/IV (n)	1/4/9/2/4	-

Controls are matched on age and gender and do therefore not significantly differ from the patients regarding these parameters. Age is expressed in year, length in cm, weight in kg, and DLCO (diffusing capacity of the lung for carbon monoxide), FEV₁, FVC (forced vital capacity) (forced expiratory volume in 1 second) in % of the predicted value based on age and gender. Data are expressed as range (mean $\pm$ SEM).

According to their chest radiographic stage, patients were subdivided into two groups, i.e. mild (stage 0-1) and severe sarcoidosis (stage 2-4). In the sarcoidosis patients, both the DLCO ($80 \pm 3\%$) and the FEV₁ ($84 \pm 6\%$) were reduced. Both the FEV₁ and DLCO values of stage 4 sarcoidosis patients were significantly lower than those of the other sarcoidosis patients ($52 \pm 10\%$ (n=4) vs $91 \pm 5\%$ (n=16) and $60 \pm 4\%$ (n=4) vs $83 \pm 3\%$ (n=16) respectively).

At the moment this study was performed, none of the sarcoidosis patients used any medication.

Controls

The control group consisted of 11 non-smoking healthy volunteers, all with dietary habits comparable to those of the patients and a quercetin

intake of approximately 15 mg per day. All controls did not use any medication or vitamin/ food supplementation.

Lung function measurement

Lung function measurements included FEV₁ and DLCO. FEV₁ was measured with a pneumotachograph, DLCO by the single-breath method (Masterlab, Jaeger, Würzburg, Germany). Values were expressed as a percentage of those predicted based on age and gender (18)

Preparation of blood samples

Blood was collected from all participants in EDTA-containing vacutainer tubes (Vacutainer, Becton-Dickinson) and kept on ice prior to processing which occurred within 1 hour after blood collection. Blood was aliquoted into eppendorfs for both the ascorbic acid and the GSH/GSSG analysis: for the former 10% TCA was added to the whole blood, whereas 1.3% SSA in 10 mM HCl was used to preserve the samples for the latter. Another aliquot of blood was used for the incubations required for the blood-based cytokine production assay as described in that section below. The remaining blood was centrifuged (3000 rpm, 5' at 4°C) to obtain plasma. Deproteinization of an aliquot of this plasma, using 10% TCA (1:1) followed by centrifugation (13.000 rpm, 5' at 4°C), was carried for the trolox equivalent antioxidant capacity measurement. All samples were stored at -80°C prior to analysis.

Trolox antioxidant capacity

The trolox equivalent antioxidant capacity (TEAC value) is a measurement for the total antioxidant status, relating the free radical scavenging properties of a solution or a compound to that of the synthetic antioxidant trolox. The assay is performed as previously described (19).

Ascorbic acid measurement

Calibrators were prepared freshly, containing the same amount of TCA as the samples. Samples and calibrators were processed identically as described previously (20).

Uric acid measurement

Uric acid was measured in the plasma of all samples as described previously (21).

GSH, GSSG and haemoglobin measurement

Both GSH and GSSG calibrators were prepared freshly and contained the same concentrations of SSA as the samples. Samples and calibrators were treated identically as described previously (22).

Blood-based cytokine production assay

Within one hour after blood collection, the blood-based cytokine production assay was performed as described previously (14). Care was taken that handling of the blood prior to LPS-stimulation did not influence the cytokine release.

Enzyme linked immune sorbent assay (ELISA) measurement

All cytokines were quantified using PeliKine Compact human ELISA kits (CLB/Sanquin, The Netherlands) based on appropriate and validated sets of monoclonal antibodies. Assays were performed as described in the manufacturer's instructions. Cytokine production was related to that of the control incubation without quercetin. The ethanol (0.5%) used to dissolve quercetin did not show any influence on the *ex vivo* LPS-induced cytokine production (data not shown).

Statistics

The data of both the patient and control group were compared using a Mann Whitney U test. A two-tailed probability value (P-value) equal to or less than 0.05 was considered to be statistically significant.

Results

All antioxidant parameters measured were decreased in the blood from sarcoidosis patients (Figure 8.1) compared to the antioxidant-levels of their age-, gender- and dietary behaviour matched controls.

Reduced glutathione (GSH) was significantly declined in the blood of sarcoidosis patients compared to the GSH-level in matched controls (25% decline). Glutathione disulphide (GSSG) levels, that are relatively low because of the efficient reduction of GSSG by endogenous glutathione reductase, did not significantly differ between the patient and control group.

The total plasma antioxidant capacity, i.e. the total sum of all plasma antioxidants that is expressed as trolox equivalents, was in sarcoidosis patients approximately 75% of that of the matched controls.

Two endogenous antioxidants that are known to contribute substantially to the total plasma antioxidant status are uric acid and vitamin C. Uric acid levels as well as vitamin C levels were significantly declined in the plasma of sarcoidosis patients (to respectively 60% and 79% of the control levels) compared to their matched controls.

Figure 8.1. Antioxidant status in sarcoidosis patients compared to that in their matched controls. The values representing the levels of the various parameters in the patient group are connected to form the dark grey area whereas the light grey area reflects the same in the control group. Total plasma antioxidant status (TEAC) is expressed as Trolox equivalents. All axes have a linear scale. The origin is zero. The value at the end of the axes is indicated by the number depicted there. Data are expressed as mean $\pm$ SEM; *= $P < 0.03$ vs the matched controls.

The TEAC value of sarcoidosis patients is significantly lower than that of their matched controls ($535 \pm 21 \mu\text{M}$ versus $652 \pm 21 \mu\text{M}$). The levels of individual antioxidants, i.e. uric acid, glutathione and vitamin C, are also significantly reduced in the patient group compared to the controls (respectively $185 \pm 16 \mu\text{M}$ vs $324 \pm 15 \mu\text{M}$; $8.2 \pm 0.6 \mu\text{M}$ vs $13 \pm 1.9 \mu\text{M}$; $52 \pm 4 \mu\text{M}$ vs $65 \pm 4 \mu\text{M}$).

Basal levels of both $\text{TNF}\alpha$ and IL-8 (Figure 8.2A), two pro-inflammatory cytokines, were significantly increased in patients suffering from sarcoidosis compared to their controls. Basal levels of the anti-inflammatory cytokine IL-10 were not different between the sarcoidosis patients and the matched controls (Figure 8.2A). As a result, the ratios of the pro- versus the anti-inflammatory cytokines $\text{TNF}\alpha/\text{IL-10}$ and $\text{IL-8}/\text{IL-10}$ were also significantly increased in the sarcoidosis patients.

The LPS-induced $\text{TNF}\alpha$ and IL-8 levels (Figure 8.2B) found in the sarcoidosis group were significantly higher compared to the controls, whereas IL-10 production was similar in both groups after LPS stimulation (Figure 8.2B).

Figure 8.2. Basal (panel A) as well as LPS-induced levels (panel B) of $\text{TNF}\alpha$, IL-8 and IL-10 in sarcoidosis patients compared to their matched controls. Basal $\text{TNF}\alpha$ - and IL-8 levels are both significantly higher in the patient group compared to their matched controls ($\text{TNF}\alpha$: 22 ± 2 pg/ml vs 5 ± 0.3 pg/ml; IL-8: 12.3 ± 1.7 pg/ml vs 7.9 ± 0.4 pg/ml). LPS-induced $\text{TNF}\alpha$ - and IL-8 levels are both significantly higher in the patients than in the controls. LPS-induced IL-8 levels are approximately 8 times higher than the LPS-induced $\text{TNF}\alpha$ - and IL-10 levels. Data are expressed as mean $\pm$ SEM; * $P \leq 0.01$ vs the matched controls for panel A; * $P < 0.04$ vs the matched controls for panel B.

The flavonoid quercetin significantly reduced the $\text{TNF}\alpha$ (Figure 8.3A) and IL-8 production (Figure 8.3B), induced in blood by LPS, in the sarcoidosis group as well as in their matched controls. The extend of this reduction depended on the quercetin concentration. Both cytokines responded more sensitive (50% for IL-8 and 80% for $\text{TNF}\alpha$) to quercetin in the sarcoidosis group compared to the matched controls (Figure 8.4A and 8.4B). The IL-10 production, induced in blood by LPS, of both patients and controls was not affected by quercetin (Figure 8.3C). As a result, the ratios of the pro- versus the anti-inflammatory cytokines $\text{TNF}\alpha/\text{IL-10}$ and $\text{IL-8}/\text{IL-10}$ were also significantly reduced by the flavonoid.

Figure 8.3 Modulating effect of quercetin on the LPS-induced TNF α , IL-8 and IL-10 production in sarcoidosis (panel A, B and C respectively) compared to their matched controls. Results are expressed in percentage, with 100% representing the cytokine-release under stimulation of LPS in the absence of quercetin. Data are expressed as mean $\pm$ SEM; * = $P < 0.01$ vs the incubation containing no quercetin; # = $P < 0.05$ vs controls and vs the incubation containing no quercetin.

Antioxidant as well as cytokine levels did not display significant differences between the two sexes. No difference was found between the mild and severe form of sarcoidosis regarding the residual plasma antioxidant capacity or the GSSG, uric acid, vitamin C, basal IL-8 and basal IL-10 levels. Compared to the mild form of sarcoidosis, the severe form tended to display slightly lower levels of GSH (6.8 ± 1.1 μ M (n=15) vs 7.6 ± 1.3 μ M (n=5)); and total plasma antioxidant capacity (519 ± 19 μ M (n=13) vs 533 ± 30 μ M (n=5)); and slightly higher levels of TNF α (24 ± 3 pg/ml (n=15) vs 19 ± 3 pg/ml (n=5)). Stage 4 sarcoidosis patients displayed a significant lower total plasma antioxidant capacity compared to the other sarcoidosis patients (473 ± 15 μ M vs 543 ± 23 μ M; $P \leq 0.05$).

Figure 8.4 The effect of quercetin (1 μ M) on LPS-induced TNF α (panel A) and IL-8 production (panel B) in both sarcoidosis patients and their matched controls. The decrease in LPS-induced cytokine production caused by quercetin (Q) (y-axis) is plotted against the LPS-induced cytokine production in the absence of the flavonoid (x-axis). The slopes of panel A indicate that on average, 1 μ M quercetin reduces the TNF α production in controls with 27% and in patients with 46%. For IL-8, this reduction is on average 19% in controls and 27% in patients (panel B). Data are expressed as individual values (n=20 for the patients; n=11 for the controls).

Discussion

The role of enhanced ROS production has been implicated in the pathology of sarcoidosis (4,5), but the influence of this increased ROS formation on the endogenous antioxidant levels in sarcoidosis have not yet been reported. To the best of our knowledge, this study is the first that demonstrates a significant decrease in the total plasma antioxidant capacity as well as in the blood levels of the important endogenous antioxidants GSH, vitamin C and uric acid in sarcoidosis patients compared to healthy controls matched for age, gender and dietary behaviour. The severe form of sarcoidosis tends to display a lower total plasma antioxidant capacity as well as a lower GSH level, indicating that the severity of the disease might be related to the level of oxidative stress occurring.

The patients and controls are matched for their dietary behavior, indicating that the observed decreased antioxidant levels are not the result of a different dietary intake. The lower endogenous antioxidant defence found, therefore, confirms the elevated production of ROS present in sarcoidosis. Low endogenous antioxidant levels combined with enhanced ROS formation is defined as oxidative stress. Oxidative stress can cause serious oxidative damage to biological macromolecules like DNA, lipids and proteins (23).

Several studies have shown that the levels of biomarkers of oxidative damage, i.e. exhaled ethane (4) and both 8-isoprostane (24) and oxidized proteins (25) in the bronchoalveolar lavage fluid (BALF), are indeed increased in sarcoidosis patients of different clinical stages. Furthermore, the transcription factor NF- κ B, of which it is known that it is activated by radical damage, is increased in alveolar macrophages (26) and mononuclear blood cells (27) of active sarcoidosis patients compared to those of healthy controls.

To strengthen the endogenous antioxidant defence and thus offer more protection against ROS, antioxidant supplementation seems a logical strategy in the treatment of sarcoidosis. Moreover, antioxidant supplementation might not only protect against ROS-mediated damage, it might also mitigate elevated inflammation since ROS can enhance inflammation. ROS are capable of promoting inflammation in the lung by activating transcription factors like NF- κ B and activator protein-1 that induce pro-inflammatory cytokines and chemokines (6,7). *In vitro* studies, using both macrophages and alveolar and bronchial epithelial cells, have demonstrated that oxidants can indeed initiate the production of inflammatory mediators like IL-8 and NO $\cdot$ (28). Because of this relation between ROS and inflammatory processes, the inflammatory status was also evaluated in sarcoidosis patients. To this end, basal levels of the pro-inflammatory cytokines TNF α and IL-8 and the anti-inflammatory cytokine IL-10 were measured.

TNF α was chosen since it is suggested to be the most prominent cytokine present in sarcoidosis (2,29). TNF α has been shown to play a pivotal role in the granula formation occurring in sarcoidosis (2). Furthermore, the individual capability of a patient to release TNF α is suggested to be linked to the progression of the disease, thereby linking this cytokine to the pathogenesis of sarcoidosis (30). TNF α is capable of activating transcription factors NF- κ B and AP-1 that subsequently will further enhance other inflammatory mediators such as IL-8 (7,31) and TNF α itself, thereby amplifying the TNF α -mediated inflammatory effects.

Our study shows that basal TNF α -levels are significantly enhanced in sarcoidosis patients compared to healthy controls. This finding is in line with previous studies (32,33). The severe form of sarcoidosis tends to display higher TNF α levels, indicating that the severity of the disease might be related to the level of inflammation occurring. Conventional therapy with glucocorticoids fails to reduce the enhanced NF- κ B activity found in sarcoidosis patients (27), whereas alternative strategies using anti-TNF α agents display a clinical beneficial effect (34).

Basal IL-8 levels are significantly increased in sarcoidosis, indicating that this cytokine is also involved in the enhanced inflammatory basal status in

this disease. This is again in line with previous studies, which show an increased expression of IL-8 in sarcoidosis (2,35,36). It should be noted that IL-8 can also be induced by $\text{TNF}\alpha$ and that the higher IL-8 level might reflect the higher $\text{TNF}\alpha$ level.

No changes were found in the basal levels of anti-inflammatory cytokine IL-10 between the sarcoidosis and control group. The results of previous studies on this cytokine are ambiguous. Some studies report enhanced IL-10 levels in the broncho-alveolar lavage fluid (BALF) of sarcoidosis patients as a compensatory mechanism (35,36), whereas other studies fail to demonstrate this increase (37,38).

To determine the anti-inflammatory potential of antioxidants, the effect of quercetin on LPS-induced production of $\text{TNF}\alpha$, IL-8 and IL-10 was quantified in blood. The employed model, unlike models using isolated cells or cultured cells grown in medium, represents a more physiological and well reproducible model to measure cytokine production *ex vivo* (14,39). The natural cell-to-cell interactions are preserved and all blood components are present in *in vivo* ratios with non-cellular components, resulting in a system that reflects the *in vivo* condition well (14,39).

In both the sarcoidosis and the control group, the antioxidant quercetin significantly reduces the LPS-induced $\text{TNF}\alpha$ and IL-8 production, whereas the LPS-induced IL-10 production is not significantly altered by the flavonoid. The inhibitory effect of quercetin on both anti-inflammatory cytokines is dose-dependent and could already be achieved at an *in vivo* attainable concentration of 1 μM (40). Interestingly, the anti-inflammatory effects of quercetin are much more pronounced in the sarcoidosis patients than in their matched controls.

The anti-inflammatory actions of quercetin observed in the present study could very well be related to its anti-oxidative activity. This association between the anti-oxidative and anti-inflammatory capabilities of flavonoids has also been suggested in several *in vitro* (41,42) and *in vivo* studies (43,44). The reduced antioxidant status may also explain both (i) why *ex vivo* LPS-induced $\text{TNF}\alpha$ and IL-8 production are significantly higher in sarcoidosis patients than in their matched controls and (ii) why quercetin exerts a more pronounced effect on this $\text{TNF}\alpha$ and IL-8 production in sarcoidosis patients compared to the controls. Therefore, sarcoidosis patients are expected to profit from the use of antioxidant supplementation. The fact that quercetin is a dietary antioxidant indicates that the diet is an important factor in sarcoidosis. However, the toxicity of quercetin should be carefully evaluated especially when it is chronically administered (45). Clinical studies are needed to substantiate the efficacy as well as the safety of quercetin supplementation.

In conclusion, our study shows for the first time that the endogenous anti-oxidant levels as well as the total plasma antioxidant capacity are significantly reduced in sarcoidosis, emphasizing that increased production of ROS is associated the pathology of this disease. Moreover, inflammation is enhanced in sarcoidosis as shown by the increased basal levels of the pro-inflammatory cytokines $\text{TNF}\alpha$ and IL-8. The antioxidant quercetin shows ex vivo significant anti-inflammatory effects in both sarcoidosis patients and controls that are, interestingly, more pronounced in the first group. Probably, this larger anti-inflammatory effect of the flavonoid in sarcoidosis is associated with the compromised antioxidant status present in these patients. This suggests that particularly in sarcoidosis patients empowering the antioxidant defense system with alimentary antioxidants, such as quercetin, might be fruitful. This is especially important since conventional treatment with glucocorticoids fails to be efficacious.

References

1. Thomeer M, Demedts M and Wuyts W. Epidemiology of sarcoidosis. *Eur Respir Mon* 2005;32:1-10.
2. Baughman RP, Lower EE and du Bois RM. Sarcoidosis. *Lancet* 2003;361:1111-1118.
3. Newman LS, Rose CS and Maier LA. Sarcoidosis. *N Engl J Med* 1997;336:1224-1234.
4. Kanoh S, Kobayashi H and Motoyoshi K. Exhaled ethane: an in vivo biomarker of lipid peroxidation in interstitial lung diseases. *Chest* 2005;128:2387-2392.
5. Halliwell B, Gutteridge JM and Cross CE. Free radicals, antioxidants, and human disease: where are we now? *J Lab Clin Med* 1992;119:598-620.
6. MacNee W. Oxidative stress and lung inflammation in airways disease. *Eur J Pharmacol* 2001;429:195-207.
7. Rahman I. Oxidative stress, transcription factors and chromatin remodelling in lung inflammation. *Biochem Pharmacol* 2002;64:935-942.
8. Papakosta D, Kyriazis G, Gioulekas D, Kontakiotis T, Polyzoni T, Bouros D and Patakas D. Variations in alveolar cell populations, lymphocyte subsets and NK-cells in different stages of active pulmonary sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2005;22:21-26.
9. Katchar K, Soderstrom K, Wahlstrom J, Eklund A and Grunewald J. Characterisation of natural killer cells and CD56+ T-cells in sarcoidosis patients. *Eur Respir J* 2005;26:77-85.
10. Baughman RP and Lower EE. Therapy for sarcoidosis. Wouters EFM. *Sarcoidosis*, Wakefield: The Charlesworth Group, 2005.
11. Raghu G, Brown KK, Bradford WZ, Starko K, Noble PW, Schwartz DA and King TE, Jr. A placebo-controlled trial of interferon gamma-1b in patients with idiopathic pulmonary fibrosis. *N Engl J Med* 2004;350:125-133.
12. Boots AW, Kubben N, Haenen GR and Bast A. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. *Biochem Biophys Res Commun* 2003;308:560-565.
13. Thorn J. The inflammatory response in humans after inhalation of bacterial endotoxin: a review. *Inflamm Res* 2001;50:254-261.
14. Swennen EL, Bast A and Dagnelie PC. Immunoregulatory effects of adenosine 5'-triphosphate on cytokine release from stimulated whole blood. *Eur J Immunol* 2005;35:852-858.
15. Fischer KD, Tedford K and Wirth T. New roles for Bcl10 in B-cell development and LPS response. *Trends Immunol* 2004;25:113-116.
16. De Groote D, Zangerle PF, Gevaert Y, Fassotte MF, Beguin Y, Noizat-Pirenne F, Pirenne J, Gathy R, Lopez M, Dehart I et al. Direct stimulation of cytokines (IL-1 beta, TNF-alpha, IL-6, IL-2, IFN-gamma and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation. *Cytokine* 1992;4:239-248.
17. Drent M, Nierop MA, van Gerssiten FA, Wouters EF and Mulder PG. Computer program using BALF analysis results as diagnostic tool in interstitial lung diseases. *Am. J. Respir. Crit. Care Med.* 1996;153:736-741.
18. Quanjer PH, Tammeling GJ, Cotes JE, Pedersom OF, Peslin R and Yernaut JC. Lung volumes and forced ventilatory flows. Report working party. Standardization of lung functions tests. European Commission for Steel and Coal. Official statement of the European Respiratory Society. *Eur Respir J* 1993;16:5-40.
19. Fischer MA, Gransier TJ, Beckers LM, Bekers O, Bast A and Haenen GR. Determination of the antioxidant capacity in blood. *Clin Chem Lab Med* 2005;43:735-740.
20. Speek AJ, Schrijver J and Schreurs WH. Fluoremetric determination of total vitamin C in whole blood by high performance liquid chromatography with pre-column derivatization. *J Chrom Biomed Appl* 1984;305:53-60.
21. Benzie IF, Chung W and Tomlinson B. Simultaneous measurement of allantoin and urate in plasma: analytical evaluation and potential clinical application in oxidant:antioxidant balance studies. *Clin Chem* 1999;45:901-904.

22. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969;27:502-522.
23. Boots AW, Haenen GR, den Hartog GJ and Bast A. Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants. *Biochim Biophys Acta* 2002;1583:279-284.
24. Montuschi P, Ciabattoni G, Paredi P, Pantelidis P, du Bois RM, Kharitonov SA and Barnes PJ. 8-Isoprostane as a biomarker of oxidative stress in interstitial lung diseases. *Am J Respir Crit Care Med* 1998;158:1524-1527.
25. Lenz AG, Costabel U and Maier KL. Oxidized BAL fluid proteins in patients with interstitial lung diseases. *Eur Respir J* 1996;9:307-312.
26. Culver DA, Barna BP, Raychaudhuri B, Bonfield TL, Abraham S, Malur A, Farver CF, Kavuru MS and Thomassen MJ. Peroxisome proliferator-activated receptor gamma activity is deficient in alveolar macrophages in pulmonary sarcoidosis. *Am J Respir Cell Mol Biol* 2004;30:1-5.
27. Drent M, van den Berg R, Haenen GR, van den Berg H, Wouters EF and Bast A. NF-kappaB activation in sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2001;18:50-56.
28. Antonicelli F, Parmentier M, Hirani N, Drost E, Rahman I, Donaldson K and MacNee W. LPS stimulation of IL-8 release is inhibited by thiol antioxidant at the transcriptional level in THP-1 macrophage cells. *Am J Respir Crit Care Med* 2000;161:A738.
29. Moller DR. Cells and cytokines involved in the pathogenesis of sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 1999;16:24-31.
30. Seitzer U, Swider C, Stuber F, Suchnicki K, Lange A, Richter E, Zabel P, Muller Quernheim J, Flad HD and Gerdes J. Tumour necrosis factor alpha promoter gene polymorphism in sarcoidosis. *Cytokine* 1997;9:787-790.
31. Rahman I and MacNee W. Role of transcription factors in inflammatory lung diseases. *Thorax* 1998;53:601-612.
32. Baughman RP, Strohofer SA, Buchsbaum J and Lower EE. Release of tumor necrosis factor by alveolar macrophages of patients with sarcoidosis. *J Lab Clin Med* 1990;115:36-42.
33. Ziegenhagen MW, Schrum S, Zissel G, Zipfel PF, Schlaak M and Muller-Quernheim J. Increased expression of proinflammatory chemokines in bronchoalveolar lavage cells of patients with progressing idiopathic pulmonary fibrosis and sarcoidosis. *J Investig Med* 1998;46:223-231.
34. Yee AM and Pochapin MB. Treatment of complicated sarcoidosis with infliximab anti-tumor necrosis factor-alpha therapy. *Ann Intern Med* 2001;135:27-31.
35. Meloni F, Caporali R, Marone Bianco A, Paschetto E, Morosini M, Fietta AM, Patrizio V, Bobbio-Pallavicini F, Pozzi E and Montecucco C. BAL cytokine profile in different interstitial lung diseases: a focus on systemic sclerosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2004;21:111-118.
36. Rutherford RM, Staedtler F, Kehren J, Chibout SD, Joos L, Tamm M, Gilmartin JJ and Brutsche MH. Functional genomics and prognosis in sarcoidosis--the critical role of antigen presentation. *Sarcoidosis Vasc Diffuse Lung Dis* 2004;21:10-18.
37. Hauber HP, Gholami D, Meyer A and Pforte A. Increased interleukin-13 expression in patients with sarcoidosis. *Thorax* 2003;58:519-424.
38. Martinez JA, King TE, Jr., Brown K, Jennings CA, Borish L, Mortenson RL, Khan TZ, Bost TW and Riches DW. Increased expression of the interleukin-10 gene by alveolar macrophages in interstitial lung disease. *Am J Physiol* 1997;273:L676-683.
39. Yaqoob P, Newsholme EA and Calder PC. Comparison of cytokine production in cultures of whole human blood and purified mononuclear cells. *Cytokine* 1999;11:600-605.
40. Conquer JA, Maiani G, Azzini E, Raguzzini A and Holub BJ. Supplementation with quercetin markedly increases plasma quercetin concentration without effect on selected risk factors for heart disease in healthy subjects. *J Nutr* 1998;128:593-597.
41. Zern TL, Wood RJ, Greene C, West KL, Liu Y, Aggarwal D, Shachter NS and Fernandez ML. Grape polyphenols exert a cardioprotective effect in pre- and postmenopausal women by lowering plasma lipids and reducing oxidative stress. *J Nutr* 2005;135:1911-1917.

42. Kostyuk VA, Potapovich AI, Vladykovskaya EN, Korkina LG and Afanas'ev IB. Influence of metal ions on flavonoid protection against asbestos-induced cell injury. *Arch Biochem Biophys* 2001;385:129-137.
43. Donnelly LE, Newton R, Kennedy GE, Fenwick PS, Leung RH, Ito K, Russell RE and Barnes PJ. Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanisms. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L774-783.
44. Comalada M, Camuesco D, Sierra S, Ballester I, Xaus J, Galvez J and Zarzuelo A. In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kappaB pathway. *Eur J Immunol* 2005;35:584-592.
45. Boots AW, Balk JM, Bast A and Haenen GR. The reversibility of the glutathionyl-quercetin adduct spreads oxidized quercetin-induced toxicity. *Biochem Biophys Res Commun* 2005;338:923-929.

9

Quercetin reduces markers of
oxidative stress and inflammation in
sarcoidosis

AW Boots, M Drent, VCS de Boer, A Bast, GRMM Haenen

Submitted

Abstract

Background

Oxidative stress and low antioxidant levels are implicated in the etiology of sarcoidosis, an inflammatory disease. Quercetin is a potent dietary antioxidant that was also found to display anti-inflammatory activities. This prompted us to examine the effect of quercetin supplementation on markers of oxidative stress and inflammation in sarcoidosis.

Methods

A double-blind intervention study has been conducted with two groups of non-smoking, un-treated sarcoidosis patients, matched for age and gender. One group was given 4x500 mg quercetin (n=12) orally in a 24-hour period, the other group was given a placebo (n=6). Plasma malondialdehyde (MDA) levels were used as a marker of oxidative damage, the ratios of $\text{TNF}\alpha/\text{IL-10}$ and $\text{IL-8}/\text{IL-10}$ in the blood of the patients as pro-inflammatory markers. *Ex vivo*, an LPS challenge in blood was performed to study the potential of the supplementation to reduce an inflammatory response.

Results

This study demonstrated that quercetin supplementation improved the antioxidant defence system, indicated by the increased total plasma antioxidant capacity. *Ex vivo* LPS-induced cytokine levels appeared to be also reduced by the supplementation. Moreover, this supplementation also reduced markers of oxidative stress and inflammation in sarcoidosis. The effects of the quercetin supplementation appeared to be more pronounced when the levels of the oxidative stress and inflammation markers were higher at baseline.

Conclusions

This study indicates that sarcoidosis patients might benefit from the use of antioxidants such as quercetin to reduce the occurring oxidative stress as well as inflammation. Long-term use of antioxidant supplementation in sarcoidosis, using e.g. quercetin, remains to be investigated.

Introduction

Sarcoidosis is a chronic inflammatory disease of which the exact cause still needs to be elucidated. There is growing evidence that sarcoidosis is associated with oxidative stress, i.e. an imbalance between the production of and the protection against reactive oxygen species (ROS). This is deduced from e.g. increased nuclear factor κ B (NF- κ B) activation (1) and increased levels of biomarkers of oxidative damage such as exhaled ethane (2) and both 8-isoprostane (3) and oxidized proteins (4) in the bronchoalveolar lavage fluid (BALF) of sarcoidosis patients. Recently, we have found that the total antioxidant capacity of sarcoidosis patients is approximately 75% of that of matched controls (unpublished results).

Quercetin is an excellent antioxidant. Within the flavonoid family, quercetin is the most active scavenger of reactive oxygen species (ROS) and reactive nitrogen species (RNS). This can be explained by the presence of two antioxidant pharmacophores within the molecule that have the optimal configuration for free radical scavenging (5). Recently, it has been shown that quercetin supplementation effectively increases the total plasma antioxidant capacity in healthy volunteers (6).

As an inflammatory disorder, sarcoidosis is characterized by increased levels of pro-inflammatory cytokines such as TNF α and IL-8 (7-10). Several studies have indicated that the flavonoid quercetin can also display anti-inflammatory effects (11-13).

Interestingly, de Boer *et al* have recently demonstrated that quercetin accumulates in the lungs of rats and pigs (14). The combination of its tissue specific distribution and potent anti-oxidative as well as anti-inflammatory capacities prompted us to examine the effect of quercetin supplementation on oxidative stress and inflammation in sarcoidosis patients. To mimic a severe inflammatory burden that might occur by incidental exposure to e.g. dust particles, cigarette smoke or other triggers, an *ex vivo* LPS challenge was applied.

Material and Methods

Chemicals

Quercetin and lipopolysaccharide (LPS, *E. coli* 0.26:B6) were purchased from Sigma Chemical Co. (St. Louis, USA). RPMI 1640 medium containing L-glutamine was obtained from Gibco (UK). Human TNF α (7300 pg/ml), human IL-10 (4000 pg/ml) and human IL-8 (10 ng/ml) were acquired from CLB/Sanquin (Amsterdam, the Netherlands). All other chemicals were of analytical grade.

Methods

General information

All participating patients were recruited via their own physician (MD). All participants were fully informed, both written and orally, about the aim and details of the study and have given their written informed consent.

The study was carried out at the University Hospital Maastricht, a tertiary referral for the Netherlands, after approval of the protocol by the Medical Ethics Committee of Maastricht University and the University Hospital Maastricht. Based on foodstuff questionnaires, it was concluded that all participants had comparable dietary habits with an average daily intake of quercetin of approximately 15 mg. None of the participants took any medication or vitamin or food supplements either prior to or during the study. Randomisation occurred by first dividing all participants into trios based on their age and gender and then by randomly giving placebo treatment to one individual out of each trio.

Participants

Eighteen non-smoking patients with symptomatic sarcoidosis (age 45 ± 10) were enrolled in this study. Sarcoidosis had been diagnosed based on both clinical features and bronchoalveolar lavage (BAL) fluid analysis results (data not shown) (15). Moreover, a biopsy confirmation of the disease had been performed in 11 out of the 18 sarcoidosis patients. The clinical symptoms of all patients included respiratory symptoms, i.e. dyspnea, coughing and chest pain. The characteristics of the study population are summarized in table 9.1.

Lung function measurement

Lung function measurements included FEV₁ and DLCO. FEV₁ was measured with a pneumotachograph, DLCO by the single-breath method (Masterlab, Jaeger, Würzburg, Germany). Values were expressed as a percentage of those predicted based on age and gender (16).

Supplementation study

Prior to the actual supplementation period, participants were subjected to a two-day wash-out period. During this period, they were not allowed to consume food rich in flavonoids in general or quercetin in particular. This food included non-organic onions, apples, red wine, tea, organic and freshly pressed fruit juices, berries (e.g. blueberries and elderberries), grapes, cherries, raisins, parsley, broccoli, cabbage, green beans and tomatoes (6). Participants also had to minimise the use of herbs and spices during this period.

The wash-out period was followed by a 24-hour supplementation period during which all participants had to take 4 capsules containing either 500 mg

quercetin or a placebo. The capsules were taken throughout the day, i.e. during lunch, during dinner, just before bedtime and the last during breakfast the following morning, three hours before the second blood withdrawal. Before and after this supplementation period, venous blood samples were drawn into EDTA-containing vacutainer tubes (Vacutainer, Becton-Dickinson, Belgium) and kept on ice prior to processing which occurred within 1 hour after blood collection. During supplementation, the same dietary restrictions as during the wash-out period were applied.

Table 9.1 Characteristics of the participants.

	Quercetin-receiving group	Placebo-receiving group
number (m/f)	12 (8/4)	6 (4/2)
age	31-69 (46 ± 3)	34-59 (44 ± 3)
length	158-186 (174 ± 3)	158-182 (175 ± 4)
weight	64-96 (81 ± 3)	69-96 (84 ± 4)
body mass index	23-32 (27 ± 1)	24-35 (28 ± 2)
time since diagnosis	1-10 (5 ± 1)	1-11 (4 ± 2)
biopsy taken	yes: 7 no: 5	yes: 4 no: 2
DLCO	55-109 (84 ± 5)	85-107 (91 ± 6)
FEV ₁	36-133 (96 ± 7)	51-101 (86 ± 7)
FVC	75-128 (104 ± 5)	65-112 (97 ± 7)
chest radiograph stage 0/I/II/III/IV (n)	3/3/3/3/1	2/2/1/1/0

Controls are matched by age and gender and do therefore not significantly differ from the patients regarding these parameters. Age is expressed by year, length by cm, weight by kg, and both DLCO (diffusing capacity of the lung for carbon monoxide) and FEV₁ (forced expiratory volume in 1 second) by % of the predicted value based on age and gender. Data are expressed as range (mean $\pm$ SEM).

Preparation of the blood samples

Blood was aliquoted into eppendorfs for both the ascorbic acid and the GSH/GSSG analysis: for the former 10% TCA was added to the whole blood, whereas 1.3% SSA in 10 mM HCl was used to preserve the samples for the latter. Another aliquot of blood was used for the incubations required for the blood-based cytokine production assay as described in that section below. The remaining blood was centrifuged (3000 rpm, 5' at 4°C) to obtain plasma. Deproteinization of an aliquot of this plasma, using 10% trichloro-acetic acid (TCA) 1:1, followed by centrifugation (13,000 rpm, 5' at 4°C), was carried for

the trolox equivalent antioxidant capacity measurement (TEAC). All samples were stored at -80°C prior to analysis.

Determination of total plasma quercetin concentration

Total quercetin (sum of quercetin aglycone and quercetin glucuronides/sulfates) concentrations in plasma were analysed by means of HPLC with coulometric array-detection after enzymatic hydrolysis as described previously (17). 3'-O-methoxy quercetin (isorhamnetin) and 4'-O-methoxyquercetin (tamarixetin) aglycone and glucuronides/sulfates were not detected in the plasma.

Total antioxidant capacity (TEAC)

The trolox equivalent antioxidant capacity (TEAC) value is a measure for the total antioxidant status, relating the free radical scavenging properties of a solution or a compound to that of the synthetic antioxidant trolox. The assay is performed as previously described (18). The relative contribution of uric acid, vitamin C and quercetin to the total TEAC value is calculated using the TEAC value described for each individual antioxidant, i.e. 1, 1 and 6.24 respectively (19).

Ascorbic acid measurement

Calibrators were prepared freshly, containing the same amount of TCA as the samples. Samples and calibrators were processed identically as described previously. (20).

Uric acid measurement

Uric acid was measured in the plasma of all samples as described previously. (21).

GSH, GSSG and haemoglobin measurement

Both GSH and GSSG calibrators were prepared freshly and contained the same concentrations of SSA as the samples. Samples and calibrators were treated identically and GSH and GSSG levels measured were related to the haemoglobin content as described previously (22).

Malondialdehyde (MDA) measurement

Malondialdehyde (MDA) was measured in the plasma of all samples as described previously (23).

Blood-based cytokine production assay

Within one hour after blood collection, the blood-based cytokine production assay was performed as described previously (24). Care was taken that handling of the blood prior to LPS-stimulation did not influence the cytokine release.

Enzyme linked immune sorbent assay (ELISA) measurement

TNF α , IL-8 and IL-10 were quantified using PeliKine Compact human ELISA kits (CLB/Sanquin, the Netherlands) based on appropriate and validated sets of monoclonal antibodies. Assays were performed as described in the manufacturer's instructions.

Statistics

The data of the quercetin and placebo group were compared using a Mann Whitney U test. The relation between the basal or LPS-induced cytokine production and the effect of the quercetin supplementation on these cytokine levels has been appraised using Spearman's rank correlation coefficient. A one-tailed probability value (P-value) equal to or less than 0.05 was considered to be statistically significant.

Results

Baseline plasma quercetin levels were below the lower limit of detection of 0.054 μM in all sarcoidosis patients prior to supplementation. After supplementation, the quercetin plasma concentration, reaching $0.27 \pm 0.04 \mu\text{M}$, could be quantified in all patients (Figure 9.1A).

Malondialdehyde (MDA), a marker of oxidative damage to lipids, was significantly reduced after supplementation with quercetin (Figure 9.1B).

Endogenous blood glutathione (GSH) levels were unaffected by supplementation in all patients (Figure 9.1C). The total plasma antioxidant capacity, i.e. the total sum of all plasma antioxidants that is expressed as Trolox equivalent, was significantly enhanced after supplementation in the quercetin-receiving patients (Figure 9.1D).

The plasma levels of the endogenous antioxidants uric acid and vitamin C were not altered by supplementation (data not shown). For the quercetin-receiving group, the relative contribution of these endogenous antioxidants, as well as that of the exogenous quercetin to the total plasma antioxidant status is depicted in Figure 9.2. This reveals that the relative contribution of quercetin to the total antioxidant capacity was extremely small (0.3%) and that a major part of the antioxidant capacity was due to plasma antioxidants other than vitamin C and uric acid, such as low molecular protein thiols (Figure 9.2A). This residual antioxidant capacity was significantly increased (3%, $P < 0.01$) after quercetin supplementation.

The ratios of pro- versus anti-inflammatory cytokines TNF α /IL-10 and IL-8/IL-10, used as inflammatory marker, were significantly decreased by supplementation (Figures 9.1E and 9.1F).

The ratios of TNF α /IL-10 and IL-8/IL-10 after *ex vivo* addition of LPS were also significantly reduced by quercetin supplementation (Figure 9.3).

Figure 9.1 The effect of quercetin supplementation on plasma quercetin concentration (panel A), plasma malondialdehyde (MDA) levels (panel B), blood glutathione (GSH) levels (panel C), the total plasma antioxidant capacity (TEAC) (panel D) and the levels of the ratios TNFα/IL-10 (panel E) and IL-8/IL-10 (panel F) in sarcoidosis patients. Data are individually expressed for all 12 sarcoidosis patients receiving quercetin and all 6 sarcoidosis patients receiving placebo; light grey bars represent the mean. * P<0.05 compared to the “before” measurement prior to the quercetin supplementation

In the placebo-receiving group, no significant differences were found between the values before and after supplementation regarding any of the parameters measured.

No correlations were found between the increase of the total quercetin plasma concentration or of the increase in total plasma antioxidant capacity

with the effect of supplementation on the markers of oxidative stress and inflammation measured. The *ex vivo* effect of quercetin supplementation on LPS-induced $\text{TNF}\alpha$ or IL-8 level was dependent on the level of the LPS-induced pro-inflammatory cytokine prior to supplementation (Figure 9.4 A-B). This indicates that the higher the cytokine production evoked by LPS is, the more prominent the inhibiting effect of quercetin on this cytokine level. Quercetin supplementation did not affect the LPS-induced production of the anti-inflammatory cytokine IL 10 (Figure 9.4C).

Figure 9.2 The relative contribution of uric acid (UA), vitamin C (vit C) and quercetin (Q) to the total plasma antioxidant capacity in sarcoidosis patients before and after quercetin supplementation. Data are expressed as mean $\pm$ S.E.M (n=12). TEACres= residual plasma antioxidant capacity.
*= $P < 0.05$ compared to the “before” measurement prior to the quercetin supplementation.

Figure 9.3 The effect of quercetin supplementation on LPS-induced cytokine production. Data are individually expressed for 11 sarcoidosis patients receiving quercetin and 5 sarcoidosis patients receiving placebo; light gray bars represent the mean. Results are expressed in ratios of percentage, with 100% representing the cytokine-release under stimulation of LPS prior to the supplementation.
*= $P < 0.05$ compared to the “before” measurement prior to the quercetin supplementation.

A similar dependency as described in the previous paragraph for the *ex vivo* effect was found for the *in vivo* effect of quercetin supplementation; the higher the $\text{TNF}\alpha$ and IL-8 levels before supplementation, the more pronounced the reduction of the plasma concentration of the cytokines by quercetin (Figure 9.4 D-E). Quercetin supplementation did not affect the plasma level of the anti-inflammatory cytokine IL 10 (Figure 9.4F).

Figure 9.4 The relation between the LPS-induced (panel A-C), the basal amount (panel D-F) of cytokine and the effect of the quercetin supplementation on these cytokine levels in sarcoidosis patients. The cytokine level (x-axis) is plotted against the effect on this level caused by the quercetin supplementation (y-axis) for $\text{TNF}\alpha$ (panel A and D), IL-8 (panel B and E) and IL-10 (panel C and F). Data are individually expressed for 11 (panel A-C) or all 12 (panel D-F) sarcoidosis patients receiving quercetin.

The efficacy of quercetin supplementation on the MDA levels is comparable to that on the pro-inflammatory cytokines; the higher the MDA level at baseline, the more pronounced the reduction of this marker of oxidative stress by quercetin supplementation (Figure 9.2B).

Figure 9.5 The relation between the amount of malondialdehyde (MDA) present and the effect of the quercetin supplementation on these MDA levels in sarcoidosis patients. MDA levels (x-axis) are plotted against the effect on these level caused by the quercetin supplementation (y-axis).

Data are individually expressed for all 12 sarcoidosis patients receiving quercetin.

Discussion

Sarcoidosis is an inflammatory disorder in which oxidative stress appears to be involved (1-3), as indicated by several biomarkers including a reduced antioxidant capacity. To the best of our knowledge, this is the first study demonstrating a positive effect of a one day antioxidant supplementation on this reduced antioxidant capacity in sarcoidosis.

After *ex vivo* LPS-stimulation of blood, $\text{TNF}\alpha/\text{IL-10}$ and $\text{IL-8}/\text{IL-10}$ levels, two markers of inflammation, are significantly reduced by the *in vivo* quercetin supplementation. These *ex vivo* results are in agreement with our previous *in vitro* findings. Quercetin decreased LPS-induced $\text{TNF}\alpha$ - and IL-8 production when added to the blood of sarcoidosis patients in the test tube (unpublished data). Interestingly, a one day quercetin supplementation also resulted in a significant decrease of the ratios of pro- versus anti-inflammatory cytokines $\text{TNF}\alpha/\text{IL-10}$ and $\text{IL-8}/\text{IL-10}$ as well as of the plasma levels of the lipid peroxidation product malondialdehyde (MDA), a marker of oxidative stress. These results confirm that quercetin is capable of

empowering the compromised antioxidant defence system of sarcoidosis patients as well as of mitigating the inflammation present in sarcoidosis.

A possible explanation for the anti-inflammatory effects of quercetin can be found in the interplay between oxidative stress and inflammation. ROS are directly involved in the occurrence of oxidative stress. Moreover, ROS are also capable of promoting inflammation by activating the transcription factors NF- κ B and activator protein-1. These transcription factors induce pro-inflammatory cytokines such as TNF α (25,26). By scavenging ROS, quercetin might not only offer protection against oxidative stress, the flavonoid may simultaneously mitigate inflammation. It has been reported that quercetin is capable of inhibiting TNF α production as well as TNF α gene expression via modulation of NF- κ B in human peripheral blood mononuclear cells (12).

During the scavenging of ROS, quercetin is converted into oxidation products. These products might react with critical sulfhydryl groups, thereby impairing vital cellular functions (27,28). The level of glutathione (GSH), the most abundant endogenous thiol, is not affected by quercetin supplementation. This indicates that the possible formation of the reactive oxidation products has no significant impact on the applied dosing regime. This outcome could have been anticipated based on the relatively low plasma quercetin concentration achieved by the current supplementation ($0.27 \pm 0.04 \mu\text{M}$) compared to the high cellular concentrations of GSH (1 to 10 mM). However, in a chronic disorder such as sarcoidosis, supplementation has to proceed over a much longer time period. The safety, tolerability and efficacy of the long-term use of quercetin in sarcoidosis remains to be investigated.

Interestingly, the effects of quercetin supplementation appear to be more pronounced when the baseline levels of MDA, TNF α and IL-8 are increased. Only when MDA levels are high does supplementation reduce this marker of oxidative stress. Similarly, only when the TNF α /IL-10 ratio or the IL-8/IL-10 ratio is high does supplementation reduce these markers of inflammation. The extent of the effect of the applied quercetin supplementation appears to be predominantly governed by the individual level of oxidative stress and inflammation in the patient. This high correlation might conceal the anticipated dependency of the effects on the degree of the quercetin plasma level. No correlation with the quercetin level was observed in the present study. These results indicate that beneficial effects of antioxidant supplementation can only be expected in people with enhanced oxidative stress or inflammation. Although this seems trivial, it is often not realised and might explain the negative outcome of many clinical studies where antioxidants were supplied to healthy subjects (29). The

present study shows that the baseline value of oxidative damage and inflammation is a major discriminator in the beneficial effect of antioxidant supplementation.

In several diseases associated with enhanced oxidative stress, the use of antioxidants, i.e. N-acetyl cysteine and lipoic acid, is proven to be clinically beneficial (30,31). In these antioxidant supplementation studies, besides having an antioxidant effect, the reduction of the occurring inflammation has also been implicated (31).

Recently, it has been demonstrated that Infliximab, a $\text{TNF}\alpha$ antibody, improved lung function in stable pulmonary sarcoidosis (32). This finding is in line with studies regarding the use of Infliximab in the treatment of other chronic inflammatory diseases such as Bechterew (33,34), Chron's disease (35) and rheumatoid arthritis (36-38). The latter studies report an inverse correlation between the effects of this anti- $\text{TNF}\alpha$ therapy and the severity of the pathologies, i.e. the more severe the disease, the less effective this anti- $\text{TNF}\alpha$ antibody therapy is. This might be due to the limited capacity of the anti- $\text{TNF}\alpha$ therapy that was restricted by the use of a fixed dosing regime independent of the $\text{TNF}\alpha$ level at baseline. In the present study, no limitation of the effect of quercetin was seen. In contrast to regular anti- $\text{TNF}\alpha$ therapy, it was found that the more outspoken the markers of oxidative stress and inflammation, the more effectively quercetin reduces these markers, including the $\text{TNF}\alpha$ level.

In conclusion, the present study indicates that the supplementation of a high dose of the antioxidant quercetin over a 24-hour period is able to reduce markers of oxidative stress and inflammation in sarcoidosis, provided these markers are elevated.

References

1. Drent M, van den Berg R, Haenen GR, van den Berg H, Wouters EF, Bast A. NF-kappaB activation in sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis.* 2001;18:50-56.
2. Kanoh S, Kobayashi H, Motoyoshi K. Exhaled ethane: an in vivo biomarker of lipid peroxidation in interstitial lung diseases. *Chest.* 2005;128:2387-2392.
3. Montuschi P, Ciabattini G, Paredi P, Pantelidis P, du Bois RM, Kharitonov SA, Barnes PJ. 8-Isoprostane as a biomarker of oxidative stress in interstitial lung diseases. *Am J Respir Crit Care Med.* 1998;158:1524-1527.
4. Lenz AG, Costabel U, Maier KL. Oxidized BAL fluid proteins in patients with interstitial lung diseases. *Eur Respir J.* 1996;9:307-312.
5. Heijnen CG, Haenen GR, Oostveen RM, Stalpers EM, Bast A. Protection of flavonoids against lipid peroxidation: the structure activity relationship revisited. *Free Radic Res.* 2002;36:575-581.
6. Wilms LC, Hollman PC, Boots AW, Kleinjans JC. Protection by quercetin and quercetin-rich fruit juice against induction of oxidative DNA damage and formation of BPDE-DNA adducts in human lymphocytes. *Mutat Res.* 2005;582:155-162.
7. Ziegenhagen MW, Schrum S, Zissel G, Zipfel PF, Schlaak M, Muller-Quernheim J. Increased expression of proinflammatory chemokines in bronchoalveolar lavage cells of patients with progressing idiopathic pulmonary fibrosis and sarcoidosis. *J Investig Med.* 1998;46:223-231.
8. Baughman RP, Iannuzzi M. Tumour necrosis factor in sarcoidosis and its potential for targeted therapy. *BioDrugs.* 2003;17:425-431.
9. Meloni F, Caporali R, Marone Bianco A, Paschetto E, Morosini M, Fietta AM, Patrizio V, Bobbio-Pallavicini F, Pozzi E, Montecucco C. BAL cytokine profile in different interstitial lung diseases: a focus on systemic sclerosis. *Sarcoidosis Vasc Diffuse Lung Dis.* 2004;21:111-118.
10. Moller DR. Cells and cytokines involved in the pathogenesis of sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis.* 1999;16:24-31.
11. Manjeet KR, Ghosh B. Quercetin inhibits LPS-induced nitric oxide and tumor necrosis factor-alpha production in murine macrophages. *Int J Immunopharmacol.* 1999;21:435-443.
12. Nair MP, Mahajan S, Reynolds JL, Aalinkeel R, Nair H, Schwartz SA, Kandaswami C. The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF-kappabeta system. *Clin Vaccine Immunol.* 2006;13:319-328.
13. Read MA. Flavonoids: naturally occurring anti-inflammatory agents. *Am J Pathol.* 1995;147:235-237.
14. de Boer VC, Dihal AA, van der Woude H, Arts IC, Wolffram S, Alink GM, Rietjens IM, Keijer J, Hollman PC. Tissue distribution of quercetin in rats and pigs. *J Nutr.* 2005;135:1718-1725.
15. Drent M, Nierop MAMF, van Gerritsen FA, Wouters EFM, Mulder PGH Computer program using BALF analysis results as diagnostic tool in interstitial lung diseases. *Am. J. Respir. Crit. Care Med.* 1996;153:736-741.
16. Quanjer PH, Tammeling GJ, Cotes JE, Pedersom OF, Peslin R, Yernaut JC. Lung volumes and forced ventilatory flows. Report working party. Standardization of lung functions tests. European Commission for Steel and Coal. Official statement of the European Respiratory Society. *Eur Respir J.* 1993;16:5-40.
17. Arts ICW, Venema DP, and Hollman PCH. Quantitative determination of flavonoids in plant foods and biological fluids. In: Santos-Buelga, C. *Methods in polyphenols analysis.* Cambridge: The Royal Society of Chemistry; 2003.
18. Fischer MA, Gransier TJ, Beckers LM, Bekers O, Bast A, Haenen GR. Determination of the antioxidant capacity in blood. *Clin Chem Lab Med.* 2005;43:735-740.
19. Arts MJTJ, Dallinga JS, Voss HP, Haenen GRMM, Bast A. A new approach to assess the total antioxidant capacity using the TEAC assay. *Food Chem.* 2004;88:567-570.

20. Speek AJ, Schrijver J, Schreurs WHP. Fluoremetric determination of total vitamin C in whole blood by high performance liquid chromatography with pre-column derivatization. *J Chrom Biomed Appl.* 1984;305:53-60.
21. Benzie IF, Chung W, Tomlinson B. Simultaneous measurement of allantoin and urate in plasma: analytical evaluation and potential clinical application in oxidant:antioxidant balance studies. *Clin Chem.* 1999;45:901-904.
22. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem.* 1969;27:502-522.
23. Lepage G, Munoz G, Champagne J, Roy CC. Preparative steps necessary for the accurate measurement of malondialdehyde by high-performance liquid chromatography. *Anal Biochem.* 1991;197:277-283.
24. Swennen EL, Bast A, Dagnelie PC. Immunoregulatory effects of adenosine 5'-triphosphate on cytokine release from stimulated whole blood. *Eur J Immunol.* 2005;35:852-858.
25. Rahman I. Oxidative stress, transcription factors and chromatin remodelling in lung inflammation. *Biochem Pharmacol.* 2002;64:935-942.
26. MacNee W. Oxidative stress and lung inflammation in airway diseases. *Eur J Pharmacol.* 2001;429:195-207.
27. Galati G, Moridani MY, Chan TS, O'Brien PJ. Peroxidative metabolism of apigenin and naringenin versus luteolin and quercetin: glutathione oxidation and conjugation. *Free Radic Biol Med.* 2001;30:370-382.
28. Boots AW, Kubben N, Haenen GR, Bast A. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. *Biochem Biophys Res Commun.* 2003;308:560-565.
29. Halliwell B. The antioxidant paradox. *Lancet.* 2000;355:1179-1180.
30. Demedts M, Behr J, Buhl R, Costabel U, Dekhuijzen R, Jansen HM, MacNee W, Thomeer M, Wallaert B, Laurent F, Nicholson AG, Verbeken EK, Verschakelen J, Flower CD, Capron F, Petruzzelli S, De Vuyst P, van den Bosch JM, Rodriguez-Becerra E, Corvasce G, Lankhorst I, Sardina M, Montanari M. High-dose acetylcysteine in idiopathic pulmonary fibrosis. *N Engl J Med.* 2005;353:2229-2242.
31. Hofmann MA, Schiekofer S, Isermann B, Kanitz M, Henkels M, Joswig M, Treusch A, Morcos M, Weiss T, Borcea V, Abdel Khalek AK, Amiral J, Tritschler H, Ritz E, Wahl P, Ziegler R, Bierhaus A, Nawroth PP. Peripheral blood mononuclear cells isolated from patients with diabetic nephropathy show increased activation of the oxidative-stress sensitive transcription factor NF-kappaB. *Diabetologia.* 1999;42:222-232.
32. Baughman RP, Drent M, Kavuru M, Judson MA, Costabel U, du Bois R, Albera C, Brutsche M, Davis G, Donohue JF, Muller-Quernheim J, Schlenker-Herceg R, Flavin S, Lo KH, Oemar B, Barnathan ES. Infliximab therapy in patients with chronic sarcoidosis and pulmonary involvement. *Am J Respir Crit Care Med.* 2006;174:795-802.
33. Baraliakos X, Listing J, Brandt J, Zink A, Alten R, Burmester G, Gromnica-Ihle E, Kellner H, Schneider M, Sorensen H, Zeidler H, Rudwaleit M, Sieper J, Braun J. Clinical response to discontinuation of anti-TNF therapy in patients with ankylosing spondylitis after 3 years of continuous treatment with infliximab. *Arthritis Res. Ther.* 2005;7:R439-444.
34. Sidiropoulos P, Kritikos HD, Siakka P, Mamoulaki M, Kouroumali H, Voudouris K, Boumpas DT. Low dose of infliximab is inadequate in most patients with spondylarthropathies. *Clin. Exp. Rheumatol.* 2005;23:513-516.
35. Menachem Y, Avidan B, Lavy A, Lang A, Bardan E, Fidler H, Bar-Meir S, Chowers Y. Increasing the infliximab dose is beneficial in Chron's disease patients who responded to a lower dose and relapsed. *Digestion* 2005;72:124-128.
36. Edrees AF, Misra SN, Abdou NI. Anti-tumour necrosis factor (TNF) therapy in rheumatoid arthritis: correlation of TNF-alpha serum level with clinical response and benefit from changing dose or frequency of infliximab infusions. *Clin. Exp. Rheumatol.* 2005;23:469-474.
37. Berger A, Edelsberg J, Li TT, Maclean JR, Oster G. Dose intensification with infliximab in patients with rheumatoid arthritis. *Ann. Pharmacother.* 2005;39:2021-2025.

38. Collantes-Estevez E, Munoz-Villanueva MC, Zarco P, Torre-Alonso JC, Gratacos J, Gonzalez C, Sanmarti R, Canete JD. Effectiveness of reducing infliximab dose interval in non-responder patients with refractory spondyloarthropathies. An open extension of a multicentre study. *Rheumatology* 2005;44:1555-1558.

A large, light gray, stylized letter 'Q' that serves as a background for the page content. It has a thick stroke and a short, curved tail.

10

Summary and general discussion

Summary and general discussion

Aerobic life forms are associated with the formation of reaction oxygen species (ROS) that can cause damage to macromolecules like lipids, proteins, DNA and RNA. Fortunately, the human body comprises an elaborated defense system against ROS-induced damage that consists of antioxidants, i.e. molecules that can neutralize ROS and other reactive species. To offer maximum protection, antioxidants work together in an intricate network. Failure of this network, either by an overload of ROS or by a decline of sufficient antioxidants, results in a situation called oxidative stress, i.e. an imbalance between the production of and the protection against ROS. Oxidative stress is implicated in the pathophysiology of various chronic inflammatory diseases like sarcoidosis. To reduce the occurring oxidative stress and thereby hopefully also the symptoms of these diseases, treatment with exogenous antioxidants might be useful. Such an antioxidant therapy might be of special interest since the treatment of several of these diseases with e.g. glucocorticoids sometimes fail to be completely efficacious. A suitable candidate for antioxidant therapy might be the flavonoid quercetin that is known for its excellent antioxidative properties. This thesis focuses on the health effects of quercetin from the mechanism of action of the flavonoid to its use as nutraceutical.

In **chapter 2** it is shown that catechol-containing antioxidants such as quercetin are indeed excellent scavengers that can offer protection against lipid peroxidation, i.e. oxidative damage to lipids. During this antioxidant activity, catechol oxidation products such as semiquinone radicals and quinones are formed. These oxidation products are capable of inactivating the GSH-dependent protection against lipid peroxidation and the calcium sequestration in liver microsomes, probably by arylating free thiol groups of the enzymes responsible for these two processes, i.e. the free radical reductase and calcium ATPase. It is therefore concluded that a catechol-containing antioxidant such as quercetin might shift radical damage from lipid peroxidation to sulfhydryl arylation.

The oxidation products of quercetin are studied in more detail in **chapter 3**. The primary oxidation product is an *ortho*-quinone, denoted as QQ, which has four tautomeric forms, i.e. the *ortho*-quinone and three quinone-methides. The reaction of QQ with two of the most important and abundant endogenous antioxidants has been investigated, i.e. its reaction with ascorbate (vitamin C) and glutathione (GSH). It appears that ascorbate is capable of recycling QQ into its parent compound quercetin. With GSH, QQ forms two glutathionylquercetin adducts (GSQ), i.e. 6-GSQ and 8-GSQ. When both GSH and ascorbate are present, QQ is converted exclusively into GSQ. In

the absence of GSH, protein sulfhydryl groups can be arylated by QQ. This protein arylation is also not prevented by ascorbate.

Since one of the most important and abundant endogenous antioxidants, ascorbate, could not offer protection against the strong thiol-reactivity of QQ, another line of defense against this QQ-induced toxicity has been examined in **chapter 4**. The enzyme DT-diaphorase (NQO1) is reported to protect against quinone-induced toxicity by reducing quinones to hydroquinones. For QQ this would mean that DT-diaphorase converts the quinone into quercetin, thereby not only preventing QQ toxicity but also regenerating the consumed antioxidant and thus boosting the antioxidant effect. It was found that QQ is indeed a substrate for DT-diaphorase. However, DT-diaphorase cannot compete with thiols; QQ reacted much faster with glutathione or protein sulfhydryl groups (>1000 times) than with the enzyme in experiments with isolated compounds as well as with human liver cytosol or blood plasma. This indicates that DT-diaphorase plays no substantial role in the protection against QQ.

Based on our studies described in chapter 2-4, it can be concluded that GSH is the main reactant of QQ. This reaction gives rise to the formation of an adduct between QQ and GSH, i.e. GSQ. As described in **chapter 5**, we have found that GSQ is not stable; it rapidly dissociates into GSH and QQ with a half life of 2 minutes. Surprisingly, GSQ incubated with 2-mercapto-ethanol (MSH), a far less reactive thiol, results in the conversion of GSQ into the MSH-adduct MSQ. MSQ appeared to be much more stable than GSQ. Incubation of GSQ with the dithiol dihydrolipoic acid (L(SH)₂) results in the formation of quercetin out of GSQ.

These results indicate that as long as the GSH concentration is high, QQ will be trapped as GSQ. In this way GSH will protect against QQ. However, when the GSH concentration is low, the dissociated QQ will react with other thiol groups, e.g. protein sulfhydryls. It is proposed that the kind of QQ-adduct that eventually will accumulate depends on (i) the reactivity of the thiols present (ii) the characteristics of the leaving group of the adducts (iii) the relative concentrations of the various thiols and (iv) the time span of the incubation period. The poor leaving group characteristics of most protein sulfhydryls indicate that, at the end, QQ might be transferred from a GSQ to a protein-thiol-Q. These findings imply that the “protection” offered by GSH against QQ might only be transient; GSH protects against QQ by scavenging QQ at the site of formation, but on the long run GSH will transfer QQ to other thiols. In that way, the initially highly focussed toxicity of QQ might be dispersed by the formation of GSQ that finally spreads QQ-induced toxicity, possible even over cells.

In order to define the delicate balance between the protective effects of quercetin and the potential toxic effects of its oxidation products, the effect of the flavonoid has been investigated in a more integrated system, viz. rat lung epithelial cells (RLEs) (**chapter 6**). Quercetin was capable of efficiently protecting RLE cells against H_2O_2 -induced DNA damage, but concurrently markers of cell toxicity (i.e. GSH loss and LDH leakage) and cell function loss (i.e. free cytosolic calcium concentration) were increased. It is therefore concluded that even in living lung cells, quercetin swaps one kind of toxicity for another. This quercetin paradox, i.e. the fact that in the process of offering protection, quercetin is converted into an oxidation product with possible toxic effects, has not been investigated in cultured cells before. Following depletion of GSH in the cells by BSO pre-treatment, this quercetin paradox becomes more pronounced, confirming that the formation of thiol reactive quercetin metabolites is involved in the quercetin paradox. Therefore, it is stated that the potential toxicity of metabolites formed during the actual antioxidant activity of free radical scavengers such as quercetin should be considered in the use of antioxidant supplementation.

Interestingly, quercetin supplementation in either healthy volunteers (**chapter 7**) or sarcoidosis patients (**chapter 9**) did not affect the GSH levels. This indicates a limited impact of potentially thiol-reactive oxidation products in the applied dosing regimes, as could be anticipated based on the relative low plasma quercetin concentrations achieved in both supplementations (respectively 0.05 and 0.27 μM) compared to the high cellular concentrations of GSH (1 to 10 mM). Apparently, quercetin supplementation will only lead to QQ-induced toxicity when, as a result of elevated oxidative stress, substantial amounts of QQ are formed and GSH levels are remarkably compromised. This might indicate that QQ-induced toxicity as a result of quercetin supplementation can be especially expected when relatively high dosages are applied to people suffering from severe oxidative stress during a relatively long period.

Because of the association between ROS and inflammation, it is often suggested that an antioxidant such as quercetin might, by scavenging ROS, also exert anti-inflammatory capacities. Therefore, in **chapter 7** the possible anti-inflammatory effects of physiologically attainable quercetin concentrations have been investigated in whole blood from healthy subjects, i.e. a model closely resembling the *in vivo* situation. Cytokine production was *ex vivo* evoked by lipopolysaccharide (LPS), a patho-physiological relevant stimulator of monocytes, neutrophils and B lymphocytes. It was found that quercetin dose-dependently inhibited *ex vivo* LPS-induced production of tumour necrosis factor α ($TNF\alpha$), an important mediator of inflammation. This anti- $TNF\alpha$ effect of quercetin already occurred at the physiological achievable concentration of 1 μM . The *ex vivo* LPS-induced IL-10 production

remained unaffected by quercetin. Consequently, quercetin also caused a significant reduction of the ratio of pro- versus anti-inflammatory cytokines $\text{TNF}\alpha/\text{IL-10}$, a frequently used diagnostic parameter of inflammation.

The *in vitro* anti- $\text{TNF}\alpha$ effect of quercetin showed a large dependency on the level of $\text{TNF}\alpha$ induced by LPS; the higher the LPS-induced $\text{TNF}\alpha$ level, the higher the inhibiting effect of quercetin on this cytokine level is. This finding indicates that the anti-inflammatory effect of quercetin is more pronounced at a higher level of inflammation.

To further elucidate the *ex vivo* anti-inflammatory effects of the flavonoid, a quercetin supplementation study for four weeks, by means of a quercetin-rich fruit juice comprising 97 mg per day, has been conducted in healthy volunteers (**chapter 7**). This supplementation increased the plasma quercetin concentration as well as the total plasma antioxidant capacity in healthy volunteers, but did not affect basal plasma (*in vivo*) or LPS-induced (*ex vivo*) $\text{TNF}\alpha$ levels. This lack of effect is probably due to the low cytokine and high antioxidant levels of the healthy volunteers at baseline, indicating that neither inflammation nor oxidative stress are present. This means that no quercetin supplementation is needed in these subjects, simply because there is no damage that needs to be reduced. Consequently, antioxidant supplementation is expected to be more fruitful in people with increased levels of inflammation and oxidative stress, e.g. patients suffering from a disease of which the pathology is associated with these two processes.

To examine whether sarcoidosis patients are more likely candidates for quercetin supplementation than healthy volunteers, the antioxidant and inflammatory status of these patients has been determined. As shown in **chapter 8**, the total plasma antioxidant capacity was decreased and the levels of the individual endogenous antioxidants GSH, vitamin C and uric acid were declined in the blood of sarcoidosis patients. Moreover, sarcoidosis patients also displayed elevated levels of the pro-inflammatory cytokines $\text{TNF}\alpha$ and interleukin-8 (IL-8). No difference in the anti-inflammatory cytokine IL-10 is observed between sarcoidosis patients versus healthy controls. As a result, the ratios of the pro- versus the anti-inflammatory cytokines $\text{TNF}\alpha/\text{IL-10}$ and $\text{IL-8}/\text{IL-10}$, frequently used diagnostic parameters of inflammation, were also significantly increased in sarcoidosis.

Determining the *in vitro* anti-inflammatory effects of quercetin on *ex vivo* LPS-induced $\text{TNF}\alpha$ and IL-8 production in both the sarcoidosis and control group showed that the inhibiting effects of the flavonoid regarding both cytokines were much more pronounced in the patients than in the matched controls. Since the LPS-induced cytokine levels were significantly higher in the patient group, this finding confirms the earlier *in vitro* result that the

anti-inflammatory effect of quercetin increases with the level of occurring inflammation.

The *in vivo* anti-oxidative and anti-inflammatory effects of quercetin in people, suffering from both oxidative stress and inflammation, have been investigated in a double blinded quercetin supplementation study (4x500 mg orally within 24 hours), conducted with 2 groups of non-smoking, non-treated sarcoidosis patients (**chapter 9**). This quercetin supplementation caused an increase of the antioxidant defence, as can be deduced from the observed increase in plasma quercetin concentration as well as in the total plasma antioxidant capacity. Additionally, this quercetin supplementation resulted in a decrease of malondialdehyde (MDA), a marker of oxidative damage to lipids. This suggests that strengthening the endogenous antioxidant shield with quercetin supplementation indeed results in more protection against oxidative damage, caused by reactive species such as ROS. Furthermore, both basal (*in vivo*) and LPS-induced (*ex vivo*) ratios of the pro- versus the anti-inflammatory cytokines, i.e. $\text{TNF}\alpha/\text{IL-10}$ and $\text{IL-8}/\text{IL-10}$, were reduced by the quercetin supplementation.

Interestingly, both the anti-oxidative and anti-inflammatory effects of quercetin appeared to be more pronounced when the level of respectively the occurring oxidative stress or inflammation is higher at baseline. This observed correlation indicates that beneficial effects of antioxidant supplementation can especially be expected in people with elevated oxidative stress, e.g. patients suffering from a disease of which the pathology is associated with this process.

Conclusion

The flavonoid quercetin is an excellent antioxidant that also possesses anti-inflammatory capacities. The excellent anti-oxidative properties of quercetin can be deduced from the fact that the flavonoid offers protection against ROS-induced oxidative damage to lipids both *in vitro* and *in vivo*. The anti-inflammatory capacities of the flavonoid are indicated by its reducing effect on the inflammatory markers $\text{TNF}\alpha$ /IL-10 and IL-8/IL-10 both *in vitro* and *in vivo*.

Interestingly, both the anti-oxidative and anti-inflammatory effects of quercetin are more pronounced when the basal levels of respectively the occurring oxidative stress and inflammation are high. This indicates that the use of quercetin supplementation is especially fruitful in people suffering from a disease that is associated with both processes, such as sarcoidosis. Moreover, the correlation between the effects of quercetin and the basal levels of both oxidative damage and inflammation may explain why, after supplementation, no anti-inflammatory effects of the flavonoid could be detected in healthy volunteers.

Up to date, many health-benefit studies on antioxidant supplementation are conducted in healthy volunteers. These volunteers will display only low levels of inflammation and oxidative stress and have high levels of endogenous antioxidants. Consequently, additional supplementation with extra antioxidants will most likely not result in any significant beneficial health effects, because there is no need for an extra defense against oxidative stress or inflammation. The design of this type of study resembles the testing of a new remedy against migraine in healthy volunteers. Inevitable nonspecific side-effects such as headaches will wrongly disqualify the new drug. This metaphor explains the disappointing, but foreseeable, negative outcome of numerous antioxidant intervention studies.

During its scavenging activities, quercetin becomes oxidized and possible toxic oxidation products are formed. The most important oxidation product of quercetin is its *ortho*-quinone, denoted as QQ. QQ is highly thiol reactive and reacts almost instantaneously with GSH or, in the absence of this thiol, with protein sulfhydryl groups, thereby impairing the function of several critical enzymes. Consequently, during *in vivo* quercetin supplementation care should be taken of the possible toxicity of its metabolites. During both our supplementation studies, no signs of this toxicity are observed. However, in a chronic disorder such as sarcoidosis, supplementation has to proceed over a much longer time period. The safety, tolerability and efficacy of the long term use of quercetin in sarcoidosis has to be established.

Implications and further research

The research conducted in the present thesis clearly indicates that *in vivo* quercetin supplementation may exert anti-oxidative and anti-inflammatory properties in people suffering from a condition that is associated with both processes such as sarcoidosis. However, only a relatively small number of patients has been included in our study and measurements comprised only biomarkers of oxidative stress and inflammation. Moreover, it is still unclear whether these anti-oxidative and anti-inflammatory properties of the flavonoid will actually lead to a reduction of the clinical symptoms present in sarcoidosis patients. Therefore, a larger quercetin supplementation study should be conducted in sarcoidosis patients to (i) confirm our results and to (ii) expand our data by measuring the effect of the supplementation on clinical parameters of the disease. In sarcoidosis, besides reduced lung function, the main symptom is an extreme fatigue. Consequently, it would be interesting to examine whether long term quercetin supplementation would improve both the lung function and the physical condition in sarcoidosis patients.

Another point that should be addressed in a larger supplementation study with quercetin is the safety of the use of this flavonoid. Since sarcoidosis is a chronic disease, it can be expected that, in order to improve the health status of patients suffering from this disease, chronic use of quercetin supplementation is required. Up to date, there are no data available regarding the safety of long term use of high dosages of antioxidants in general and quercetin in particular. As is described in the current thesis, quercetin becomes oxidized while exerting its anti-oxidative capacities and potentially toxic oxidation products are formed. The long term cytotoxic effects of these oxidation products should be monitored in detail in order to evaluate the safety of long term quercetin supplementation. This can be achieved, for example, by screening the health status of cells that are expected to be exposed to the flavonoid the most, i.e. blood and lung cells. Alternatively, the thiol reactivity of these oxidation products can be quantified by, for example, measuring the activity of various enzymes of which it is known that they can be affected by the oxidation products of quercetin, i.e. calcium ATPase or glutathione transferases. Prevention of toxicity, induced by the thiol reactive oxidation products, might be achieved by supplementing quercetin together with a dithiol that is capable of capturing, and thus detoxifying, these products.

Optimal effects of any supplementation can only be expected when the compound that needs to be supplemented is administered in the most appropriate way. For quercetin, various ways of supplementing are possible.

In the current thesis, two variants are used, i.e. a pure supplement and a diet intervention using a juice with a high quercetin content. The supplement only contains the aglycone form of quercetin, whereas the juice comprises high amounts of various quercetin derivatives that might have a better biological availability than the aglycone itself. Another advantage of a dietary supplementation versus a 'conventional' supplement might be a better compliance, especially in long term use. A good compliance is mandatory in chronic diseases such as sarcoidosis. Conversely, a disadvantage of using the diet is that the intake of alimentary antioxidants is relatively low, i.e. ~100 mg a day versus up to several grams a day using an antioxidant supplement. As a result, the plasma quercetin concentrations achieved by the quercetin supplement were higher compared with the plasma levels obtained by the dietary intervention in our studies. Consequently, larger beneficial health effects might be expected when supplementation is applied via a supplement instead of via the diet. However, in order to make a balanced overall comparison between both ways of supplementation, more research is necessary.

Finally, it should be noted that sarcoidosis is a multi-system disorder that can manifest throughout the body by influencing various pathways. It is, therefore, not surprising that the current treatment of sarcoidosis with only anti-inflammatory agents, such as glucocorticoids, fails to be completely efficacious. Instead, better results can be expected from a multi-factorial treatment that could tackle the various aspects of sarcoidosis. The flavonoid quercetin displays not only the anti-inflammatory and anti-oxidative properties investigated in the current thesis. Additionally, immuno-suppressive, anti-fibrotic, anti-coagulative and anti-proliferative capacities of quercetin have been reported as well. Interestingly, all these activities have also been suggested for the treatment of sarcoidosis. This complimentary activity appears to make quercetin an even more promising candidate for the treatment of sarcoidosis than could be expected based on the results presented in the current thesis.

Samenvatting en algemene discussie

Samenvatting en algemene discussie

Aërobe levensvormen zijn geassocieerd met de vorming van reactieve zuurstof species (RZS) die schade kunnen berokkenen aan macromoleculen zoals vetten, eiwitten, DNA en RNA. Gelukkig beschikt het menselijk lichaam over een uitgebreid verdedigingssysteem tegen RZS-geïnduceerde schade. Dit verdedigingsmechanisme bestaat uit antioxidanten, ofwel moleculen die RZS en andere reactieve species kunnen neutraliseren. Om maximale bescherming te kunnen bieden werken antioxidanten samen in een fijnmazig netwerk. Falen van dit netwerk als gevolg van een overdaad aan RZS of een te lage spiegel van geschikte antioxidanten resulteert in een situatie die oxidatieve stress genoemd wordt. Oxidatieve stress kan daarom gedefinieerd worden als een disbalans tussen de productie van en de bescherming tegen RZS. Oxidatieve stress speelt een belangrijke rol in de pathofysiologie van verschillende chronische, ontstekingsgerelateerde ziektebeelden waaronder sarcoïdose. Behandeling met exogene antioxidanten zou dan ook raadzaam kunnen zijn om niet alleen deze optredende oxidatieve stress, maar hopelijk ook de bijbehorende symptomen van de diverse ziektebeelden te kunnen verminderen. Dergelijke antioxidant therapie zou met name interessant zijn omdat de huidige behandeling van aantal van deze ziektebeelden met bijvoorbeeld glucocorticoïden soms te wensen overlaat. Een geschikte kandidaat voor antioxidant therapie zou het flavonoid quercetine, dat bekend staat om zijn uitstekende antioxidatieve eigenschappen, kunnen zijn. In dit proefschrift staat het effect van quercetine op de gezondheid centraal, van het werkingsmechanisme tot het gebruik van het flavonoid als nutraceutical.

In **hoofdstuk 2** wordt aangetoond dat catechol-bevattende antioxidanten zoals quercetine uitstekend in staat zijn om RZS en andere vrije radicalen weg te vangen en op deze manier bescherming te bieden tegen oxidatieve schade aan vetten, ook wel lipide peroxidatie genoemd. Tijdens het uitoefenen van deze antioxidant activiteit worden er diverse catechol oxidatieproducten gevormd, zoals bijvoorbeeld semiquinon radicalen en quinonen. Deze oxidatieproducten zijn in staat zowel de GSH-afhankelijke bescherming tegen lipide peroxidatie als de calcium binding in lever microsomen te inactiveren. Deze inactivatie vindt vermoedelijk plaats door de arylatie van de vrije thiolgroepen van de enzymen die verantwoordelijk zijn voor deze twee processen, namelijk het vrije radicaal reductase en het calcium ATPase. Vandaar dat geconcludeerd wordt dat een catechol-bevattende antioxidant zoals quercetin in staat kan zijn om radicaal schade te verschuiven van lipide peroxidatie naar sulfhydryl arylatie.

De oxidatieproducten van quercetine zijn nader bestudeerd in **hoofdstuk 3**. Het belangrijkste oxidatieproduct van quercetine is een *ortho*-quinone,

weergegeven als QQ. QQ heeft vier tautomeren, te weten het *ortho*-quinone en drie quinone-methides. De reactie van QQ met twee van de meest belangrijke en veelvoorkomende antioxidanten, namelijk vitamine C and GSH, is nader onderzocht. Het is gebleken dat vitamine C QQ kan recyclen tot zijn moederstof quercetine. De reactie van GSH met QQ daarentegen leidt tot de vorming van twee glutathionyladducten (GSQ), namelijk 6-GSQ en 8-GSQ. In de aanwezigheid van zowel GSH als vitamine C wordt QQ exclusief omgezet in GSQ. In de afwezigheid van GSH aryleert QQ thiolgroepen van eiwitten. Ook deze reactie kan niet voorkomen worden door de aanwezigheid van vitamine C.

Aangezien één van de meest belangrijke en veelvoorkomende endogene antioxidanten, te weten vitamine C, geen bescherming kon bieden tegen de sterke thiol-reactiviteit van QQ is in **hoofdstuk 4** een andere verdedigingslijn tegen QQ-geïnduceerde toxiciteit nader onderzocht. Het enzym DT-diaphorase (NQO1) staat in de literatuur bekend om zijn bescherming tegen quinone-geïnduceerde toxiciteit door de reductie van quinonen tot hydroquinonen. Voor QQ zou dit impliceren dat DT-diaphorase in staat zou zijn om dit quinone te converteren tot quercetine. Middels deze reactie zou DT-diaphorase niet alleen QQ-geïnduceerde toxiciteit kunnen voorkomen, maar tevens de geconsumeerde antioxidant quercetine kunnen regenereren waardoor het totale antioxidant effect eveneens versterkt kan worden. Onze resultaten hebben inderdaad laten zien dat QQ een substraat is voor DT-diaphorase. Het enzym kan echter niet rivaliseren met thiolen; QQ reageerde veel sneller met GSH of eiwit-thiolen (>1000 maal) dan met het enzym in experimenten met geïsoleerde stoffen, humaan lever cytosol of bloed plasma. Deze resultaten suggereren dat DT-diaphorase geen substantiële rol speelt in de bescherming tegen QQ.

Gebaseerd op onze studies, beschreven in hoofdstuk 2-4, kan geconcludeerd worden dat GSH de belangrijkste reactant van QQ is. Deze reactie leidt tot de vorming van een adduct tussen QQ en GSH, ook wel GSQ genoemd. In **hoofdstuk 5** hebben we aangetoond dat dit GSQ niet stabiel is; het dissocieert snel tot GSH en QQ met een halfwaardetijd van 2 minuten. Verrassend genoeg resulteert de incubatie van GSQ met 2-mercapto-ethanol (MSH), een veel minder reactief thiol, in de omzetting van GSQ in het MSH-adduct MSQ. MSQ bleek veel stabielere te zijn dan GSQ. Incubatie van GSH met een dithiol dihydro-lipoïne zuur ($L(SH)_2$) resulteert in de vorming van quercetine uit GSQ.

Deze resultaten geven aan dat, zolang de GSH concentratie hoog is, QQ gevangen zal worden in GSQ. Op deze manier beschermt GSH tegen QQ. Bij lage GSH concentraties zal QQ echter reageren met andere thiolgroepen, zoals bijvoorbeeld eiwit sulfhydryl groepen. Er wordt geopperd dat het soort

QQ-adduct dat uiteindelijk zal accumuleren afhankelijk is van (i) de reactiviteit van de aanwezige thiolen (ii) de eigenschappen van de “vertrekkende groep” (leaving group) van de adducten (iii) de relatieve concentraties van de verschillende thiolen en (iv) de lengte van de incubatie periode. De veronderstelde slechte “vertrekkende groep” eigenschappen van de meeste eiwit-sulphydryl groepen geven aan dat op de lange termijn QQ wellicht van GSH naar een eiwit-thiol-Q adduct getransporteerd zal worden. Deze bevindingen impliceren dat de “bescherming” van GSH tegen QQ wellicht slechts van tijdelijke aard is; GSH beschermt tegen QQ door het quinone in te vangen op de plaats waar het gevormd wordt, maar op de lange termijn zal GSH QQ slechts doorgeven aan andere thiolen. Op deze manier zou de QQ-geïnduceerde toxiciteit, die in eerste instantie op cellulair niveau lokaal ontstaat, uiteindelijk verspreid kunnen worden door de vorming van GSQ. Deze verspreiding zou zelfs plaats kunnen vinden over cellen heen.

Om de gevoelige balans tussen de beschermende effecten van quercetine en de mogelijk toxische effecten van zijn oxidatieproducten beter te kunnen beschrijven, is het effect van dit flavonoid bestudeerd in een systeem dat dichterbij de *in vivo* situatie staat, te weten long epitheel cellen van een rat (RLE cellen) (**hoofdstuk 6**). Quercetine was in staat om RLE cellen effectief te beschermen tegen H₂O₂-geïnduceerde DNA schade, maar deze bescherming ging gepaard met een verhoging van markers van celtoxiciteit (bijvoorbeeld GSH-verlies en LDH lekkage) en celfunctie verlies (bijvoorbeeld vrije cytosolische calcium concentratie). Vandaar dat er geconcludeerd kon worden dat zelfs in gekweekte longcellen quercetine in staat is om de ene vorm van toxiciteit uit te wisselen voor een andere. Deze quercetine paradox, die gekenmerkt wordt doordat gedurende het bieden van bescherming quercetine wordt omgezet in een oxidatieproduct met mogelijk schadelijke effecten, is nog niet eerder aangetoond in gekweekte cellen. Deze quercetine paradox komt nog duidelijker naar voren als de cellen geen GSH meer bevatten omdat ze van tevoren met BSO zijn behandeld. Deze bevinding bevestigt een mogelijke rol voor de vorming van thiolreactieve quercetine metabolieten in de quercetine paradox. Daarom wordt er in dit hoofdstuk gesuggereerd dat er tijdens het gebruik van antioxidant suppletie rekening gehouden moet worden met de mogelijke toxiciteit van metabolieten, die gevormd kunnen worden tijdens de eigenlijke antioxidant activiteit van stoffen die vrije radicalen invangen zoals quercetine.

In onze humane studies had quercetine suppletie in zowel gezonde vrijwilligers (**hoofdstuk 7**) als sarcoïdose patiënten (**hoofdstuk 9**) geen invloed op de GSH niveaus. Deze bevinding impliceert dat het effect van mogelijke thiolreactieve oxidatieproducten in de gebruikte doseringschema's van beide studies slechts beperkt is. Dit beperkt effect van oxidatieproducten

had ook al voorspeld kunnen worden op basis van de relatief lage quercetine plasmaconcentraties die in beide studies bereikt zijn (respectievelijk 0.05 en 0.27 μM) in vergelijking met de hoge cellulaire GSH concentraties (1 tot 10 mM). Klaarblijkelijk zal quercetine suppletie alleen tot QQ-geïnduceerde toxiciteit leiden als, ten gevolge van verhoogde oxidatieve stress, substantiële hoeveelheden QQ gevormd zullen worden terwijl de GSH spiegel relatief laag is. Dit zou kunnen impliceren dat QQ-geïnduceerde toxiciteit als gevolg van quercetine suppletie in het bijzonder verwacht kan worden als, gedurende een lange periode, hoge doseringen toegediend zullen worden aan mensen die lijden aan ernstige oxidatieve stress.

Aangezien RZS en ontsteking geassocieerd zijn, wordt regelmatig gesuggereerd dat een antioxidant zoals quercetine, middels het invangen van RZS, ook ontstekingsremmende eigenschappen bezit. Daarom zijn in **hoofdstuk 7** de ontstekingsremmende effecten van klinisch haalbare quercetine concentraties onderzocht. Deze experimenten zijn uitgevoerd in het bloed van gezonde vrijwilligers om de *in vivo* situatie zoveel mogelijk na te bootsen. In deze experimenten is de cytokine productie opgewekt middels *ex vivo* toediening van lipopolysaccharide (LPS), een fysiologisch relevante stimulator van monocyten, neutrofielen en B lymfocyten. De resultaten laten zien dat quercetine in staat was om dosis-afhankelijk de *ex vivo* LPS-geïnduceerde productie van tumor necrose factor α ($\text{TNF}\alpha$), een belangrijke mediator van ontsteking, te remmen. Dit anti- $\text{TNF}\alpha$ effect van quercetine trad al op bij een concentratie van 1 μM . De *ex vivo* LPS-geïnduceerde interleukin (IL)-10 productie werd niet beïnvloed door quercetine. Vandaar dat de ratio van de ontstekingsbevorderende versus -remmende cytokine $\text{TNF}\alpha/\text{IL}-10$, een regelmatig gebruikte maat voor ontsteking, ook significant verlaagd werd door quercetine.

De grootte van het *in vitro* anti- $\text{TNF}\alpha$ effect van quercetine was afhankelijk van de $\text{TNF}\alpha$ productie door LPS; hoe hoger het LPS-geïnduceerde $\text{TNF}\alpha$ niveau, des te groter het remmende effect van quercetine op dit cytokine niveau. Deze bevinding betekent dat het ontstekingsremmende effect van quercetine afhangt van de ernst van de optredende ontsteking.

De *ex vivo* ontstekingsremmende effecten van het flavonoid zijn nader bestudeerd door middel van een quercetine suppletie studie van vier weken met gezonde vrijwilligers, waarin gebruik werd gemaakt van een quercetine-rijke fruitdrink die 97 mg quercetine per dag leverde (**hoofdstuk 7**). Deze suppletie verhoogde zowel de quercetine plasmaconcentratie als de totale plasma antioxidant capaciteit in de gezonde vrijwilligers, maar had geen effect op de basale plasma (*in vivo*) of LPS-geïnduceerde (*ex vivo*) $\text{TNF}\alpha$ niveaus. Dat hierop geen effect gevonden wordt is waarschijnlijk te verklaren door de lage cytokine en hoge antioxidant niveaus van de gezonde

vrijwilligers op het beginpunt (baseline), die aangeven dat er geen ontsteking of oxidatieve stress aanwezig is. Dit betekent dat quercetine suppletie niet nodig is bij deze vrijwilligers omdat er simpelweg nog geen schade aanwezig is die verminderd moet worden. Verwacht kan worden dat antioxidant suppletie meer effect zal hebben bij mensen bij wie de niveaus van ontsteking en oxidatieve stress verhoogd zijn, zoals bijvoorbeeld patiënten die lijden aan een ziekte waarvan de pathologie geassocieerd is met deze twee processen.

Om na te gaan of sarcoïdose patiënten mogelijk wel baat hebben bij quercetine suppletie zijn dan gezonde vrijwilligers, is de antioxidant- en ontstekingsstatus van deze patiënten bepaald. Zoals beschreven in **hoofdstuk 8** vertoonde het bloed van sarcoïdose patiënten niet alleen een verlaagde totale plasma antioxidant capaciteit, maar ook verlaagde spiegels van de afzonderlijke endogene antioxidanten GSH, vitamine C en urinezuur. Bovendien waren de concentraties van de ontstekingsbevorderende cytokines $\text{TNF}\alpha$ en IL-8 verhoogd in het bloed van sarcoïdose patiënten. Het niveau van de ontstekingsremmende cytokine IL-10 was niet verschillend in de sarcoïdose patiënten ten opzichte van gezonde controles. Hierdoor zijn de ratio's van de ontstekingsbevorderende versus -remmende cytokines $\text{TNF}\alpha/\text{IL-10}$ en $\text{IL-8}/\text{IL-10}$, regelmatig gebruikte parameters van ontsteking, ook verhoogd in sarcoïdose.

Gedurende het bepalen van de *in vitro* ontstekingsremmende effecten van quercetin op de *ex vivo* LPS-geïnduceerde $\text{TNF}\alpha$ en IL-8 productie in bloed van zowel de sarcoïdose patiënten als de gezonde controles viel op dat de remmende effecten van het flavonoid op beide cytokines veel sterker waren in de patiënten dan in de gezonde controles. Aangezien de LPS-geïnduceerde cytokine niveaus ook hoger waren in de patiënten groep, bevestigt deze bevinding het eerder verkregen *in vitro* resultaat dat het ontstekingsremmende effect van quercetine toeneemt met de mate van de optredende ontsteking.

De *in vivo* antioxidatieve en ontstekingsremmende effecten van quercetine in mensen die lijden aan zowel oxidatieve stress als ontsteking zijn onderzocht in een dubbelblinde quercetine suppletie studie (4x500 mg oraal binnen 24 uur) die is uitgevoerd met twee groepen niet-rokende, niet-behandelde sarcoïdose patiënten (**hoofdstuk 9**). Deze quercetine suppletie verhoogde de antioxidant bescherming, zoals afgeleid kan worden uit de waargenomen verhoging in zowel de quercetine plasmaconcentratie als de totale plasma antioxidant capaciteit. Bovendien resulteerde de quercetine suppletie in een afname van malondialdehyde (MDA), een marker van lipide peroxidatie. Deze laatste bevinding suggereert dat het versterken van het

endogene antioxidant netwerk middels quercetine suppletie inderdaad resulteert in meer bescherming tegen oxidatieve schade, veroorzaakt door RZS. Tenslotte zorgde de quercetine suppletie ook voor een verlaging van zowel de basale (*in vivo*) als de LPS-geïnduceerde (*ex vivo*) ratio's van de ontstekingsbevorderende versus -remmende cytokines $\text{TNF}\alpha$ /IL-10 en IL-8/IL-10.

Een interessant gegeven is dat zowel de antioxidatieve als de ontstekingsremmende effecten van quercetine beduidend sterker leken te zijn wanneer het niveau van respectievelijk de oxidatieve stress of de ontsteking hoger was op het beginpunt (baseline). Deze correlatie suggereert dat positieve effecten van antioxidant suppletie met name verwacht kunnen worden in mensen die lijden aan een ziekte waarvan de pathologie geassocieerd is met oxidatieve stress.

Conclusie

Het flavonoid quercetine een uitstekende antioxidant is die tevens ontstekingsremmende capaciteiten bezit. De uitstekende antioxidatieve eigenschappen van quercetine kunnen afgeleid worden uit het feit dat het flavonoid zowel *in vitro* als *in vivo* bescherming biedt tegen RZS-geïnduceerde lipide peroxidatie. De ontstekingsremmende capaciteiten van het flavonoid werden zowel *in vitro* als *in vivo* aangetoond door zijn remmende effect op twee markers van ontsteking, te weten $\text{TNF}\alpha$ /IL-10 en IL-8/IL-10.

Een interessant gegeven is dat zowel de antioxidatieve als de ontstekingsremmende effecten van quercetine beduidend sterker blijken te zijn als de basale niveaus van respectievelijk de aanwezige oxidatieve stress of ontsteking hoog zijn. Deze bevinding suggereert dat het gebruik van quercetine suppletie met name effect zal hebben in mensen die lijden aan een ziektebeeld dat geassocieerd is met beide processen zoals bijvoorbeeld sarcoïdose. Bovendien zou deze correlatie tussen de effecten van quercetine en de basale niveaus van zowel de aanwezige oxidatieve schade als de ontsteking ook kunnen verklaren waarom, na suppletie, geen ontstekingsremmende effecten van het flavonoid aangetoond konden worden in gezonde vrijwilligers.

Tot op de dag van vandaag worden veel studies naar de gezondheidsbevorderende effecten van antioxidant suppletie uitgevoerd in gezonde vrijwilligers. Deze vrijwilligers vertonen slechts lage niveaus van ontsteking en oxidatieve stress en hebben hoge endogene antioxidant niveaus. Bij deze mensen zal additionele suppletie met extra antioxidanten niet leiden tot significante gezondheidsbevorderende effecten, aangezien zij simpelweg geen behoefte hebben aan extra bescherming tegen oxidatieve stress of ontsteking. De opzet van dit soort studies komt overeen met het testen van een nieuw medicijn tegen migraine in gezonde vrijwilligers. Onvermijdelijke niet-specifieke neveneffecten zoals hoofdpijn zullen ten onrechte leiden tot het stoppen van de verdere ontwikkeling van dit nieuwe medicijn. Deze metafoor verklaart de teleurstellende maar voorspelbare negatieve uitkomst van vele interventiestudies met antioxidanten.

Tijdens het invangen van vrije radicalen door quercetine ontstaan er oxidatieproducten van het flavonoid die mogelijk toxisch zijn. Het meest belangrijke oxidatieproduct van quercetine is het *ortho*-quinone, weergegeven als QQ. QQ is uitermate thiol reactief en reageert vrijwel direct met GSH of, in afwezigheid van dit thiol, met eiwit sulfhydryl groepen. Deze laatste reactie kan leiden tot functieverlies van verscheidene belangrijke enzymen. Vandaar dat er tijdens *in vivo* quercetine suppletie aandacht besteed moet worden aan de mogelijke toxiciteit van zijn metabolieten. Tijdens onze twee suppletie studies zijn geen tekenen van toxiciteit

waargenomen. Bij een chronische aandoening zoals sarcoïdose zal suppletie echter over een veel langere periode moeten plaatsvinden. De veiligheid, bijwerkingen en effectiviteit van quercetine gebruik op de lange termijn moeten daarom nader worden vastgesteld.

Implicaties en verder onderzoek

Het onderzoek beschreven in dit proefschrift duidt erop dat *in vivo* quercetine suppletie antioxidatieve en ontstekingsremmende effecten kan hebben in mensen die lijden aan een aandoening die geassocieerd wordt met beide processen zoals bijvoorbeeld sarcoïdose. Het aantal patiënten waarmee deze studie is uitgevoerd is relatief klein en de uitgevoerde metingen behelzen alleen biomarkers van oxidatieve stress en ontsteking. Bovendien is het onzeker of deze antioxidatieve en ontstekingsremmende effecten van het flavonoid uiteindelijk ook zullen leiden tot een afname van de klinische symptomen van de sarcoïdose patiënten. Vandaar dat een grotere quercetine suppletie studie uitgevoerd dient te worden bij sarcoïdose patiënten om (i) onze resultaten te bevestigen en (ii) onze data uit te breiden door het meten van het effect van deze suppletie op klinische parameters van het ziektebeeld. Bij sarcoïdose is, naast een verlaagde longfunctie, extreme vermoeidheid het belangrijkste symptoom. Vandaar dat het belangrijk is om na te gaan of langdurige quercetine suppletie zowel de longfunctie als de algemene lichamelijke toestand van sarcoïdose patiënten verbetert.

Een ander aandachtspunt binnen een grotere suppletie studie met quercetine zou de veiligheid van het gebruik van dit flavonoid moeten zijn. Aangezien sarcoïdose een chronische ziekte is kan verwacht worden dat quercetine langdurig gebruikt zal moeten worden om de gezondheid van patiënten die lijden aan deze ziekte te verbeteren. Tot op de dag van vandaag zijn er nog geen gegevens beschikbaar omtrent de veiligheid van het lange termijn gebruik van hoge doseringen antioxidanten in het algemeen en quercetine in het bijzonder. Zoals beschreven in dit proefschrift wordt quercetine geoxideerd tijdens het uitoefenen van zijn antioxidatieve capaciteiten en worden er mogelijk schadelijke oxidatieproducten gevormd. De cytotoxische effecten van deze oxidatieproducten zouden grondig bestudeerd moeten worden om de veiligheid van chronische quercetine suppletie te bepalen. Dit kan bijvoorbeeld bereikt worden door het evalueren van de gezondheidsstatus van cellen waarvan verwacht wordt dat ze het meest blootgesteld zullen worden aan het flavonoid, zoals bijvoorbeeld bloed- en longcellen. Daarnaast kan de toxiciteit van deze oxidatieproducten gekwantificeerd worden door het meten van de activiteit van enzymen waarvan bekend is dat ze aangetast kunnen worden door de oxidatieproducten van quercetine, zoals bijvoorbeeld calcium ATPase of glutathione transferases. Om het optreden van dergelijke toxiciteit, veroorzaakt door thiolreactieve oxidatieproducten, te voorkomen zou quercetine eventueel gecombineerd kunnen worden met een dithiol dat in staat is om deze producten in te vangen en dus onschadelijk te maken.

Bij suppletie is de wijze waarop de suppletie plaats vindt van groot belang. Voor quercetine zijn er meerdere manieren van suppletie mogelijk. In dit proefschrift worden twee varianten gebruikt, te weten een conventioneel supplement en een voedingsinterventie waarbij gebruik gemaakt wordt van een vruchtensap dat een hoge concentratie quercetine bevat. Het supplement bevat alleen de aglycone vorm van quercetine, terwijl het sap hoge concentraties bevat van diverse quercetine derivaten met een betere biologische beschikbaarheid dan het aglycone zelf. Een ander voordeel van het suppleren van antioxidanten via de voeding, met name bij chronisch gebruik, is een verhoogde therapietrouw. Een nadeel van het suppleren via de voeding is dat de inname van antioxidanten via de voeding relatief laag is, ongeveer 100 mg per dag versus meerdere grammen per dag indien gebruik wordt gemaakt van een supplement. Dit verschil wordt weerspiegeld in de quercetine plasmaspiegels bereikt na toediening van het supplement, die hoger waren dan de spiegels na inname van het quercetine-verrijkt sap. Vandaar dat grotere gezondheidsbevorderende effecten verwacht zouden kunnen worden indien de suppletie uitgevoerd wordt via een supplement in plaats van via de voeding. Meer onderzoek is echter nodig om een uitgebalanceerde totaalvergelijking te kunnen maken tussen beide manieren van suppletie.

Tenslotte dient opgemerkt te worden dat sarcoïdose een multi-systemische aandoening is die zich kan manifesteren door het hele lichaam en daarbij diverse processen kan beïnvloeden. Het is daarom ook niet verwonderlijk dat de huidige behandeling van sarcoïdose met alleen ontstekingsremmende middelen, zoals bijvoorbeeld glucocorticoïden, niet volledig effectief blijkt te zijn. Betere resultaten zijn daarentegen te verwachten van een multi-factoriële behandeling die de diverse aspecten van sarcoïdose zou kunnen aanpakken. Het flavonoid quercetine bezit niet alleen de antioxidatieve en ontstekingsremmende eigenschappen die onderzocht zijn in dit proefschrift. Quercetine heeft tevens immuunsuppressieve, antifibrotische, anticoagulatieve en antiproliferatieve activiteiten. Al deze facetten zijn ook van belang in de behandeling van sarcoidose. Dit lijkt des te meer te rechtvaardigen dat nader onderzoek dient te worden gedaan naar het effect van Q in sarcoidose.

Dankwoord

Dankwoord

Na al die jaren onderzoek en werken aan “je boekje” komt dan toch opeens nog onverwacht snel het moment waarop het schrijven van je dankwoord echt niet langer uitgesteld kan worden. Maar wat blijkt het lastig te zijn om in een paar bladzijden samen te vatten wat de mensen uit je naaste omgeving voor je betekend en gedaan hebben gedurende die jaren... Ik kan dan ook alleen maar hopen dat ik met dit dankwoord iedereen recht doe en niemand vergeet, want zonder jullie was het me nooit gelukt!!

Allereerst wil ik natuurlijk mijn promotor Prof. Dr. A. Bast en co-promotor Dr. G.R.M.M. Haenen bedanken. Beste Aalt, mijns inziens kan niemand zo enthousiasmerend en positief uit de hoek komen als jij! Op de momenten dat ik even vastzat of dringend behoefte had aan een objectieve kijk op mijn data kon ik altijd bij jou terecht. Bedankt dat jij op de juiste momenten wist bij te sturen en je vertrouwen in mij en mijn project nooit bent verloren! Beste Guido, het is onmogelijk hier te beschrijven wat jij allemaal voor mij hebt gedaan en betekend tijdens mijn promotietijd! Je wist echt altijd tijd te maken om te brainstormen, data te analyseren, manuscripten te corrigeren of mij op te vangen als ik het even niet meer zag zitten. Hoe onmogelijk het tijdstip ook was waarop ik mijn problemen per mail op je afvuurde, vrijwel altijd volgde er direct een reactie! Enorm bedankt voor deze toewijding en betrokkenheid. Het lijkt mij niet overdreven te stellen dat ik mijn vorming tot betere wetenschapper voor een groot deel aan jou te danken heb!

Ook wil ik mijn officieuze begeleider uit de kliniek, Prof. dr. M. Drent, bedanken. Beste Marjolein, jij stapte mijn onderzoek in op het moment dat ik de vertaalslag naar de kliniek probeerde te maken en daarin eigenlijk direct vastliep. Je aarzelde geen moment maar bood gelijk je expertise en hulp aan, niet wetende dat het zoveel meer van je tijd in beslag zou gaan nemen en er zoveel leuke nieuwe ideeën uit geboren zouden worden! Je enthousiasme en betrokkenheid hebben een grote indruk op mij gemaakt en een belangrijke rol gespeeld in mijn keuze om meer klinisch werk te gaan doen in de toekomst... ik kijk uit naar onze verdere samenwerking!

Vervolgens wil ik graag mijn collega's van de vakgroep Farmacologie en Toxicologie bedanken voor hun steun en betrokkenheid de afgelopen jaren. Gertjan, bedankt omdat jij degene bent die me als eerste wist te overtuigen door te gaan binnen dit vakgebied en vervolgens ook degene was die me vanaf de zijlijn altijd weer terug op de goede weg wist te zetten als ik het spoor even bijster was. Onze gesprekken, die gelukkig behalve over werk ook vaak ergens anders over gaan (Kimi!), betekenen veel voor me! Rachelle,

bedankt voor je opbeurende peptalks als ervarings-deskundige en natuurlijk ook voor de talloze shop- en bijklets-avondjes! Mariken, helaas zijn we maar kort kamergenootjes geweest maar als ergens de uitdrukking “kort maar krachtig” op kan slaan.... bedankt voor je ongeremde energie en positivisme, na een gesprek met jou komt de glimlach altijd weer bovendrijven! Els, met name in de laatste periode van mijn aio-periode heb je je ontpopt tot een ware steun en toeverlaat. Of het nou ging om een helpende hand op het lab, het meedenken bij het oplossen van een probleem of een luisterend oor toen de laatste loodjes daadwerkelijk zwaar bleken... voor onze “huis-belg” is niks teveel gevraagd, ge zijt ene engel! Shufan, our “roomies” period started just recently, but I’ve enjoyed our “small” talks (that are never actually small) from the beginning! Marie-José, bedankt voor de labtechnische ondersteuning tijdens mijn klinische studie. Marc, bedankt voor al je hulp en troubleshooting op het lab en omdat je deur altijd voor me openstond om “even” te spuien! Roger, bedankt voor het eindeloos aanleveren van stabiele GSQ (“Waarom wordt ie bij mij nou nooit zo geconcentreerd?!”), maar vooral voor de gezelligheid en sfeer op het lab... en daarbuiten, Themby’s binnenkort?! Evi, het labwerk is een stuk gezelliger én interessanter met jou erbij! Els, Mariëlle en Mia, bedankt voor de secretariële ondersteuning, maar vooral ook omdat jullie er altijd op bleven letten hoe het met de persoon achter het onderzoek zelf gaat!

Leden van de beoordelingscommissie, Prof. dr. C. Stehouwer, Prof. dr. W. Buurman, Prof. dr. M. Drent, Prof. Dr. J. Kleinjans en Prof. dr. I Rietjens, hartelijk dank voor de snelle beoordeling van het manuscript.

Ook de leden van het “quercetine-netwerk” wil ik graag bedanken. De aio’s Vincent de Boer, Ashwin Dihal, Lonneke Wilms en Hester van der Woude en hun begeleiders Gerrit Alink, Ilja Arts, Peter Hollman, Jos Kleinjans, Ivonne Rietjens, Rob Stierum en Ruud Woutersen: bedankt voor de vruchtbare bijeenkomsten en de zinvolle discussies, ik heb veel van jullie geleerd! Lonneke, “*hakuna matata*”... bedankt voor het delen van het lief en leed dat promoveren heet, van het samenwerken in jouw (mega!)interventie studie tot de “lange” ritten naar Wageningen (die door het keihard meezingen toch echt minder lang werden!) tot het delen van het schrijfleed in de laatste fase. Het was geweldig jou als collega te hebben!

Stagiair Nard Kubben wil ik graag bedanken voor zijn tomeloze ijver en inzet, onze samenwerking en de mogelijkheid om te leren wat het is om iemand te begeleiden: hoofdstuk drie is van jou! Succes met je eigen promotie, ik weet zeker dat jij gemaakt bent voor de wetenschap!

Gedurende mijn onderzoek ben ik zo bevoorrecht geweest om, behalve op het lab van mijn eigen vakgroep, ook op andere laboratoria te mogen werken. Veel dank ben ik verschuldigd aan de mensen van de Vakgroep “Fibre and Particle Toxicology” in Düsseldorf. Roel Schins, bedankt voor de mogelijkheid om op jullie lab aanvullende metingen te mogen doen! Ook bedankt voor de vele brainstorm sessies, je hulp op het lab en de mogelijkheid tot verdere samenwerking in de toekomst... hopelijk hoef je ook daarin voorlopig je schoen nog niet op te eten! Rodger, *“heb je even voor mij”*... labwork will never be the same without you! Thank you for all your help but most of all for your “gezelligheid” and sense of humour. Hui Li, thank you very much for teaching me the Comet assay, the cell culture and so much more... it was an honour to work with you! Furthermore I would like to thank Astrid, Seema, James and Catrin for their help and hospitality. Ook wil ik graag de mensen van de vakgroep Biochemie bedanken voor de samenwerking en hun gastvrijheid. Johan Heemskerk, bedankt voor jouw deskundige hulp bij de calcium-metingen en je “geen woorden maar daden” mentaliteit..., briljant dat je altijd even tijd voor me wist vrij te maken! Imke en Marion, bedankt voor jullie hulp als ik weer eens iets verkeerd had ingesteld of niet precies meer wist wat ik nou eigenlijk aan het doen was...

Mijn patiëntenstudies zouden natuurlijk nooit tot stand gekomen zijn zonder de medewerking en het enthousiasme van alle betrokken patiënten! Verder ook een speciaal woord van dank aan de mensen van de longpoli, in het bijzonder Janine, voor de hulp bij het recruteren en includeren. Arne van Belle, bedankt voor je hulp bij het COPD gedeelte van mijn onderzoek!

Gé en Esther, zonder jullie toewijding en professionaliteit had ik nooit in zo’n korte termijn twee patiëntenstudies kunnen uitvoeren. Naast de zekerheid dat alles tot in de puntjes goed verloopt, betekent samenwerken met jullie vooral ook veel lol en gezelligheid. Absolute bekroning van onze samenwerking was natuurlijk het WASOG congres in Sicilië, dat mij bijzonder veel goede herinneringen en bovendien ook een extra moeder heeft opgeleverd! “Mamma Gé”, onze trip door Sicilië was echt onvergetelijk!! Ik kijk uit naar mijn verdere samenwerking met jullie!

Tiny Wouters wil ik heel graag bedanken voor de geweldige lay-out! Vanaf onze eerste afspraak was het meteen overduidelijk dat jij deze minstens zo belangrijk vond als ik. Door jouw inzet, ervaring en waanzinnige werktempo is mijn boekje mooier geworden dan ik voor ogen had gehouden!

Vervolgens een speciaal woordje van dank voor mijn paranimfen. Lieve Harald, *“look at the stars, look how they shine for you”*... waar moet ik

beginnen met bedanken?! Er is zo ongelooflijk veel wat je voor me gedaan hebt: bloed prikken onder vrijwel onmogelijke omstandigheden, bijspringen op het lab als mijn briljante planning toch weer net iets te krap bleek, eindeloos prutsen aan de lay-out van mijn figuren en de kraft en zo kan ik nog wel even doorgaan. Maar het meest wil ik je toch bedanken voor je steun, engelengeduld en relativiseringsvermogen! Lieve Marieke, wie had kunnen denken dat onze vriendschap zo snel zo sterk zou worden? We hebben de laatste tijd lief en leed gedeeld en zonder jouw lieve mailtjes, kaartjes en opbeurende verwenpakketten was de eindspurt zeker een stuk zwaarder voor me geweest! Voor jullie allebei geldt dat ik vereerd ben dat ik jullie mijn paranimfen maar vooral ook mijn vrienden mag noemen!

Lieve Anne, *“I’ll be there for you, cause you’re there for me too”*....ook al kunnen we dit niet vaak meer naast elkaar op de bank meezingen, de strekking ervan is door jouw verhuizing naar Noorwegen gelukkig nooit veranderd! Mijn leven zou zo onnoemelijk veel saaier en leger zijn geweest als ik jou niet had leren kennen in die eerste onderwijsgroep bijna 10 jaar geleden! Jouw humor, wijze raad en onvoorwaardelijke steun hebben mij in al die jaren al door heel veel siutaties heen gesleept. Als ik er iemand vandaag graag bij had willen hebben... jeg savner deg, tusen hjertelig takk !

Lieve Daniëlle (miep), bedankt voor de vele bier- en wijnavondjes waarop ik heerlijk kon spuien en mocht genieten van jouw ervaring als aio maar vooral van jouw bijzondere vriendschap! Lieve Lin, samen begonnen als stagiaires bij GRAT en na al die jaren gelukkig nog steeds bevriend! Ik ben blij dat onze “drie musketier” etentjes weer in ere hersteld zijn, bedankt voor alle gezelligheid en ontspanning!

Verder natuurlijk een big thanx voor al mijn vrienden! Bedankt voor jullie aanwezigheid, luisterende oor, afleiding en vooral ook begrip als ik het vanwege werk weer eens moest laten afweten! Lieve Chiel, bedankt voor al je computerhulp en voor het feit dat je in al die jaren gewoon jij bent gebleven en op die manier vaak voor ontspanning en afleiding hebt gezorgd! Lieve Mischa, Robin en Natasja: bedankt voor de hervonden vriendschappen en goede gesprekken, it means a lot to me! Lieve Lies, bedankt voor de vele lunchdates, spui-uurtjes en gezellige kletsmomenten... ik heb het nodig!

Uiteraard wil ik ook mijn (schoon)ouders en zussen bedanken voor hun steun. Lieve pappa en mamma, *“you’re simply the best”*... bedankt voor alle mogelijkheden die jullie me gegeven hebben en jullie steun en vertrouwen om deze mogelijkheden op mijn eigen manier optimaal te benutten!! Ook jullie concrete hulp in de afrondingsfase was geweldig! Lieve Clazien en Alex,

bedankt voor jullie altijd aanwezige belangstelling, steun en begrip, zeker toen ik mezelf genoodzaakt zag om mijn schrijfblok zelfs naar Egypte mee te nemen! Lieve opa, ik vind het bijzonder dat je er vandaag bij bent en ben je enorm dankbaar voor je nimmer aflatende interesse in alles wat ik doe. Het is zeker niet vanzelfsprekend dat iemand van jouw leeftijd nog zo scherp en bij de tijd is, getuige ook je stelling, en ik hoop dan ook dat ik daar nog een stuk langer van mag genieten! Lieve tante Gon, *“soms is 't beater iets moeis te verleeze, beater verleeze dan dat ge 't nooit het gehad”*... het doet pijn dat je er vandaag niet bij kan zijn, maar in mijn hart leef je voort en ik hoop dat je vanaf je eigen plekje vandaag toch meekijkt en een beetje trots bent op je petekind!

En dan tenslotte “mijn” Tim. Lief, *“ik wil nooit wennen aan jou”*... jij bent voor mij het begin en het einde, alles komt samen bij jou. Onvoorwaardelijk, maar nooit vanzelfsprekend. Zonder jou kan ik niet maximaal functioneren, maar ook niet volledig loslaten en genieten van alles! Bedankt voor je oneindige steun, rust en liefde; met jou kan ik alles aan!!

List of publications

List of publications

Full papers

Boots AW, Haenen GRMM, den Hartog GJM, Bast A. Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants. *BBA* 2002;1583:279-284

den Hartog GJM, Boots AW, Haenen GRMM, van der Vijgh WJF, Bast A. Lack of inhibition of endothelial nitric oxide synthase in the isolated rat aorta by doxorubicin. *Toxicol in Vitro* 2003;17:165-167

Boots AW, Kubben N, Haenen GRMM, Bast A. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. *BBRC* 2003;308:560-565

Boots AW, Haenen GRMM, Bast A. Oxidant metabolism in COPD. *ERJ* 2003;46:14s-27s

van Schooten FJ, Boots AW, Knaapen AM, Godschalk RW, Maas LM, Borm PJ, Drent M, Jacobs JA. Myeloperoxidase (MPO) -463 G→A reduces MPO activity and DNA adduct levels in bronchoalveolar lavages of smokers. *Cancer Epidemiol Biomarkers Prev* 2004;13:828-833

Boots AW, Bast A, Haenen GRMM. No role of DT-diaphorase (NQO1) in the protection against oxidized quercetin. *Febs Letters* 2005;579:677-682

Wilms LC, Hollman PCH, Boots AW, Kleinjans JCS. Protection by quercetin and quercetin-rich fruit juice against induction of oxidative DNA damage and formation of BPDE-DNA adducts in human lymphocytes. *Mutation Research* 2005;582:155-162

Boots AW, Balk J, Bast A and Haenen GRMM. The reversibility of the glutathionyl-quercetin adduct spreads oxidized quercetin-induced toxicity. *BBRC* 2005;338:923-929

Full papers (submitted)

Boots AW, Lui H, Schins RPF, Duffin R, Bast A and Haenen GRMM. The quercetin paradox. Submitted, 2006

den Hartog GJM, Boots AW, Haenen GRMM and Bast A. Erythritol is a sweet antioxidant. Submitted, 2006

Boots AW, Wilms LC, Swennen ELR, Kleinjans JCS, Bast A and Haenen GRMM. The anti-inflammatory activity of quercetin. Submitted, 2006

Boots AW, Drent M, Swennen ELR, Moonen HJJ, Bast A and Haenen GRMM. Anti-oxidant status associated with inflammation in sarcoidosis: a potential role for antioxidants. Submitted, 2006

Boots AW, de Boer VCS, Drent M, Bast A and Haenen GRMM. The *in vivo* effects of quercetin supplementation in onset sarcoidosis patients. Submitted, 2006

Wilms LC, Boots AW, de Boer VCS, Maas LM, Pachen DMFA, Gottschalk RWH, Ketelslegers H, Godschalk RWL, van Schooten FJ, Haenen GRMM, Kleinjans JCS. Interindividual differences in effects of a four-week blueberry juice intervention in human volunteers on ex vivo induced DNA damage in peripheral lymphocytes. In preparation, 2006

Boots AW, Drent M, Swennen ELR, Moonen HJJ, Bast A and Haenen GRMM. Antioxidant and inflammatory status in patients suffering from idiopathic pulmonary fibrosis: a possible role for quercetin. In preparation, 2006

Boots AW, van Belle A, Wouters EFM, Bast A and Haenen GRMM. Antioxidant and inflammatory status in COPD patients: the effect of quercetin. In preparation, 2006

Abstracts

Boots AW, Haenen GRMM, Bast A. Shift of oxidative damage by quercetin. Proceedings of the 1st Seminar European Graduate school of Toxicology, Maastricht, the Netherlands, November 2002

Boots AW, Haenen GRMM, Bast A. Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants. Proceedings of the scientific meeting of the Netherlands Society of Toxicology, De Bilt, the Netherlands, January 2003

Boots AW, Haenen GRMM, den Hartog GJM, Bast A. Catechol-containing antioxidants shift oxidative damage toward thiol arylation. Abstracts of the 41st Congress of the European Societies of Toxicology, Florence, Italy, September 2003. Toxicology Letters 2003;144(S1):s118.

Boots AW, Haenen GRMM, Bast A. From lipid peroxidation to thiol arylation by the flavonoid quercetin. Proceedings of the scientific meeting of the Netherlands Society of Toxicology, Veldhoven, the Netherlands, October 2003

Boots AW, Kubben N, Haenen GRMM, Bast A. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. Proceedings of the 2nd Seminar European Graduate School of Toxicology, January 2004

Boots AW, M. Drent M, Bast A and Haenen GRMM. Reduced antioxidant status in interstitial lung diseases (ILD): the relation with enhanced inflammatory status? Proceedings of the 3rd International WASOG Conference on Diffuse Lung Diseases, Catania, Sicily, June 2006

Boots AW, Drent M, Bast A and Haenen GRMM. The effect of the antioxidant quercetin on oxidative stress and inflammation in sarcoidosis. Proceedings of the 3rd International WASOG Conference on Diffuse Lung Diseases, Catania, Sicily, June 2006

Curriculum vitae

Curriculum vitae

Agnes Wilhelmina Boots was born on February 13th 1979 in Heerlen, the Netherlands. From 1991 to 1997, she attended high school (gymnasium) at the Grotius College in Heerlen. After graduation she moved to Maastricht to study Health Sciences at the Maastricht University from 1997 to 2001 with specialization Biological Health Sciences. Her M. Sc thesis entitled “The role of -463G→A MPO polymorphism on smoking-induced DNA adduct formation in bronchoalveolar lavage cells” was based on research conducted at the Department of Health Risk Analysis and Toxicology (GRAT). The supervisors were Prof. dr. F.J. van Schooten (GRAT) and Dr. J.A. Jacobs (Department of Microbiology, Academic Hospital Maastricht). After obtaining her M Sc in 2001, she started as a PhD-student at the Department of Pharmacology and Toxicology under the supervision of Dr. G.R.M.M. Haenen and Prof. dr. A. Bast. Her PhD study was part of a larger project, entitled “Benefit-risk evaluation of flavonoids in foods and their use as functional food ingredients”, that was conducted at 5 departments throughout the Netherlands by 5 PhD-students. This study was financed by the Netherlands Organisation for Health Research and Development (ZonMw) as a part of their “Functional Food” programme. The investigations described in the current thesis have been carried out during this PhD-project from 2001 to 2006. During this period, she also spent several short research stays in the laboratory at the Institut für Umweltmedizinische Forschung (IUF) at the Heinrich-Heine Universität Düsseldorf, under supervision of Dr. R.P.F. Schins, to perform part of the experiments described in chapter 6 of this thesis. During her last year as PhD student, she has written a research proposal entitled “Oxidative stress in the development of interstitial lung diseases: sources and regulation” that was awarded with a Kootstra Fellowship for talented PhD-students. Furthermore, she obtained a Stensen Stipendium for her research proposal entitled “Oxidative stress in sarcoidosis”. Both this Stipendium and Fellowship allow her to continue her research, partially in the Netherlands at the Department of Pharmacology and Toxicology in collaboration with Prof. dr. M. Drent at the Department of Respiratory Medicine, Maastricht University, partially in the USA in collaboration with Dr. A. van der Vliet at the Department of Pathology, University of Vermont and partially in Germany in collaboration with Dr. R.P.F. Schins at the Institut für Umweltmedizinische Forschung, Heinrich-Heine Universität Düsseldorf.