Antioxidants and their metabolites: some toxicological aspects

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Antioxidants and their metabolites:

Some toxicological aspects

Bashir Mahmoud Rezk

The Graduate School

The study presented in this thesis was performed at 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.

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Antioxidants and their metabolites: Some toxicological aspects

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

In The Name Of ALLAH
The Most Gracious, The Most Merciful

To my parents, teachers, brothers and sisters

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Preface

Preface

The fundament of toxicology is the risk-benefit analysis. This means that the positive as well as the negative health effects of all compounds, including antioxidants, have to be assessed and related to each other. Only in this way the rational for the use of a compound can be evaluated.

The beneficial effects of antioxidants are due to their ability to protect against free radical damage. In the diseases and other pathological conditions where free radicals are implicated, the biochemistry may greatly vary, i.e. the radicals involved, their flux, the site where they are generated and the target they attack can differ. Thus for each type of radical damage specific criteria need to be fulfilled by an antioxidant in order to be active.

Regarding safety, in the 16th century Paracelsus already stated that all compounds are toxic, provided the dose is high enough. There is no reason to assume that antioxidants should be an exception to this rule. On the contrary, the mega-dosages sometimes recommended make safety a very relevant issue for antioxidants. The biological origin of most antioxidants helps to increase the acceptance of antioxidant supplementation by the general public. The perception is that the biological origin guaranties that antioxidants are not harmful; biological and natural is incorrectly thought to be synonymous to safe.

The adage on the use of antioxidant supplementation seems to be: The more, the better. Indeed, antioxidant supplements are taken frequently by a great part of the public at a relatively high dose despite our limited knowledge on their beneficial health effect and safety. From a toxicological point of view, the free radical processes as well as the profiles of antioxidants have to be elucidated to a greater extend to be able to further rationalize and optimize antioxidant therapy (1).

An important issue in the use of antioxidants is metabolism (2). Like other bioactive compounds, metabolites formed by phase 1 and phase 2 enzymes can contribute to the beneficial and toxic effect of an antioxidant. More importantly, during the actual antioxidant activity of a free radical scavenging antioxidant, it is converted into a metabolite. The effect of such a metabolite on a biological system is relevant since the metabolite usually contains some residual reactivity of the radical that has been scavenged.

In the present thesis, antioxidants are studied from a toxicological point of view. Therefore, the general introduction reviews radicals and four antioxidants that were selected based on biological interest. Both the antioxidant and non-antioxidant effects of these antioxidants are discussed as well as the relative importance of the different chemical forms and metabolites of them. In the following chapters, original research on the four selected antioxidants is described. The focus is on the mechanism of action of these

compounds. The part of the molecule responsible for the biological effect is studied. This will contribute to a more accurate risk-benefit analysis of antioxidants

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Chapter 1

Antioxidants and their metabolites: Some toxicological aspects

Bashir M. Rezk, Wim J.F. van der Vijgh, Aalt Bast and Guido R.M.M. Haenen

1. Free radicals in biology

A free radical is defined as an atom or molecule that contains one or more unpaired electron(s). The presence of an unpaired electron results in a relatively high reactivity of the free radical compared to the corresponding non-radical since the spin of the unpaired electron is not compensated by another one. In aerobic organisms free radicals appear to be formed sometimes intentionally. The phagocytes (neutrophils, eosinophils, macrophages, monocytes) that defend against e.g. bacteria, generate large amounts of reactive species such as superoxide, $O_2^{\bullet-}$. Also other reactive species such as hydrogen peroxide, H_2O_2 and hypochlorous acid, $HOCl$ are generated. These reactive species are formed to kill bacteria (1-4), however, they also induce collateral damage to healthy tissue at the site of infection.

Another example of an intentionally generated radical is the nitric oxide radical, $NO^{\bullet}$. It is produced by nitric oxide synthases (NOS) and plays a key role in different physiological events such as vasodilatation and neurotransmission (5, 6). $NO^{\bullet}$ readily reacts with $O_2^{\bullet-}$, and the product formed, i.e. peroxynitrite ($ONOOH$) is highly reactive (7). Peroxynitrite is also generated during the bactericidal action of phagocytes and again this reactive compound may induce collateral damage (8).

Free radicals are also generated accidentally in living cells as the result of numerous processes such as endogenous metabolic reactions. Exogenous triggers of radical formation include pollutants, xenobiotics, and radiation (9). Excessive free radical formation plays a role in the etiology of many pathological processes such as cancer, cardiovascular disease, neurological disease, rheumatoid arthritis, cataract, emphysema, diabetes, and Alzheimer's disease (10-12).

Free radicals of biological interest are often divided into oxygen centred and nitrogen centred radicals. Reactive oxygen species (ROS) is a collective term that includes both oxygen centred radicals and certain oxygen containing non-radicals that are oxidizing agents or easily converted into radicals. In the reactive nitrogen species (RNS) the reactivity of the species is located on or near a nitrogen atom. Typical physiological relevant ROS and RNS are given in Table 1. The adjective "reactive" is not always appropriate; H_2O_2 , $O_2^{\bullet-}$ and $NO^{\bullet}$ quickly react only with very few molecules. $OH^{\bullet}$ reacts instantaneously with almost everything. $RO_2^{\bullet}$, $RO^{\bullet}$, $HOCl$, $NO_2^{\bullet}$, $ONOOH$, and O_3 have a reactivity that lies in between these extremes (10-12).

$NO^{\bullet}$ and $O_2^{\bullet-}$ are probably the most studied free radicals in biology. In 1997, the Journal of Nitric Oxide: Biology and Chemistry, a journal on $NO^{\bullet}$ research, has been founded. This reflects the physiological, toxicological and medical appreciation of $NO^{\bullet}$, which has not yet appeared for $O_2^{\bullet-}$. Despite many similarities between both radicals, $NO^{\bullet}$ research, after a relatively short time span, has been awarded a Nobel Prize in 1998

(13,14), whereas research on $O_2^{\bullet-}$, a radical with a much larger track record in science, has not received such an appreciation.

Table 1. Radical and non-radical reactive species

Reactive species	Radicals	Non-radicals
ROS	Superoxide, $O_2^{\bullet-}$	Hydrogen peroxide, H_2O_2
	Hydroxyl, $OH^{\bullet}$	Hypochlorous acid, $HOCl$
	Peroxyl, $RO_2^{\bullet}$	Hypobromous acid, $HOBr$
	Alkoxy, $RO^{\bullet}$	Hypoiodous acid, HOI
	Hydroperoxyl, $HO_2^{\bullet}$	Ozone, O_3
	Trioxocarbonate, $CO_3^{\bullet-}$	Singlet oxygen, 1O_2
RNS	Nitric oxide, $NO^{\bullet}$	Nitrous acid HNO_2
	Nitrogen dioxide, $NO_2^{\bullet}$	Dinitrogen trioxide, N_2O_3
		Dinitrogen tetroxide, N_2O_4
		Nitryl chloride, NO_2Cl
		Nitronium cation, NO_2^+
		Nitrosyl cation, NO^+
		Nitrosyl anion, NO^-
		Peroxynitrite, $ONOO^-$
		Peroxynitrous acid, $ONOOH$
		Nitrosoperoxycarbonate anion, $O=N-OOCO_2^-$
		Nitrocarbonate anion, $O_2N-OCO_2^-$
		Alkyl peroxynitrites, $ROONO$

This reflects the physiological, toxicological and medical appreciation of $NO^{\bullet}$, which has not yet appeared for $O_2^{\bullet-}$. Despite many similarities between both radicals, $NO^{\bullet}$ research, after a relatively short time span, has been awarded a Nobel Prize in 1998 (13,14), whereas research on $O_2^{\bullet-}$, a radical with a much larger track record in science, has not received such an appreciation.

As stated above, when $NO^{\bullet}$ and $O_2^{\bullet-}$ meet, they react fast with each other and form the non-radical peroxynitrite ($ONOOH$). In biological systems carbon dioxide (CO_2) is present in relatively high concentrations. The rate of the reaction of CO_2 with $ONOOH$ clearly exceeds the rate of reaction of $ONOOH$ with most biological substrates (15). The reaction of $ONOO$ with CO_2 generates an adduct, the nitrosoperoxycarbonate anion ($ONOOCO_2^-$). $ONOOCO_2^-$ is, like $ONOOH$, very reactive. In biological systems $ONOOCO_2^-$ is the species that is ultimately responsible for the toxicity that starts when $NO^{\bullet}$ and $O_2^{\bullet-}$ combine. $ONOOCO_2^-$ fragments into the reactive free radicals $CO_3^{\bullet-}$ and $NO_2^{\bullet}$. Alternatively, $ONOOCO_2^-$ can be rearranged into nitrocarbonate anion

($\text{O}_2\text{NOCO}_2^-$) which, in turn, undergoes hydrolysis by water to give the relatively non-toxic products nitrate (NO_3^-) and carbonate (CO_3^{2-}).

Hydrogen peroxide (H_2O_2) is from a toxicological point of view a very important non-radical ROS because of its ability to penetrate biological membranes. It is easily converted into more reactive species. Stimulated neutrophils generate $\text{O}_2^{\bullet-}$ and its dismutation product, H_2O_2 , and release the heme enzyme myeloperoxidase. Myeloperoxidase catalyses the oxidation of halide ions (Cl^- , Br^- , I^-) to hypohalous acid at the expense of H_2O_2 . It has been estimated that up to 80% of the H_2O_2 generated by activated neutrophils is used to form HOCl (1-4).

Hypochlorous acid (HOCl) is a highly reactive species and is a strong 2 electron oxidator but a weak 1 electron oxidator (16). It is capable of oxidizing many important biomolecules such as plasma membrane ATPases, collagen, α_1 -antiproteinase, nucleotides, RNA, DNA and DNA repair enzymes (4, 17). HOCl can react with nitrite (NO_2^-) to form nitryl chloride (NO_2Cl) (18,19). NO_2Cl is capable of nitrating, chlorinating, dimerising phenolic compounds such as tyrosine as well as protein modification (18,19).

The hydroxyl radical ($\bullet\text{OH}$) is extremely reactive, it is probably capable of doing more damage to biological systems than any other ROS (10,11,20). The radical is formed from H_2O_2 in a reaction catalysed by metal ions (Fe^{2+} or Cu^+) (21,22). $\bullet\text{OH}$ can be also formed by the reaction of HOCl with $\text{O}_2^{\bullet-}$ (23).

Organic peroxy radicals ($\text{RO}_2^{\bullet}$) also have been identified as important reactive species in physiology (24). They are generated throughout the body when organic molecules, e.g. polyunsaturated fatty acids, react with radicals. The presence of double bonds in a polyunsaturated fatty acid weakens the carbon-hydrogen bond of the carbon atom adjacent to a double bond. Reactive free radicals ($\text{R}^{\bullet}$), e.g. $\bullet\text{OH}$, have the capacity to abstract the hydrogen atom from the carbon-hydrogen bond, preferably located between two double bonds (Fig. 1). Because only the hydrogen atom is abstracted, an unpaired electron is left on the carbon. This lipid radical undergoes molecular rearrangement resulting in a conjugated diene (25). The conjugated diene radical rapidly reacts with oxygen to form a peroxy radical. Subsequently, the lipid peroxy radical abstracts a hydrogen atom from a second polyunsaturated fatty acid resulting in a lipid hydroperoxide and a new lipid radical. This new lipid radical, on its turn, rearranges into a conjugated diene and the chain reaction is continued by its reaction with oxygen and generation of a second lipid hydroperoxide and a third lipid radical. The formed carbon centred lipid radical continues the process.

Fig. 1. Reactions taking place in the process of lipid peroxidation. $R^\bullet$ is a radical that starts lipid peroxidation.

This chain reaction or propagation will stop when one of the substrates (the lipid or oxygen) is consumed or the reaction is terminated because two radicals combine. Under physiological conditions, peroxy radicals can react with $NO^\bullet$, generating a non-radical that has been identified as alkyl peroxynitrite ($ROONO$) (26). If the $ROONO$ simply rearranges to an organic nitrate, $NO^\bullet$ may serve as an antioxidant (27). Alternatively, $ROONO$ can decompose to nitrogen dioxide (NO_2) and an oxyl radical ($RO^\bullet$) (26). In this case, $NO^\bullet$ will induce a more reactive species and act as a prooxidant (26). The chain reaction can also be interrupted by antioxidants such as vitamin E (28).

2. Antioxidant defence

As described above, free radicals are continuously produced in the living cells and they may damage almost any cellular component. Fortunately, the cell is equipped with an elaborate antioxidant defence system, from either endogenous or exogenous origin, that protects cellular components from free radical induced damage. An antioxidant is defined as a compound which, in a relatively low concentration, prevents or delays the oxidation of another compound. Directly acting antioxidants do so by reacting with radicals or other reactive species. The directly acting antioxidants can be divided into enzymatic antioxidants and non-enzymatic antioxidants.

2.1 Enzymatic antioxidants

2.1.1 Catalase

Catalase consists of four protein subunits, each containing a heme group and NADPH (29). The enzyme catalyses the conversion of hydrogen peroxide to water and oxygen (29,30). Catalase is largely located within cells in the peroxisomes, which also contain most of the enzymes capable of generating hydrogen peroxide. The amount of catalase in cytoplasm and subcellular compartments has not been established accurately, because peroxisomes are easily ruptured during manipulation. The highest activity is present in liver and erythrocytes but some catalase is found in all tissues. Glucose 6-phosphate dehydrogenase deficiency is a genetic disorder in which NADPH is lowered in mature erythrocytes. It has been suggested that the hemolysis observed in glucose 6-phosphate dehydrogenase deficiency is derived from inhibition of catalase rather than from less glutathione peroxidases or glutathione reductase activity (31).

2.1.2 Glutathione peroxidase and glutathione reductase

There are at least four different glutathione peroxidases (GPx) in mammals, all of them contain selenium at the active site. Deficiency might occur as a result of selenium deficiency (32,33). The highest concentrations are found in the liver, although GPx is widely distributed in almost all tissues. GPx catalyses the reduction of hydrogen peroxide or other hydroperoxides such as a lipid hydroperoxide using reduced glutathione (GSH) as a cofactor (34). The catalytic mechanism for the reduction of hydroperoxides by GPx involves oxidation of the active site selenolate (Se^-) to selenenic acid (SeOH). In this reaction a hydroperoxide (ROOH) is converted into the corresponding, far less reactive alcohol (ROH). Upon addition of one molecule of GSH, the SeOH is transformed to a selenenylsulfide adduct with GSH (Se-SG), which can be regenerated to the active Se^- and glutathione disulfide (GSSG) by addition of a second molecule of GSH (34). Thus, in the reaction, two molecules of GSH are oxidized to GSSG. GSSG can be reduced by the NADPH-dependent flavoenzyme glutathione reductase (GR), the major mammalian GSSG-reducing enzyme which has a similar tissue distribution as GPx (35-37). The activity of GPx is dependent on the constant availability of GSH and the ratio of GSH/GSSG is usually kept very high as a result of the activity of GR (36,37).

2.1.3 Superoxide dismutase

Superoxide dismutase (SOD) was the first antioxidant enzyme characterized to have a free radical as a substrate. It catalyses the dismutation of $\text{O}_2^{\bullet -}$ to H_2O_2 and O_2 (38,39).

There are three forms of superoxide dismutase in mammalian tissues, each with a specific subcellular location and different tissue distribution.

(I) Manganese superoxide dismutase (Mn-SOD), an 80-kDa tetrameric enzyme, is found in mitochondria of almost all cells in relatively high amounts. It forms a primary line of defence against $O_2^{\bullet -}$ that is formed due to the leakage of electrons from the respiratory chain (40,41).

(II) Copper zinc superoxide dismutase (CuZn-SOD) is a cytosolic 32-kDa dimeric enzyme (38,42). Mice with deletion of the gene that codes for CuZn-SOD, develop normally but show enhanced susceptibility to central nervous system injury (43). A specific form of the lethal neurodegenerative disease amyotrophic lateral sclerosis (ALS) is caused by mutations in the gene for cytosolic CuZn-SOD (44).

(III) Extracellular superoxide dismutase (EC-SOD) has been identified by Marklund (45) and is a secretory copper and zinc containing SOD distinct from the CuZn-SOD described above. EC-SOD is highly synthesized in fibroblasts and endothelial cells and it is released into the circulation upon the injection of heparin (46). EC-SOD might play a role in the regulation of the vascular tone, because $NO^{\bullet}$ or another closely related endothelial derived relaxing factor is inactivated in the plasma by $O_2^{\bullet -}$ (47).

2.2 Non-enzymatic antioxidants

Directly acting, non enzymatic antioxidants usually act by directly scavenging free radicals. These antioxidants can receive an electron from a radical or donate an electron to a radical with the formation of relatively stable products. They can be divided into lipophilic and hydrophilic antioxidants.

2.2.1 Vitamin E

Lipophilic antioxidants scavenge radicals in membranes and lipoprotein compartments and are crucial in preventing lipid peroxidation. The most important lipophilic antioxidant is probably vitamin E (VE) (28). VE was discovered in 1922 by Evans and Bishop (48) and recognized as a dietary factor in animal nutrition which was needed for normal fertility. They found that in pregnant rats deprived of VE, the fetuses do not develop normally but are resorbed. Rat fetal resorption is still the bioassay for the determination of the vitamin E activity of a preparation. In 1936 the chemical diversity of VE was observed when two vitamers were isolated and characterized from wheat germ oil (49). These two vitamers were assigned as α - and β -tocopherol. The name was derived from Greek words 'tokos' (childbirth) and 'phorein' (to bring forth).

In the following years two additional tocopherols, γ - and δ -tocopherol (50,51) as well as the tocotrienols (52) were isolated from edible plant oils. The tocopherols (α , β , γ , and δ) have a hydrocarbon tail and a chromanol head (with two rings: one phenolic and the

other heterocyclic) in which the number and position of the methyl groups differ. The tocotrienols (α , β , γ , and δ) are structurally similar to tocopherols but have an unsaturated hydrocarbon tail. In biomembranes, the hydrocarbon tail of VE intercalates with fatty acid residues of phospholipids, while the chroman head faces the cytosol, although it is still located in the lipophilic region.

The 4 tocopherols each have 3 independent asymmetrical carbon atoms giving rise to 8 different stereoisomers of each tocopherol. The 4 tocotrienols have one asymmetrical carbon atom, and thus two stereoisomers of each of these vitamers exist. This makes a total of 40 different vitamers of VE. The vitamer of VE that has the highest vitamin activity *in vivo* is RRR- α -tocopherol.

2.2.1.1 Bioavailability of vitamin E

VE is a vitamin and thus it has to be provided by foods and supplements to maintain good health. Major dietary sources of VE are vegetable oils, nuts, foods such as grains and dairy products (53,54). Because of its hydrophobicity, VE requires special transport mechanisms in the aqueous environment of plasma, body fluids and cells.

The absorption of tocopherols and tocotrienols follows that of lipids and they are taken up in chylomicrons. The chylomicrons are transported via the lymph and they reach the blood stream in the ductus thoracicus (55). Only free tocopherols and tocotrienols appear in chylomicrons and in blood, VE esters are hydrolysed in the gastrointestinal tract e.g. by pancreatic esterases (56). The triacylglycerols present in chylomicrons are hydrolysed in the blood by the endothelial-bound lipoprotein lipase. The chylomicron remnant formed in this way still contains the VE. In hepatocytes, the α -tocopherol transfer protein (α -TTP) specifically filters RRR- α -tocopherol from all incoming tocopherols and transfers it into nascent very low-density lipoproteins (VLDL) (57).

About half of the resulting VLDL is transformed into low-density lipoproteins (LDL) by hepatic lipase. LDL is the major carrier for RRR- α -tocopherol in blood, supplying it to tissues in a transport process involving the LDL receptor. The intracellular transport of vitamin E is mediated by tocopherol-associated proteins (TAPs). Again RRR- α -tocopherol is the vitamer with the highest affinity for these proteins. TAP protects tocopherol against oxidation and might be involved in VE cell signalling. As a consequence of its high affinity to these proteins, the half life and the biological activity of RRR- α -tocopherol are much higher than those of the other stereoisomers which are more readily excreted from the plasma and secreted with the bile (57,58).

2.2.1.2 Antioxidant activity of vitamin E

The best-known biochemical activity of VE is the protection of biomembranes against endogenous and exogenous oxidative stress. VE inhibits lipid peroxidation by scavenging

ing of lipid peroxy radicals. In its role as a chain-breaking antioxidant, the hydrogen atom from the aromatic hydroxyl group of VE is rapidly abstracted by the lipid peroxy radical resulting in formation of a lipid hydroperoxide and a VE radical (Fig. 2) (59). Beside lipid peroxy radicals, VE is also able to efficiently scavenge a number of other reactive species such as singlet oxygen (60), peroxyntirite (61), nitrogen dioxide (62), ozone (63) and superoxide (64).

The VE radical produced during its antioxidant activity, is relatively stable due to delocalization of the unpaired electron over the chroman head (Fig 2). This makes the radical relatively un-reactive. The oxygen in the heterocyclic ring of the chroman head is fixed in such a way that there is a considerable overlap between the 2p-type orbital of a lone pair of the oxygen and the aromatic π -system (59). This allows stabilization of the VE radical by interaction of the unpaired electron with a lone pair of that oxygen, and in this way the delocalisation is enhanced (59).

Fig. 2. Oxidation and regeneration of α -tocopherol.

In the antioxidant network, it has been proposed that regeneration of VE from VE radicals can occur by interaction of the VE radical with vitamin C (65), GSH or reduced Q_{10} (66). Alternatively, the VE radical can be further oxidized into a quinone (Fig. 2). In this oxidation reaction, the heterocyclic ring of the chroman head is opened (67). Interestingly, *in vivo*, the quinone is readily reduced into the corresponding hydroquinone (67-69). The antioxidant activity of α -tocopherol hydroquinone is superior to that of α -tocopherol (59, 69, 70). Kohar et al. (68) suggested that *in vivo* not VE itself but instead VE-hydroquinone displays the actual antioxidant activity ascribed to VE. VE-quinone administered to human volunteers is partly converted into VE (71).

Also prooxidant activities have been ascribed to VE. VE can reduce transition metals like iron and reduced iron might generate free radicals by reacting with lipid hydroper-

oxides which are present in arterial plaques. This mechanism was put forward as an explanation for the rise in fatal myocardial infarctions observed in a clinical study on VE supplementation (72).

The VE radical that is formed during the antioxidant activity still has sufficient residual reactivity to promote radical damage. For example, the VE radical can react with a polyunsaturated fatty acid, thus starting the chain reaction again that was interrupted once VE scavenged the lipid peroxy radical. As also mentioned above, elimination of the VE radical can occur by interplay with other antioxidants present in the so-called antioxidant network; Vitamin C, GSH or reduced Q₁₀ have the ability to regenerate VE from its VE radical.

VE quinone that is formed by the two-electron oxidation of VE (Fig. 2), is cytotoxic due to its ability to generate oxygen radicals and to form an adduct with thiols (59). Thornton et al. (73) has reported that γ -tocopherol quinone and δ -tocopherol quinone are more toxic than α -tocopherol quinone. This toxicity involves the formation of Michael adducts with cellular thiols (73).

2.2.1.3 Non-antioxidant activity of VE

Various non-antioxidant activities have been ascribed to VE. Some of them are summarized in Table 2.

Beside the protection of membranes against free radical damage, VE can also directly interact with membranes. Despite its low concentration, VE stabilizes membranes by forming complexes with products of membrane lipid hydrolysis, such as lysophospholipids and free fatty acids (85). Patel and Edwards (86) have reported that vitamin E affects membrane fluidity, at low concentration it increases membrane fluidity, whereas at high concentration it has the opposite effect.

Table 2. Some of the non-antioxidant effects of VE.

Compound	Activity	Reference(s)
α -Tocopherol	Inhibition of protein kinase C- α	74-79
	Activation of protein phosphatase 2A	80
	Inhibition of platelet aggregation	75
	Reduction of the expression of CD36 scavenger receptor	81
	Activation of eNOS	82
	Inhibition of glutathione-transferase P1-1	83
Tocotrienols	Inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase	84
All vitamers	Stabilisation of lipid membranes	85

The inhibition of PKC activity by α -tocopherol has gained much attention. This effect is perceived to occur at concentrations of α -tocopherol close to those measured in healthy adults (77,78). β -Tocopherol does not inhibit PKC. This indicates that the mechanism involved in the inhibition of PKC is not directly related to the antioxidant activity since both compounds have a similar direct antioxidant effect (79). Ricciarelli et al. (81) have demonstrated that treatment of rat aortic smooth-muscle cells with α -tocopherol resulted in a time- and dose-dependent inhibition of PKC- α , whereas the other isoforms, PKC- β , PKC- δ , PKC- ϵ , and PKC- ζ are not affected by α -tocopherol. The inhibition of PKC- α by α -tocopherol has been found to be associated with its dephosphorylation (80). α -Tocopherol activates protein phosphatase 2A (PP2A). This enzyme might mediate the observed dephosphorylation and subsequent deactivation of PKC α induced by α -tocopherol (80).

The antiproliferative effect of α -tocopherol has been linked to the inhibition of PKC- α . β -Tocopherol has no antiproliferative activity (80). The inhibition of cell proliferation by α -tocopherol is blocked by addition of β -tocopherol (80). It has also been suggested that the tocopherol-binding protein TAP, that selectively binds α -tocopherol, initiates a cascade of events once α -tocopherol binds that leads to the inhibition of cell proliferation. (79)

Despite a long history of extensive research on the compound, new activities of VE still emerge. For example, in human aortic smooth muscle cells α -tocopherol but not β -tocopherol interferes with the oxLDL uptake by reducing the expression of the CD36 scavenger receptor. This receptor mediates the uptake of oxLDL (81). Heller et al. (82) have demonstrated that α -tocopherol primes eNOS by increasing the phosphorylation of its residue serine 1177. VE can also directly interact with membranes. In our opinion, the complete picture on the role of VE in nutrition, physiology, pathology, biochemistry and toxicology still has to be revealed.

2.2.2. Ester derivatives of VE

Because of the oxidizability of VE, supplementation with VE often occurs by adding more stable VE esters to VE formulations. The phenolic hydroxyl group is essential for the antioxidant activity and esterification of this group improves the stability of the compound. Various esters have been prepared (Fig 3). The esters themselves do not display a free radical scavenging activity (87). The ester of VE most commonly used in foods and cosmetic products is vitamin E acetate (VEA). Cheeseman et al. (88) found that in humans the availability of VE from VEA is higher than that of other VE esters such as vitamin E succinate (VES). In lambs, the concentrations of VE were significantly higher after supplementation with VEA compared to that after supplementation with VES (89). The bioavailability of vitamin E nicotinate (VEN) was lower than that of VES (89). VES is taken up more readily than free VE in neuroblastoma cells (90). As also stated

above, after oral administration the esters are hydrolysed in the intestine and the released VE is absorbed (91).

As shown in Table 3, the rank order of the antioxidant activity of VE and its esters is variable. The differences in potency of VE and its derivatives in the reported studies are probably due to differences in the assay and the experimental conditions.

In contrast to its antioxidant effect, VES also induces apoptosis and promotes cell death. Allready in 1982 (98), it was noted that treatment of murine B-16 melanoma cells with VES induced a more potent morphological differentiation and growth inhibition in comparison to treatment of these cells with VE, VEA, and VEN.

Table 3. The reported rank order of the antioxidant activity of VE and its ester derivatives.

Rank order	Assay	Reference(s)
VEA > VE	Rat fetal resorption	92
VEA > VE	Rat fetal resorption	93
VEA > VE	Rat fetal resorption	94
VES > VE	Microsomal lipid peroxidation	95
Trolox > VE >>> VEA and VES	Lipid peroxidation in cultured endothelial cells	96
VES > VE $\approx$ VEA	Lipid peroxidation in hepatocytes	97
Trolox > VE > VEA > VES	Microsomal lipid peroxidation	87

One year later, Rama and Prasad (99) reported that VES inhibited growth and caused morphological changes in mouse melanoma (B-16), mouse neuroblastoma (NBP2), and rat glioma (C-6) cells in culture. In 1986, it was established that VES enhanced the growth inhibitory effects of adriamycin on a variety of cancer cells in vitro (100). In 1989, Slack and Proulx (90) reported that VES can inhibit the growth of neuroblastoma N1E 115 cells. Recently, several studies have shown that VES induces growth inhibition and apoptotic activity on a variety of human and rodent cancer cell lines without affecting the proliferation of normal cells in vitro (101-108) and in vivo (109-112).

The mechanism by which VES induces apoptosis is not fully understood. It might involve a combination of membrane destabilising activity and dysregulation of signalling pathways. Destabilisation of the membrane can occur due to the detergent-like action of VES, since VES has a hydrophobic tail, essential for membrane docking of the compound, and a hydrophilic succinyl moiety, in membranes located in the water-membrane interface (105).

VES can exist as a deprotonated, charged species, or the protonated, uncharged counterpart (105). The ratio of these two forms depends on the pH. Almost 99% of the total VES is charged at neutral pH. The part that is uncharged will increase to 25% at pH 6.2

(105). Of the two, only the uncharged species will enter cells by passive diffusion. This might contribute to the apparent selective toxicity of VES towards malignant versus normal cells (104,110), since the pH of the interstitium in most tumours is 6.2-6.5, whereas the pH of most normal tissues is 7.0-7.4 (113). Interestingly, the cytotoxic effect of VES in Jurkat and MCF-7 cell lines was greater at acidic pH, which might also be due to the high diffusion rate of VES over cell membranes at acidic pH (105).

Birringer et al. (106) found that neither trolox succinate nor phytol succinate caused apoptosis. This indicates that all parts of the molecule (i.e. the succinate group, the chroman head and the phytol tail) are essential for the induction of apoptosis by VES. Vitamin E glutarate has a very poor apoptotic activity compared to that of VES, vitamin E maleate and vitamin E fumarate (Fig 3). Apoptotic potency also differs between vitamin E oxalate, vitamin E malonate and VES. The relative potency of these VE esters correlates with the flexibility of the terminal carboxyl containing moiety (108); the longer the terminal dicarboxylic moiety, the more flexible this moiety and the lower the apoptotic effect. Non-apoptotic and apoptotic VE analogues are shown in Figure 3.

The signalling pathways that have been suggested to play a role in the VES-induced apoptosis are diverse. One of the mechanisms that has been proposed is that VES can activate the caspase cascade and in this way modulates the mitochondrial membrane function which finally leads to apoptosis (103).

2.2.3. Vitamin E phosphate

Only recently, it has been discovered that vitamin E phosphate (VEP) is present in plant and animal tissues, including human. The concentrations of VEP are in general similar to those of VE. In rat and pig liver VEP levels were substantially higher (114). In food-

Table 4. Some physiological effects of VEP.

Compound	Activity	Reference(s)
Vitamin E phosphate	Reduction of the intensive rate of metabolism in rabbit muscles	116-119
	Activation of cAMP phosphodiesterase	120
	Increasing the rate of phenylalanine hydroxylase	121
	Protection against irradiation in rat intestine and cultured mouse skin	115,122
	Increase in sperm survival	123
	Improvement of acetylcholine-dependent relaxation in the aged aortic strips exposed to oxidative stress	124
	Protection against LPO in non-heated and heated rat liver microsomes	87

stuffs such as chocolate and cheese the concentrations of VEP are 10 to 30 times higher than those of VE (114). Interestingly, supplementation of VEP (30 mg/kg/day) in rats for 32 days significantly increased the levels of VEP (62%) as well as of VE (40%) in liver indicating the possible absorption of this VE-ester and conversion of VEP into VE (87,114,115). Table 4 summarizes some of the physiological effects of VEP.

The research on VEP started some six decades ago. In 1942, Houchin and Mattill (116) have shown that the administration of VEP to a dystrophic rabbit lowers the excessive metabolic rate of the muscle tissue to a normal level within 4 hours. The level of creatine in the liver was not affected (119). Furthermore, VEP increased the maximum velocity of phenylalanine hydroxylase about 25-fold in the presence of tetrahydrobiopterin (BH₄), while the increase by VES was only 3-fold (121). It was stated that VEP is intimately connected with complicated and unknown enzyme systems that maintain the physiological integrity of muscle tissue (116).

During the last ten years, VEP has been promoted as a radioprotector. Felemovicius et al. (122) reported that a chronic oral supplementation of VEP (5 mg/ml) significantly protected against acute irradiation in rat intestine. This protection was slightly superior to that of VEA (5 mg/ml) (122). Pre-treatment of cultured mouse skin with 0.5% of VEP (9.4 mM) substantially protected against UV-B triggered skin damage in comparison to that of VEA (9.4 mM) (115). The radioprotective effect of VEP has been ascribed to its ability to protect against lipid peroxidation, cell damage due to sunburn and DNA degradation (115). This protection was suggested to be due to the conversion of VEP to VE in mouse skin by alkaline phosphatase (115).

Another remarkable function of VEP was reported by Pérez-Pé et al. (123). They found a significant beneficial effect of VEP in combination with semen plasma protein on sperm survival. There was no protective effect with VEA (123). They suggested that this difference might be due to a more potent inhibition of lipid peroxidation by VEP compared to VEA (123). Recently, we have shown that - in contrast to VEA - VEP had a potent antioxidant activity of its own, without conversion into VE (Chapter 2) (87). VEP was not consumed during its protection against lipid peroxidation. VEP also induced a potent hemolysis in erythrocytes and reduced membrane fluidity (87). Our results indicated that VEP might act as a detergent and forms a barrier in biomembranes and in this way inhibits the transfer of radicals from one polyunsaturated fatty acid to another (87).

We have also found that VEP has a very potent apoptotic activity (Chapter 3) (125). VEP potentially induced DNA fragmentation and nuclear condensation in osteosarcoma cells (125). As stated above, in a series of dicarboxylic esters of VE, the ester with the least flexible dicarboxylic moiety displayed the highest apoptotic effect (106,108). The

phosphate group is not flexible and, based on analogy with the VE esters, this might be an important feature for the apoptotic activity of VEP.

Recently, Munteanu et al. (126) reported that a mixture of VEP (VEPm) containing VEP (55.3%), di-VEP (30.6%), VE (5%), inorganic phosphate (2.6%), water (1.9%), oleic acid (1%) and some unidentified products (3.5%) modulated cell proliferation and gene expression in rat aortic smooth muscle cells (RASMC) at a relatively low concentration. At higher concentrations, VEPm induced apoptosis in human THP-1 monocytic leukaemia cells. Munteanu et al. (126) focussed on a beneficial role of VEPm in atherosclerosis and inflammation. In our opinion, the apoptotic effect of VEP on monocytic leukaemia cells might also be of interest.

Fig. 3. Chemical structures of non-apoptotic (A) and apoptotic (B) analogues of VE and its ester derivatives.

2.2.4. *Flavonoids*

Several plant pigments have been identified as flavonoids during the last century. Also other polyphenolic compounds which are widely distributed in foods of plant origin belong to this group. Over 5000 different naturally occurring flavonoids have been described and the list is still growing. Flavonoids are divided into eight main groups based on the degree of saturation of the C-ring, the hydroxylation pattern of the compound and the substitution in the 3-position (Fig. 4). These groups include flavanols (e.g., epicatechin), flavonols (e.g., quercetin), flavones (e.g., luteolin), flavanones (e.g., naringenin), isoflavones (e.g., genistein), anthocyanidins (e.g., cyanidin), chalcones (e.g., chalcone naringenin) and dihydrochalcones (e.g., phloretin) (Table 5).

Humans have consumed flavonoids and other dietary phenols ever since the emergence of human life on earth. They are present in fruits, vegetables, nuts, plant-derived beverages such as tea and wine, as well as in some traditional herbal-containing medicines. Much attention is given to the potential health-beneficial properties of flavonoids. They are reported to be powerful antioxidants *in vitro* (127-133) and *in vivo* (134,135). Furthermore, flavonoids interfere in several of the steps that lead to the development of different types of cancer, including protection of DNA from oxidative damage, inhibition of carcinogen activation and activation of carcinogen-detoxifying systems (136-138). Understanding the mechanisms of action of flavonoids, either as antioxidants (127-135) or as modulators of cell signalling (139), and the influence of absorption and metabolism (140-142) on these properties are fundamental to determine the effect of these compounds on health.

Dietary flavonoids have been shown to act as pro-oxidants in systems containing redox-active metals. In the presence of O₂, transition metals such as copper and iron catalyze the redox cycling of some flavonoids leading to the formation of ROS and phenoxyl radicals that can damage DNA, lipids, and other biological molecules (143-145). Quercetin, the most ubiquitous of the dietary flavonoids, contains a catechol ring and is oxidized readily to a quinone/quinone methide. This oxidation product readily reacts with GSH resulting in quercetin glutathionyl adducts (146). Boots et al. (147) have shown that the oxidation product also readily reacts with protein thiols and this is a key event in quercetin toxicity. Consumption of large amounts of flavonoids present in various supplements might be harmful due to the reaction of flavonoid oxidation products with cellular constituents.

Fig. 4. Basic structure of flavonoids.

Table 5. *Subclasses of flavonoids with some examples of each class.*

Class	Compound	Substituents					
		3	5	7	3'	4'	5'
Flavonol	Quercetin	OH	OH	OH	OH	OH	H
	Kaempferol	OH	OH	OH	H	OH	H
Flavanone	Naringenin	H	OH	OH	H	OH	H
	Naringin	H	OH	ORu	H	OH	H
Flavone	Apigenin	H	OH	OH	H	OH	H
	Diosmin	H	OH	ORu	OH	OMe	H
Isoflavone	Genistein	H(2)	OH	OH	H	OH	H
	Biochanin A	H(2)	OH	OH	H	OMe	H
Flavanol	Catechin	OH	OH	OH	OH	OH	H
	3'-O-methyl epicatechin	OH	OH	OH	OMe	OH	H
Anthocyanidin	Cyanidin	OH	OH	OH	OH	OH	H
	Pelargonidin	OH	OH	OH	OH	H	H
Chalcone	Chalcone naringenin	OH(2)	OH(4)	OH(6)	H	OH	H
Dihydrochalcone	Phloretin	OH(2)	OH(4)	OH(6)	H	OH	H
	Phloridzin	OGl(2)	OH(4)	OH(6)	H	OH	H

Ru = Rutinose (=Glu-Rha), Me = CH₃, Gl = Glucose

2.2.4.1. Phloretin

An apple a day keeps the doctor away! This saying might be related to the presence of the dihydrochalcones, phloretin and phloridzin, in apples. These compounds are present predominantly in the peel of apples but they are also found in the pulp (148). Significant quantitative differences between the apple varieties have been found. The Golden Delicious variety has the lowest content of dihydrochalcones and the Green Reineta variety the highest (148). Generally, apples contain as much as 2 g of polyphenols per kilogram wet weight.

Fig. 5. Scheme of the metabolic degradation of the flavonoid kaempferol, showing chalcone as a metabolic product.

2.2.4.2. Bioavailability and metabolism of phloretin

Due to the large chemical heterogeneity of flavonoids, bioavailability and metabolism of flavonoids is diverse. In Figure 5, the metabolic pathway of the flavonoid kaempferol, showing chalcone as a metabolic product, is depicted. For phloridzin, Malathi and Crane (149) reported that in the brush border of the hamster small intestine, the enzyme β -glucosidase hydrolysed phloridzin into phloretin and glucose. This enzyme was identified in the study of Kraml et al. (150) as lactase-phloridzin hydrolase (LPH).

The degradative metabolism of dihydrochalcones aglycons involves splitting of the molecule, giving products that only contain the A- or B-ring (Fig. 5) (151). Monge et al. found that in rat about half of the administered phloretin was excreted in the urine within two days (152). Most of the metabolites were degradation products, including phloroglucinol and, in larger amounts, phloretic acid and related metabolites formed by dehydrogenation, beta-oxidation and glycine conjugation. Phloroglucinol, administered in a similar experiment, was rapidly (90% within 24 h) excreted in the urine, either unchanged or as conjugates (glucuronide/sulphate) (152). Crespy et al. (153) reported that the same compounds are found in 24-h urine of the rat fed either phloretin or phloridzin. This is in line with the conversion of phloridzin into phloretin in the intestine before absorption. The time needed for this conversion explains why phloretin appeared more

rapidly in plasma after phloretin administration compared to phloridzin administration. These results are in good agreement with a previous study of the same group (154), showing that phloretin was absorbed efficiently in an *in situ* intestinal perfusion model. Clearance of the conjugated metabolites of phloretin was higher than that of unconjugated phloretin (153).

2.2.4.3. Antioxidant activity of phloretin

The antioxidant activity of phloretin has been demonstrated in different assays with different reactive species, such as $\text{OH}^\bullet$ (155-157), ONOOH (157) and the 1,1-diphenyl-2-picrylhydrazyl radical (158-160). Various activities e.g. the radioprotective effect of phloretin in mice, has been attributed to its scavenging potency (156). The position of hydroxyl groups in phloretin plays an important role in its antioxidant activity (155-160). Formation of intramolecular hydrogen bonds is of great importance for the antioxidant activity (160). In Chapter 4 (157), it is shown that the activity of phloretin does not reside only in the three hydroxyl groups of ring A. The carbonyl group plays a key role in the potent antioxidant activity of phloretin (157). Phloretin also possesses some prooxidant activity since it can oxidize human erythrocyte oxyhemoglobin and cause erythrocyte hemolysis (144).

2.2.4.4. Non-antioxidant activity of phloretin

The reported non-antioxidant activities of phloretin and phloridzin are summarized in Table 6. The effect of phloretin or phloridzin on glucose uptake suggests the use of both compounds as an antidiabetic agent (161,162). Interestingly, the phloretin induced apoptosis in melanoma cells was suggested to proceed through the inhibition of glucose transmembrane transport and inhibition of PKC activity (163).

Table 6. Non-antioxidant activity of phloridzin and phloretin.

Compound	Activity	Reference(s)
Phloridzin	Competitive inhibition of intestinal glucose uptake	164
Phloretin	Insulin-like activity on glucose transport	165
	Antagonist of the prostaglandin $\text{F}_{2\alpha}$ receptor	166
	Anti-inflammatory activity	167
	Inhibition of tyrosine kinase and protein kinase C	163,168
	Induction of apoptosis in melanoma cells	163

2.2.5. Lipoic acid

Lipoic acid (LA), a naturally occurring dithiol, is present in the cells of most prokaryotes and eukaryotes. In foodstuff, LA is present in a relatively high amount in meat and

liver and to a lesser extent in fruits and vegetables. In humans, LA is present in all parts of the body as a cofactor for several mitochondrial enzymes which are required for energy metabolism. In the late 1940's, LA was identified as a growth factor and as a protein-bound cofactor to activate acyl-moiety transfer reactions. Based on this biological activity, LA has been named "acetate-replacing factor", "pyruvate oxidation factor" and "protogen A". In 1951, 30 mg of LA was purified as a yellow crystalline substance from 100 kg liver by Reed et al. (169). The chemical structure was elucidated (170,171) and the substance was identified as 1,2-dithiolane-3-pentanoic acid (Fig. 6). Because of its lipophilicity and acidity, LA obtained its name. The non-official name 6,8-thioctic acid emphasizes the presence of the two sulfur atoms in the molecule. Other names of lipoic acid are α -lipoic acid, 5-(1,2-dithiolane-3-yl)-valeric acid and 5-3-(1,2-dithiolanyl)-pentanoic acid.

LA is not a vitamin because its biosynthesis occurs in man. In *E. coli*, LA is synthesized from octanoic acid by the addition of two sulfur atoms to an octanoyl group bound to the acyl carrier protein (ACP) in mitochondria. Miller et al. (172) showed that this reaction is catalyzed by an enzyme, lipoic acid synthase. The actual source(s) for the two sulfhydryl moieties in the biosynthesis of LA is currently unknown. In mammals LA is synthesized in mitochondria by lipoic acid synthase, but it can also be absorbed from the diet (173).

2.2.5.1. Bioavailability and metabolism of lipoic acid

Several bioavailability studies have shown that LA is well absorbed from the gastrointestinal tract after oral administration. This rapid uptake is followed by an equally rapid clearance, suggesting both transport into tissues as well as glomerular filtration and renal excretion (174). Biewenga et al. (175) revealed that after oral administration of 1 g of LA to a human volunteer, LA appeared in the blood plasma after 30 min and its elimination showed first order kinetics with a half life of 56 min. In a comparable study, Teichert et al. reported that LA was absorbed rapidly with peak plasma concentrations at 10 to 45 min after administration. It was eliminated rapidly, with a mean plasma elimination half-life of 0.56 hours (176). Similarly, Smith et al. (177) also found a rapid uptake and clearance in human subjects following oral LA administration.

Besides LA, its reduced form, dihydrolipoic acid (DHLA), as well as several metabolites could be detected in plasma after oral administration of 1 g of LA to human volunteers. The maximum concentration of LA (1154 ng/ml), 3-ketolipoic acid (2092 ng/ml), and bisnorlipoic acid (704 ng/ml) were observed after 92 min, 119 min, and 189 min respectively (175). Bisnorlipoic acid, β -hydroxy-bisnorlipoic acid, and tetranorlipoic acid were the first discovered metabolites of LA in rats (174,178). 4,6-Bismethylthio-hexanoic acid was found as the main metabolite in urine samples of healthy volunteers after oral administration of LA, whereas both 6,8-bismethylthio-

octanoic acid and 2,4-bismethylthio-butanoic acid were minor metabolites (179,180). It was suggested that 3-ketolipoic acid has a major contribution to the antioxidant activity and therapeutic effect of LA (175). Most metabolites are produced via β -oxidation of the LA pentanoic side chain (175). Harrison and McCormick (174) found that 25% of the radioactivity of LA, ^{14}C labelled on the 1 and 6 position, was exhaled as $^{14}\text{CO}_2$ within 2 h after administration, reaching a total amount of 30% after 24 h. This indicates that LA is metabolised via β -oxidation. Some of the metabolites which are generated via this process are depicted in Figure 6.

The LD_{50} after oral administration in rats and mice is 1130 and 502 mg/kg, respectively, and after intraperitoneal administration this is 200 and 160 mg/kg, respectively (175). This indicates that LA is relatively non toxic (175). The therapeutic dose of LA in the treatment of diabetic polyneuropathy is three times 600 mg per day (175).

Fig. 6. Lipoic acid, dihydrolipoic acid, isomers of β -lipoic acid (A) and β -oxidation products of lipoic acid (B).

2.2.5.2. Antioxidant activity of lipoic acid

The successful therapeutic application of LA as antioxidant has boosted scientific research on LA, especially directed to understand the beneficial effect of LA on pathophysiological processes. LA and DHLA were found to be highly reactive against several ROS and RNS in the different in vitro (181-188) and in vivo (189-192) assays. During

this scavenging activity, LA is oxidized to β -lipoic acid (β -LA) (Fig. 6) (175,185,188). LA efficiently protected against hypochlorous acid (182) and peroxynitrite (185,186) induced α 1-antiproteinase inhibition, while another disulfide such as GSSG has no effect.

The explanation for the relatively potent scavenging activity of the disulfide LA can be found in the dihedral angle of C-S-S-C moiety (φ). In the open chain disulfide GSSG, φ approximates 90° (182,193). At this angle, the energetically unfavourable interaction between the sulfur lone pairs is kept at a minimum and an interchange of these electrons with the free d-levels of the neighbouring sulfur atoms is possible to give the S-S bond a partial double bond character. Indeed, GSSG is a poor scavenger. The disulfide group in LA is located in a dithiolane, a strained, five membered ring in which φ is 35° . This results in an energetically unfavourable interaction of the orbital of the lone pz electron pairs of the sulfur atoms (182,193). The relatively high energy content of the disulfide group in LA is responsible for the relatively high antioxidant activity.

In addition to direct scavenging activity of LA, the LA/DHLA redox couple appears to be favourable to regenerate other antioxidants in the antioxidant network such as VE, VC and GSH (193-195). Considering a redox potential of -0.32 V for the LA/DHLA redox couple compared to that of the GSH/GSSG couple (-0.24 V), DHLA is able to reduce GSSG to GSH (193-195). Increasing of the intracellular availability of cysteine was proposed to be the underlying mechanism of LA to increase GSH synthesis (196). Cysteine is a major building block for the synthesis of GSH.

Various studies have shown an antioxidant effect of LA *in vivo*. LA supplementation (600 mg/day, 2 months) to healthy volunteers decreases urinary F2-isoprostane levels, a biomarker of lipid peroxidation, and increased the lag time of LDL oxidation (189). Intragastric LA administration (150 mg/kg/day, 2 months) to rats protects against exercise-induced oxidative lipid damage in heart, liver, and skeletal muscle, increased total GSH levels in liver and blood and also prevented the exercise-induced decline in heart GST activity (190). Cardiac myocytes from old, LA-supplemented rats (0.2% w/w) exhibited a markedly lower rate of oxidant production that was no longer significantly different from that in cells from control, young rats (191). LA supplementation also restored myocardial ascorbic acid levels and reduced oxidative DNA damage (191). Intraperitoneal administration of LA (100 mg/kg body weight/day for 7 and 14 days) attenuated cerebral lipid peroxidation and prevented age-associated decrease in the levels of VC, VE, and GSH (192). Dietary supplementation of LA for 2 weeks completely reversed the age-related decline in hepatocellular GSH levels and the increased susceptibility to tert-butylhydroperoxide.

Interestingly, in an animal model of Wilson's disease, which results in hepatic copper overload and chronic necroinflammatory disease, LA lowered the mitochondrial copper content with no effect on the cytosolic levels (197). Despite the potential benefits of LA

and DHLA to ameliorate metal-catalysed oxidative stress, DHLA accelerated iron-dependent hydroxyl radical generation and lipid peroxidation in liposomes, probably by reducing ferric to ferrous ions (181,198). Under certain circumstances, DHLA accelerated the loss of activity of alpha-antiproteinase exposed to ionising radiation under a N_2O/O_2 atmosphere and also the loss of creatine kinase activity in human plasma exposed to gas-phase cigarette smoke (198,199).

2.2.5.3. *Non-antioxidant activity of lipoic acid*

LA is successfully used in the treatment of type II diabetes induced polyneuropathy (200). In experimental animal models of diabetes, LA treatment improved neural blood flow, endoneural glucose uptake and metabolism as well as nerve conduction (201). Mechanistic studies conducted in insulin-responsive cells in culture demonstrate that LA (2.5 mM) rapidly stimulates glucose uptake by activating the insulin-signalling pathway (202). The increase in glucose uptake has also been explained by a stimulatory effect of LA on the glucose transport by GLUT-4 (193,203). Since the increased glucose transport is not accompanied by an increase in GLUT-4 mRNA, it is supposed that the degradation of GLUT-4 is decreased by LA (193, 203).

LA reduced the expression of adhesion molecules of Jurkat T cells and of vascular endothelial cells stimulated by TNF- α or phorbol myristate acetate (204,205). LA also attenuated the NF κ B-induced expression of TNF- α in macrophages treated with lipopolysaccharide (206). Kim et al, (207) showed that LA exerted anti-obesity effects by suppressing hypothalamic AMP-activated protein kinase activity.

2.2.6. *Folic acid*

Folic acid (FA), a water soluble B vitamin, is the most oxidised and stable form of folate. FA consists of an aromatic pteridine ring linked through a methylene bridge to *p*-aminobenzoic acid and then to one L-glutamic acid residue. In the plant folate-synthesis pathway, the pteridine moiety, hydroxymethyldihydropterin, is formed from guanosine-5'-triphosphate (GTP), whereas the *p*-aminobenzoic acid is formed from chorismate. Folates present in body tissues and foods are mainly 5,6,7,8-tetrahydropteroylpolyglutamates, which contain a fully-reduced pteridine ring together with additional glutamic acid molecules linked by γ -peptide bonds. The vitamin activity of these reduced polyglutamates is present as long as the essential subunit structure of folic acid remains largely intact. Folates are usually carbon substituted at the N-5 (e.g. 5-methyltetrahydrofolate) or N-10 (e.g. 10-formyltetrahydrofolate) positions, or have a single carbon bridge spanning these positions (e.g. 5,10-methylenetetrahydrofolate). Apparently, there are many chemical forms of folic acid that exhibit vitamin activity (as folates) (208,209).

Plants, fungi, and many microorganisms synthesize folates *de novo*, but humans and other higher animals do not and therefore require a dietary supply (210,211). The B vitamin was first isolated from spinach leaves. Therefore, the name folate, derived from the Latin word *folium*, meaning leaf, was given to this compound. Folate deficiency has been implicated in cardiovascular and hematological diseases, (212,213) neurological and neuropsychiatric disorders, (214,215) neural tube defects (NTD) (216) and cancer including cervical, lung, brain, pancreatic, colorectal and breast cancer (217,218).

2.2.6.1. Bioavailability and metabolism of folic acid

The metabolism of folates is schematically depicted in Figure 7. Plant folates have a short γ -linked chain of glutamyl residues attached to the first glutamate, and most folate-requiring enzymes prefer such polyglutamylated forms (210). Before absorption in the intestine can take place, dietary folates need to be hydrolysed to the folate monoglutamate in the gut lumen by a brush-border γ -glutamyl hydrolase or conjugase (folyl-polyglutamate carboxypeptidase) (219). Human brush-border folate conjugase is a Zn-dependent exopeptidase that catalyses the stepwise hydrolysis of folate polyglutamates (220). The mucosal cells of the proximal small intestine (jejunum) then take up these monoglutamates by a saturable, active process at “normal” concentrations, and also by passive diffusion at higher concentrations (221). Many foods contain inhibitors of the intestinal brush-border folate conjugase enzyme or the folate transport system, which can reduce the efficiency of absorption (222). Some foods, such as milk and other dairy products, contain folate-binding proteins that also reduce folate absorption (223). The efficiency of deconjugation and absorption in the gut lumen can vary considerably with the folate form, the composition of the diet and various physiological factors. Absorption is not affected by ageing (224) but it is markedly influenced by pH. Maximum absorption is at pH 6.3 and sharply declines from pH 6.3 to 7.6 (225). Small amounts of folate may also be synthesised by the intestinal flora in the body, but in man (unlike the rat) this source of absorbable folate is of minor importance. Absorbed folate monoglutamates are converted into 5-methyltetrahydrofolic acid (5-MTHF) during transit through the intestinal mucosa. The only folate form normally entering the human circulation from intestinal cells is 5-MTHF. The liver removes a sizeable proportion of the absorbed dose (226).

Fig. 7. Metabolism of folate and homocysteine. PG, polyglutamate; MG, monoglutamate; FPGC, folylpolyglutamate carboxypeptidase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; THF, tetrahydrofolate; TS, thymidylate synthetase; dUMP, deoxyuridylate; dTMP, thymidylate; MTHFR, methylenetetrahydrofolate reductase; 5-MTHF, 5-methyltetrahydrofolate; MT, methionine synthetase; VB₁₂, vitamin B₁₂; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; DNA-MT, DNA methyltransferase; BH₂, dihydrobiopterin; BH₄, tetrahydrobiopterin.

2.2.6.2. Antioxidant activity of folic acid

Supplementation with FA improves endothelial dysfunction in patients with established coronary heart disease independent on the levels of homocysteine (212, 227). It has been suggested that the reduction of oxidative stress by folic acid might be involved in this effect (212,228). 5-MTHF when given intra-arterially, abolished the homocysteine-induced increase in intracellular $O_2^{\bullet}$ generation. FA and H₄B also abolished the homocysteine-induced intracellular $O_2^{\bullet}$ production in cultured cells (227). This suggests that FA, 5-MTHF, and H₄B (a cofactor essential of NO[•] formation by NOS) restore endothelial function by suppressing $O_2^{\bullet}$ production and increasing NO[•] half-life. This is supported by the observation that 5-MTHF has no direct effect on in vitro NO[•]

production by eNOS but induces a dose-dependent reduction in eNOS- and xanthine oxidase-mediated $O_2^{\cdot -}$ generation. The reduced $O_2^{\cdot -}$ production might be responsible for the impaired endothelium-dependent vasodilation in patients with familial hypercholesterolemia (228). Additionally, it has been reported that 5-MTHF has a direct stimulatory effect on the enzymatic activity of NOS in recombinant eNOS and cultured endothelial cells (229). It is also interesting to note that oral FA supplementation (10 mg/d) to healthy human volunteers not only enhances NOS activity but also prevents nitrate tolerance to continuous treatment with nitroglycerin, possibly by restoring or stimulating endogenous regeneration of H_4B (230). In vitro, 5-MTHF and THF inhibit lipid peroxidation and scavenge ONOOH (Chapter 6) (231). Also the ability of FA to scavenge and repair of thiyl radicals makes it possible to consider this vitamin as a physiologically relevant antioxidant (232).

2.2.6.3. *Non-antioxidant activity of folic acid*

The non-antioxidant effect of FA has gained more attention than its potential antioxidant effect. A too high homocysteine level has been implicated in the development of cardiovascular diseases. Hyperhomocysteinemia involves both genetic and nutritional causes (233) and has been found in patients with coronary arterial disease, (234) cerebrovascular (235) and peripheral vascular diseases and thrombosis (236).

The metabolism of FA and homocysteine are interrelated (Fig. 7). Folic acid catalyses methylation of homocysteine, leading to a reduction of plasma total homocysteine (233). Conversion of homocysteine into methionine can occur in a reaction catalyzed by methionine synthetase, which uses 5-MTHF as a methyl donor and cobalamin (vitamin B_{12}) as an essential cofactor (237). Several studies have shown a protective role of periconceptional intake of folic acid in reducing both the occurrence and recurrence of neural tube defects. Goldenbergh, et al.(238) showed that folic acid improves the outcome of pregnancy not only by decreasing NTD but by decreasing growth retardation and increasing birth weight. In 1992, the U.S. Public Health Service issued a recommendation that all women of childbearing age capable of becoming pregnant should consume 400 μg of folic acid each day. Homocysteine might directly be involved in NTD by causing vasculopathy leading to an inadequate maternal-fetal circulation. This is based on the observed relationship between high levels of total homocysteine and defective chorionic villous vascularisation in mothers with recurrent early pregnancy loss (239).

Currently, it is believed that the beneficial effect of folate is due to its ability to maintain DNA stability (240-245). One of the mechanisms suggested is through DNA methylation. Folate, in the form of 5-MTHF enhances the formation of methionine, which can be converted into *S*-adenosylmethionine (SAM). SAM methylates specific cytosines in DNA, and this regulates gene transcription. As a consequence of folate deficiency, cel-

lular SAM is depleted, which potentially induces proto-oncogene expression leading to cancer (217,246).

Another mechanism through which folate might enhance DNA stability has received less attention. Folate, as 5,10 methylene THF, donates a methyl group to uracil converting it into thymine, which is used for DNA synthesis and repair (Fig. 7). When folate is lacking, uracil misincorporation into DNA may occur which may lead to double strand breaks and chromosomal damage (247).

Conclusion

Free radicals are implicated in various pathophysiological processes. Protection is provided by both enzymatic and non-enzymatic antioxidants.

The biological effects of antioxidants are diverse. First of all, an antioxidant is often not a clear defined compound, consists of various chemical forms and in the body it is converted into various metabolites. Each of these different forms of the antioxidant displays a different biological effect. Additionally, antioxidants do not only display various antioxidant activities, they often possess some prominent non-antioxidant effect. The free radical processes in the body as well as the biological effects of antioxidants have to be elucidated to a greater extent in order to further rationalise and optimise antioxidant therapy.

3. Outline and scope of the thesis

As indicated in the Introduction a more accurate risk-benefit analysis of antioxidants is needed. Therefore, the mechanism of action of vitamin E phosphate, phloretin, lipoic acid and folic acid was studied. These antioxidants were chosen because they represent different chemical classes and are widely used as food constituents, food supplements or as a medicament. Moreover, the studied compounds are of interest from a toxicological point of view.

Vitamin E phosphate (VEP) is of interest because very recently it has been discovered that its levels in the human body are equal to or higher than those of vitamin E (VE). The question is whether VEP has its own intrinsic activities or has to be converted into VE to display a prominent biological effect. Therefore we investigated the antioxidant activity of VEP (Chapter 2) and its apoptotic properties (Chapter 3).

Phloretin is representative for the compounds in the group of dihydrochalcones, i.e. flavonoids without a heterocyclic C ring. It is abundantly present in apples. Therefore it is likely that phloretin might contribute to the positive health effect associated with a high intake of fruit and vegetables. To understand its antioxidant activity, the part of the molecule responsible for this activity was identified (Chapter 4).

Lipoic acid (LA) is a naturally occurring dithiol and present in all parts of the human body. Additionally, it is the only antioxidant that has been registered as such. In Germany LA is widely used in the treatment of polyneuropathy in diabetics. LA appears to be a very potent protector against one type of ONOOH- mediated damage as well as a very poor protector against another type of damage mediated by the same reactive species. This controversy was further investigated by examining the antioxidant activity of LA and other sulfur-containing compounds in the presence of different detectors and different oxidants (Chapter 5).

Folic acid (FA) supplementation can significantly reduce the etiological risk of a number of diseases. In these pathologies the production of reactive oxygen species plays a role. The antioxidative potential of FA itself and of its physiological metabolites was assessed and the part of the molecule which is important for this activity was determined (Chapter 6).

Finally, the results and impact of our findings are summarised in Chapter 7.

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Chapter 2

The extraordinary antioxidant activity of vitamin E phosphate

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Abstract

The antioxidant activity of RRR-vitamin E (VE), all-*rac*-vitamin E (all-*rac*-VE), trolox, RRR-vitamin E acetate (VEA), all-*rac*-vitamin E phosphate (VEP) and RRR-vitamin E succinate (VES) were compared. In this study, the rank order in the inhibition of lipid peroxidation of VE and its derivatives was trolox > VE $\approx$ all-*rac*-VE > VEA > VES. VE and trolox inhibited lipid peroxidation in non-heated and heated rat liver microsomes. It has generally been accepted that this is due to scavenging of free radicals by these antioxidants, and during this protection the antioxidants are oxidized. VEA and VES have to be converted into VE by esterases to obtain antioxidant activity against lipid peroxidation. VEP, however, had a potent antioxidant effect of its own without conversion to VE. In contrast to VE, VEP is not consumed during this protection. Of the compounds tested, VEP is the most potent in induction of hemolysis of erythrocytes. EPR experiments using the spin label 16-doxylstearic acid showed that VEP reduces membrane fluidity, in contrast to VE. This indicates that VEP acts as a detergent and forms a barrier that might inhibit the transfer of radicals from one polyunsaturated fatty acid to another. This new mechanism may form the basis for a new class of antioxidant.

Introduction

Free radicals are continuously produced within living cells as a result of metabolic reactions. In addition, numerous exogenous sources, including xenobiotics and radiation can induce free radicals. Although cells have an elaborate defense system that provides protection against these free radicals, the development of cancer, cardiovascular and neurological diseases, and other oxidative stress mediated dysfunctions occur (1-3).

Vitamin E (VE) is one of the most important lipid-soluble primary defense antioxidants. Vitamin E is a generic term used for several naturally occurring tocopherols and tocotrienols. The vitamin E molecule can be divided into two parts, a hydroxyl-bearing aromatic system (one phenolic and one heterocyclic ring, called the chroman head) that is responsible for its antioxidant properties, and either a saturated (tocopherols) or polyunsaturated (tocotrienols) hydrocarbon tail for the orientation of VE in the lipid membrane (4).

In its function as a chain-breaking antioxidant, VE rapidly transfers its phenolic H-atom to a lipid peroxy radical, converting it into a lipid hydroperoxide and a VE radical (5, 6). The VE radical can be reduced to VE by vitamin C or reduced glutathione or alternatively it is further oxidized to VE quinone (6). VE is often supplemented as a chemically stable ester derivative such as RRR-vitamin E acetate (VEA), all-*rac*-vitamin E phosphate (VEP) or RRR-vitamin E succinate (VES). These esters are expected to possess no antioxidant effect due to the shielding of the phenolic active antioxidant group by acetate, phosphate or succinate, respectively (Fig.1).

The aim of this study is to compare the intrinsic antioxidant activity of VE and its derivatives, trolox, VEA, VEP and VES in non-heated and heated microsomes.

2. Materials and Methods

2.1. Chemicals

The tested compounds, RRR- α -tocopherol (VE), all-*rac*- α -tocopherol (all-*rac*-VE), RRR- α -tocopherol succinate (VES), RRR- α -tocopherol acetate (VEA), all-*rac*- α -tocopherol phosphate (VEP) and trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) in addition to alkaline phosphatase (Orthophosphoric-monoester phosphohydrolase[alkaline optimum]: EC 3.1.3.1), 16-doxylstearic acid and ABTS (2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) were obtained from Sigma (St. Louis, MO, USA). All other used chemicals were of analytical grade purity.

Fig. 1. Chemical structure of vitamin E (VE) and its derivatives vitamin E acetate (VEA), vitamin E succinate (VES), vitamin E phosphate (VEP) and Trolox. The assymetrical carbon atoms are indicated by an asterisk.

2.2. Isolation of rat liver microsomes

Male Lewis rats, 200-250 g, were killed by decapitation. Microsomes were prepared according to Haenen and Bast (7) with a slight modification. Livers were removed and homogenized (1:2 w/v) in ice cold phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA. The homogenate was centrifuged at 10,000 g for 30 min at 4 °C. Subsequently, the supernatant was centrifuged at 65,000 g for 60 min at 4 °C. The microsomal pellet was resuspended in the phosphate buffer (2 g liver/ml), corresponding with 1-1.3 mg protein/ml, and stored at - 80 °C. Before use the microsomes were thawed and di-

luted 5-fold with ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing 150 mM KCl and washed twice followed by centrifugation at 65,000 *g* for 40 min at 4 °C. Finally, the pellet was resuspended in 4 ml Tris-HCl buffer and then used. The protein concentration was measured according to Smith *et al.* (8).

2.3. Lipid peroxidation (LPO) assay

2.3.1. Incubation conditions.

At 37 °C, 2150 µl of Tris-HCl/KCl buffer was added to 1 ml non-heated microsomes or heated microsomes (3 min at 90 °C), the final concentration was 0.5 g liver tissue/ml. Twentyfive µl of a stock solution of a test compound were added giving a final concentration of 5, 20, or 40 µM (VE, trolox, VEA, and VES were dissolved in absolute ethanol, and VEP was dissolved in deionized water). Control experiments showed that at the used concentration, the solvent has no effect on the lipid peroxidation (LPO). Five hundred µl of an ascorbate solution were added giving a final concentration of 0.2 mM. The reaction started by adding 300 µl of a freshly prepared ferrous sulfate solution giving a final concentration of 10 µM. The reaction was stopped after 20 min of incubation as described in section 2.3.2. The concentration that reduces LPO by 50% (IC₅₀) was calculated using the lowest concentration tested giving a protection more than 50% and the concentration tested that was just below that concentration. A linear relationship between the concentration of the antioxidant and the inhibition of LPO was used in the calculation.

2.3.2. Spectral measurements.

LPO was assayed by measuring thiobarbituric acid (TBA) reactive material as described previously (7). An aliquot of the incubation mixture (0.3 ml) was stopped by adding 2 ml of an ice-cold TBA-trichloroacetic acid-HCl-butylhydroxytoluene (BHT) solution. The mixture was heated for 15 min at 90 °C and then centrifuged for 5 min. The absorbance was determined at 535 nm vs 600 nm. The TBA-trichloroacetic acid-HCl-BHT solution was prepared by dissolving 41.6 mg TBA/10 ml trichloroacetic acid (16.8 w/v in 0.125 N HCl). To 10 ml TBA-trichloroacetic acid-HCl 1 ml BHT (1.5 mg/ml ethanol) was added. The added chemicals did not interfere with the assay in the concentrations used.

2.4. The Trolox Equivalent Antioxidant Capacity (TEAC) assay

The TEAC assay described by Re *et al.* (9) was used with minor modifications. The ABTS[•] stock solution was prepared by dissolving 30 mg of ABTS in 7.8 ml of 2.46 mM potassium peroxodisulfate. The ABTS[•] stock solution was diluted with 100 mM phos-

phate buffer (pH 7.4). The absorbance of this radical solution at 734 nm had to be between 0.68 and 0.73 at 37 °C. For measuring the antioxidant capacity 50 µl of the test compounds were mixed with 950 µl of the ABTS* solution. Absorbance was monitored at 734 nm for 6 min. The antioxidant capacity was calculated according to van den Berg *et al.* (10).

2.5. Vitamin E determination

2.5.1. Conversion of VEP into VE.

VEP (500 µg/ml) was incubated with alkaline phosphatase (3 U/ml) at 37 °C. The reaction was carried out in a solution, containing 8.3 mM sodium carbonate, 5.5 mM glucose, 1.2 mM magnesium chloride, 0.5 mM potassium chloride and 0.2 mM zinc sulfate (pH 7.4). To stop the reaction, 250 µl of the reaction mixture was added to a cold solution of absolute ethanol (2 ml) with 750 µl of deionized water on ice. VE was extracted and determined as described in section 2.5.3.

2.5.2. VE content of microsomes.

One ml of non-heated and heated microsomes (1-1.3 mg protein) was incubated with 100 µM of the vitamin E derivatives for 20 min at 37 °C. The reaction was stopped by addition of 100 µl of 2.5 % SDS, and 2 ml ethanol containing 1 mg BHT to prevent further oxidation. VE was extracted and determined as described in section 2.5.3. Vitamin E nicotinate (200 µg/ml) was used as an internal standard.

2.5.3. VE extraction and HPLC determination.

The mixture (from section 2.5.1, 2.5.2. or 2.6) was shaken for 5 min, then 3 ml of hexane was added. Again the mixture was shaken for 15 min. After centrifugation the upper hexane layer was aspirated in a conical glass tube and evaporated under nitrogen. The residue was dissolved in 200 µl isopropylalcohol for HPLC analysis according to Burton *et al.* (11) using a Nucleosil C-18 column and absolute methanol as a mobile phase, with a flow rate of 2 ml/min. VE was detected at 295 nm.

2.6. Vitamin E phosphate content of microsomes

One hundred µM of VEP was added to microsomes as described in section 2.3.1. After 1 h incubation, the samples were centrifuged at 65,000 g for 40 min. The pellet was re-suspended in 4 ml Tris-HCl and 250 µl of 4 M KOH and then samples were heated at 90 °C for 3 min for complete rapture of microsomal membrane. After cooling (room temperature), the samples were neutralized (pH 7.4). At 37 °C, 10 U/ml alkaline phos-

phatase was added and the samples were incubated for 30 min to convert VEP into VE. Extraction and determination of VE was carried out by the procedure described above in section 2.5.3. Experiments without the addition of alkaline phosphatase were performed as blank.

2.7. Membrane fluidity of human erythrocytes.

The membrane fluidity of erythrocytes from healthy donors was measured by means of electron paramagnetic resonance (EPR) spectroscopy. Blood samples were obtained by venipuncture. Heparin was used as anti-coagulant. The plasma and buffy coat were carefully removed after centrifugation at 2000 g for 10 min at 4 °C. Erythrocytes were washed three times with phosphate-buffered saline (PBS) and finally resuspended in PBS at a haematocrit of 45%. Half ml of PBS with the test compound (final concentration; 50 µM) was added to 0.5 ml of the erythrocyte containing PBS. After 90 min incubation at 37 °C, spin labeled 16-doxylstearic acid was added and the samples were incubated for 90 min at 37 °C. Finally, EPR measurements were performed using spectrometer (Bruker EMX, GmbH, Freiburg, Germany) under the following condition: microwave power; 2 mW, modulation amplitude; 1 G, scan width; 50 G, modulation frequency; 100 kHz and temperature; 30 °C. Membrane fluidity was calculated from the peak height ratio (h_0/h_{-1}) as described previously (12).

2.8. Erythrocyte Hemolysis

Erythrocyte hemolysis was assessed as described by Neuzil *et al.* (13) with minor modifications. Erythrocytes were prepared from fresh heparin-treated human blood. One ml blood was diluted 80 times with saline, followed by centrifugation at 2000 g for 10 min at 4 °C. The pellet was resuspended with 45 ml saline. One ml of the erythrocyte suspension was incubated with different concentrations of the test compounds at 37 °C. After 2 h incubation the samples were centrifuged for 10 min at 2000 g. Supernatant was diluted 2 times with saline, and the absorbance at 546 nm was determined.

2.9. Statistics analysis

All results are expressed as mean $\pm$ SD. Differences were tested a Student t-test with a p value of 0.05.

3. Results

The TEAC of trolox is by definition 1. The TEAC of VE and all-*rac*-VE were 0.97 ± 0.05 and 0.96 ± 0.03 respectively. The TEAC of VEA, VES, and VEP were 0.01 ± 0.02 , 0.01 ± 0.01 and 0.01 ± 0.01 respectively.

Alkaline phosphatase (3U/ml) can hydrolyze VEP (500 μ g/ml) into VE (Fig. 2) and thus unshield the OH moiety. The rate of VE formation was $0.718 \pm 0.048 \mu\text{g} \cdot \text{min}^{-1} \cdot \text{U}^{-1}$.

Fig. 2. Hydrolysis of vitamin E phosphate by alkaline phosphatase. Experimental conditions are given in the materials and methods, section 2.5.1.

Fig. 3. Time-course of the effect of 20 μ M of vitamin E (VE), Trolox, vitamin E acetate (VEA), vitamin E succinate (VES), and vitamin E phosphate (VEP) on lipid peroxidation in non-heated microsomes. Experimental conditions are given in the materials and methods, see section 2.3. Values are presented as mean of at least three separate experiments.

Fig. 4. Time-course of the effect of 20 μ M of vitamin E (VE), Trolox, vitamin E acetate (VEA), vitamin E succinate (VES), and vitamin E phosphate (VEP) on lipid peroxidation in heated microsomes. Experimental conditions are given in the materials and methods, see section 2.3. The insert shows the percentage of VE and VEP consumption. Values are presented as mean of at least three separate experiments.

VE, trolox, VEA, and VES induced a lag time in the time course of LPO in non-heated microsomes (Fig. 3). The antioxidant activities of VE, VEA and VES were approximately the same. The antioxidant activity of trolox was superior to that of VE, VEA or VES. VEP was also a potent antioxidant but showed another profile, i.e. it reduced the extent of LPO without inducing a lag time (Fig. 3).

The protection provided by the test compounds in non-heated microsomes against LPO was dose dependent. The IC_{50} values of VEP, trolox, VE, VEA, and VES were 25 ± 2 μ M, 20 ± 1 μ M, 30 ± 2 μ M, 35 ± 2 μ M, and > 40 μ M, respectively.

Heating of the microsomes did not greatly affect the antioxidant activity of VE and trolox (Fig. 4), whereas the antioxidant activity of VEA and VES almost completely disappeared. The potent antioxidant effect of VEP in heated microsomes is comparable to that in non-heated microsomes. In heated microsomes the IC_{50} values of VEP, trolox, VE, VEA, and VES were 25 ± 1 μ M, 23 ± 1 μ M, > 40 μ M, $>> 40$ μ M and $>> 40$ μ M, respectively.

Formation of VE from VEA, VEP and VES in non-heated and heated microsomes was also measured. VEA showed a larger conversion into VE in non-heated microsomes compared to VES and VEP. No conversion of the esters into VE in heated microsomes could be observed (Fig. 5).

To further investigate the mechanisms of protection, the consumption of VE and VEP during the protection against LPO was assessed. It was found that addition of 100 μM VE to the microsomes was fully consumed after 1 h incubation. In contrast 100 μM VEP was not consumed during the 1 h incubation with heated microsomes (Fig. 4, insert).

Fig. 5. Formation of vitamin E (VE) from vitamin E acetate (VEA), vitamin E succinate (VES) and vitamin E phosphate (VEP) in non-heated and heated microsomes after 20 min incubation. Experimental conditions are given in the materials and methods, see section 2.5.2. Values are presented as mean $\pm$ SD of at least three separate experiments. * Indicates a significant change vs control ($p < 0.05$).

To determine the effect of VEP on biomembranes, membrane fluidity of erythrocytes was monitored. Membrane fluidity is determined using EPR by calculating the ratio of h_0/h_{-1} [12]. At a concentration of 50 μM , VEP induced a significant decrease in membrane fluidity. The peak height ratio with VEP is 8.1 ± 0.7 compared to that of control, h_0/h_{-1} is 7.2 ± 0.2 . Both VE and all-*rac*-VE at concentration 50 μM have no significant effect on the erythrocyte membrane fluidity (Fig. 6).

Fig. 6. Effect of VE, all-rac-VE and VEP on membrane fluidity of erythrocytes. Membrane fluidity is expressed as the ratio of the peak height (h_0/h_{-1}) [12]. The higher the peak height ratio, the lesser freedom of motion of the spin label in biomembrane bilayer, indicating a lower membrane fluidity [12]. Experimental conditions are given in the materials and methods, section 2.7. The data are presented as mean $\pm$ SD of at least 3 separate experiments. * Indicates a significant change of VEP vs control ($p < 0.05$).

Fig. 7. Induction of hemolysis by vitamin E (VE) and VE esters. Isolated erythrocytes were incubated for 2 h with different concentrations of compounds at 37 °C. The data are presented as mean $\pm$ SD of at least 3 separate experiments. * Indicates a significant change vs control ($p < 0.05$).

Hemolysis of erythrocytes by VE, all-*rac*-VE, VEA, VES and VEP were also determined (Fig. 7). At a concentration of 80 μM , VEP induced 100% erythrocyte hemolysis, whereas the same concentration of VES induced only 50% erythrocyte hemolysis. VE, all-*rac*-VE and VEA were not effective in inducing erythrocyte hemolysis.

4. Discussion

In the present study the antioxidant action of trolox, VE and several VE esters was compared. Firstly, the ability of these VE-derivatives to scavenge a stable ABTS[•] radical was measured. The obtained data were normalized to trolox, which has a TEAC of 1. The TEAC value of VE was also approximately 1 for either VE or all-*rac*-VE. This was expected since VE and all-*rac*-VE contain the same antioxidant moiety as trolox. In contrast, the TEAC of VEA, VES, and VEP was almost 0. This could be anticipated because the antioxidant moiety in these compounds is shielded by acetate, succinate and phosphate, respectively. The small antioxidant capacity of the esters is possibly due to a small fraction ($< 1\%$) of free VE.

Our results showed that, the phosphate group of VEP can be removed by alkaline phosphatase which results in the formation of VE (Fig. 2) as has previously been demonstrated by Nakayama *et al.*, (14). Esterases can hydrolyze VEA and VES into VE (15, 16).

As expected, VE and trolox decreased LPO induced by the iron/ascorbate system, both in non-heated and heated rat liver microsomes (Fig. 3, 4). This is due to the free radical scavenging property of the free hydroxyl group. Once VE and trolox are consumed, LPO starts. During the antioxidant activity the chroman head is finally converted into a quinone (6). The higher efficacy of trolox may be due to the absence of the hydrocarbon tail, which makes it less lipophilic in comparison with VE. Similarly, Kagan *et al.* (17) showed that, decreasing the length of the phytyl chain of α -tocopherol from 16 carbon atoms to 11, 6 or 1 decreased the concentration needed to inhibit LPO *in vitro*. No difference in antioxidant effect was found between VE and all-*rac*-VE indicating that the stereochemistry of the hydrocarbon tail has no major influence. This is of importance since VEP is only available as all-*rac*-VEP and the stereochemistry of its tail is probably of minor importance in the *in vitro* experiments.

The inhibition of LPO by VEA and VES in non-heated microsomes (Fig. 3) occurs as a secondary event following the enzymatic conversion of VEA and VES into VE (Fig. 5) (15, 16). In non-heated microsomes the higher antioxidant activity of VEA in comparison to VES is probably due to a more extensive conversion of VEA into VE compared to VES. The lack of inhibition of LPO by VEA and VES in heated microsomes (Fig. 4) corroborates this explanation because denaturation of the enzymes in the microsomes by heating prevented hydrolysis of VEA and VES into VE.

The rate of conversion of VEP into VE was less than that of VEA in non-heated microsomes (Fig. 5). VEP had no direct radical scavenging activity as demonstrated in the TEAC assay. Nevertheless, VEP was a more efficient antioxidant than the other esters and VE not only in non-heated microsomes but also in heated microsomes. Also the profile of the time course of inhibition of LPO points to different mechanism of inhibition. VE, trolox, VEA, and VES induced a lag time in the onset of LPO. VEP induced no lag time, it reduced the extent of LPO. Additionally, in heated microsomes VEP is not consumed during the LPO (Fig. 4, insert). Apparently, the antioxidant effect of VEP is not due to scavenging of free radicals but rather to another mechanism. A clue for this mechanism may be found in the chemical structure of VEP. VEP has a hydrophilic polar phosphate group attached to the chroman head and the hydrophobic hydrocarbon tail (Fig.1). This might enable VEP to be incorporated into biomembranes in a such way that its polar lipophobic phosphate group is in the water-membrane interface, and that the lipophilic hydrocarbon chain tail is embedded inside the lipid membrane. In this way VEP could act as a barrier for the transfer of radicals from one polyunsaturated fatty acid to another one. This may shorten the propagation phase of lipid peroxidation, re-

sulting in less peroxidation. This is in line with the finding that VEP reduces membrane fluidity (Fig. 6). Results from this study also revealed that VEP induces hemolysis in erythrocytes much more efficiently as the other compounds (Fig. 7).

Mabile et al., (15) reported that, trolox exhibited a more potent cytoprotective effect than VE against oxidized LDL induced endothelial cell toxicity, whereas VEA and VES were almost completely ineffective. In contrast, Carini et al., (16) emphasized that VES exerted a more effective antioxidant protection than an equivalent amount of VE against iron/ascorbate mediated peroxidation in microsomes. In the present study, the rank order of the antioxidant activity of VE and its derivatives in microsomal LPO is trolox > VE $\approx$ all-*rac*-VE > VEA > VES. Recently, Nakayama et al., (14) reported that pretreatment of cultured mouse skin with VEP provided significant protection greater than VEA against UV-B induced skin damage characterized by reduced LPO, sunburn cell formation, and DNA degradation. Nakayama et al. (14) hypothesized that the potent protective effect of VEP might be due to the high conversion of VEP into VE in cultured mouse skin compared to that of VEA. Our study showed that in lipid membranes VEP has a potent antioxidant effect of its own which is not due to the conversion into VE. The differences in antioxidant activity of the compounds and different mechanisms in the reported studies are probably due to differences in the experimental conditions.

With respect to the antioxidant effect of VES it should be noted that in control microsomes VES and VEP yielded the same amount of VE. VES did induce a small lag time in the time course of LPO. VEP did not induce a lag time. This indicates that the antioxidant effect of VES cannot be explained only by its conversion to VE (15,16). Possibly a moderate detergent like effect of VES, as observed in the hemolysis of erythrocytes, is involved. An antioxidant effect of VES was, however, not found in heated microsomes. The reduction of membrane fluidity by VEP can be explained both the protection against LPO and induction of erythrocyte hemolysis. In various pathological conditions, LPO plays a major role in the etiology. It is speculated that, the extraordinary antioxidant effect of VEP against LPO might be of importance in the treatment of these pathologies. Data on bioavailability and metabolism are needed to support this possible use.

In conclusion, VEP has a potent antioxidant effect of its own in contrast to VEA and VES. The antioxidant mechanism differs from that of VE. The mechanism used by VEP may lead to the development of a new class of antioxidants that might strengthen our therapeutic arsenal against free radical mediated disorders.

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Chapter 3

Vitamin E phosphate is a novel apoptotic agent

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Submitted

Abstract

Vitamin E succinate (VES) is a well-known potent and selective apoptotic agent. This apoptotic activity has been ascribed to its detergent-like activity. The structurally related compound vitamin E phosphate (VEP) also has a detergent-like activity. In this study we investigated the effect of VEP on the osteosarcoma cell line MG-63 using VES as a reference compound. It was found that the antiproliferative effect of VEP on the MG-63 cancer cell line is superior to that of VES. Also the ability of VEP to induce apoptosis in the MG-63 cell line was better than that of VES. The cytotoxic effect of VEP and VES seems to be due to the effect of the intact compounds, since only a minor conversion into VE could be detected. EPR experiments showed that VES and VEP reduce membrane fluidity, whereas vitamin E (VE) has no effect. In addition, induction of erythrocyte hemolysis by VEP depends on the pH. Our results indicate that the detergent-like activity of these compounds might be involved in their biological effect. VEP meets the structural requirements that have been described for the apoptotic activity of VE esters, i.e. the combination of three structural domains, a functional, a signalling and an orientation domain.

Introduction

Recently, it has been discovered that vitamin E phosphate (VEP) is endogenously present in plant and animal tissues, including human tissues. The concentrations of VEP are in general similar to those of vitamin E (VE). In rat and pig liver VEP levels even surpass those of VE by far (1). In foodstuffs such as chocolate and cheese the concentrations of VEP are 10 to 30 times higher than that of VE (1). We recently demonstrated that VEP interacts with biomembranes in a particular way, i.e. it acts as a potent antioxidant by its own, without conversion into VE (2). Moreover, VEP is not consumed during its protection against lipid peroxidation (2). Because VEP reduced membrane fluidity and induced erythrocyte hemolysis, it was suggested that the antioxidant effect of VEP is due to its detergent-like activity (2). VEP contains a charged phosphate group and a long aliphatic group. In biomembranes, the hydrocarbon tail intercalates with fatty acid residues of phospholipids, while the charged phosphate group faces the cytosol. In this way it acts as a detergent.

In 1982, Prasad and Edwards-Prasad reported that vitamin E succinate (VES) induced inhibition of proliferation and cell death of murine melanoma B-16 cells in culture (3). Subsequently, it has been shown that VES is a potent growth inhibitor of a variety of malignant cell types in vitro (4-11) and in vivo (12-15). The antitumour activity of VES seems to be selective for cancer cells, since VES treatment is not toxic to normal cells (7,13). Although the precise mechanism for the growth inhibition activity by VES remains unclear, it might be attributed to the detergent-like action of VES (8,12). The detergent activity of VES is due to the charged succinyl group and the long aliphatic hydrocarbon tail (8,12).

Due to the structural similarity between VEP and VES and the similar detergent-like activity, VEP may display a similar anti-proliferative activity as VES. The present study has been designed to investigate the effect of VEP on the osteosarcoma cell line MG-63, using VES as a reference compound.

Materials and Methods

Chemicals

Vitamin E (VE), vitamin E succinate (VES), vitamin E phosphate (VEP) and 16-doxylstearic acid were obtained from Sigma (St. Louis, MO, USA). All other used chemicals were of analytical grade purity.

Cell Culture

Osteosarcoma cells (MG-63) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ in air.

Viability assay

MG-63 cells were seeded into 6-well plates at 0.5×10^6 cells per well and were cultured for 24 h in DMEM. Then the medium was replaced with 2.7 ml of balanced saline solution and 300 µl of medium, and in addition 24 µl of the tested compounds was added lead to the desired final concentration of 80 µM. Control cells were treated with 24 µM of both solvents, i.e. either deionised water (for VEP) or ethanol (for VE or VES). After 6 h of incubation the cells were collected and tested for viability. Determination of cell number viability was assessed by the exclusion of trypan blue. Cells stained blue were counted as non-viable, whereas those excluding the dye were regarded as viable. Results were expressed as percentage cell death, which - because the controls mainly contained a neglectable number of dead cells - was expressed as number of dead cells/(number of dead cells + viable cells).

Proliferation assay (qualitative and quantitative)

MG-63 cells were plated in flasks with a bottom of 25 cm² to 90 – 97% confluence. The medium was replaced with 4.5 ml of balanced saline solution and 500 µl of medium, and in addition 40 µl of the tested compounds was added to lead a final concentration of 80 µM. Control cells were treated with both solvents, i.e. either deionised water (for VEP) or ethanol (for VE or VES). After 4 h of incubation the balanced saline solution was replaced by medium and the cells were incubated further for 26 h. Photomicrographs were taken for a qualitative examination. For a quantitative determination, cells were harvested and then counted with a hemocytometer. The antiproliferative effect was calculated as (number of control cells – number of treated cells)/number of control cells.

DNA fragmentation assay for apoptosis

MG-63 cells were seeded into Lab-Tek Chamber Slide at 0.1×10^6 cells/ml per chamber and cultured in DMEM. After 24 h the medium was replaced with 900 µl of balanced saline solution and 100 µl of medium, and in addition 8 µl of the test compound was added to lead a final concentration of 80 µM. Control cells were treated with both solvents, i.e. either deionised water (for VEP) or ethanol (for VE or VES). After 4 h incubation the balanced saline solution was replaced by medium and further incubated for

26 h. Cells were washed with PBS and then fixed with 3.7% formaldehyde. After 30 min of incubation the cells were treated with 0.2 % triton X-100 and finally, stained with DAPI. Slides were evaluated microscopically. Cells with nuclei containing clearly condensed chromatin or cells with fragmented nuclei were counted and defined as apoptotic cells. Because the controls did not contain a noticeable number of apoptotic cells, the percentage of apoptotic cells was calculated as number of apoptotic cells / (number of apoptotic cells + number of non-apoptotic cells).

Vitamin E determination

MG-63 cells were seeded into 6-well plates at 0.5×10^6 cells per well and were cultured for 24 h in DMEM. Then the medium was replaced with 2.7 ml of balanced saline solution containing 300 μ l of medium, and the cells were treated with 24 μ l of the tested compounds leading to a final concentration of 80 μ M. After 2 h of incubation the reaction was stopped by addition of 100 μ l of 2.5 % SDS, and 2 ml ethanol containing 1 mg BHT to prevent further oxidation. VE was extracted and determined as described in the following paragraph. Vitamin E nicotinate (200 μ g/ml) was added as an internal standard.

The mixture was shaken for 5 min, then 3 ml of hexane was added. Again the mixture was shaken for 15 min. After centrifugation the upper hexane layer was aspirated into a conical glass tube and evaporated under nitrogen. The residue was dissolved in 200 μ l isopropylalcohol for HPLC analysis according to Burton *et al.* (16) using a Nucleosil C-18 column and absolute methanol as the mobile phase, with a flow rate of 2 ml/min. VE was detected at 295 nm.

Membrane fluidity of human erythrocytes.

The membrane fluidity of erythrocytes from healthy donors was measured by means of electron paramagnetic resonance (EPR) spectroscopy. Blood samples were obtained by venipuncture. Heparin was used as an anticoagulant. The plasma and buffy coat were carefully removed after centrifugation at 2000 g for 10 min at 4 °C. Erythrocytes were washed three times with phosphate-buffered saline (PBS) and finally resuspended in PBS to a hematocrit of 45%. PBS (0.5 ml) with the test compound (final concentration 50 μ M) was added to 0.5 ml of the erythrocyte suspension. Controls were treated with both solvents, i.e. deionised water (for VEP) and ethanol (for VE or VES). After 90 min of incubation at 37 °C, spin labelled 16-doxylstearic acid was added and the samples were incubated for 90 min at 37 °C. Finally, EPR measurements were performed using an EPR spectrometer (Bruker EMX, GmbH, Freiburg, Germany) under the following conditions: microwave power 2 mW; modulation amplitude 1 G; scan width 50 G; modulation frequency 100 kHz and temperature 30 °C. Membrane fluidity was calcu-

lated from the peak height ratio (h_0/h_{-1}) as described previously (17). The higher the peak ratio, the lesser the freedom of the motion of the spin label in the biomembrane bilayer, indicating a lower membrane fluidity (17)

Erythrocyte Hemolysis

Erythrocyte hemolysis was assessed as described by Neuzil *et al.* (8) with minor modifications. Erythrocytes were prepared from freshly obtained heparin-treated human blood. One ml blood was diluted 80 times with of PBS (pH 6.4 or pH 7.4) followed by centrifugation at 2000 g for 10 min at 4 °C. The pellet was resuspended with 45 ml PBS either of pH 6.4 or pH 7.4. One ml of the erythrocyte suspension was incubated with different concentrations of VEP at 37 °C. Controls were treated with both solvents, i.e. either deionised water (for VEP) or ethanol (for VE or VES). After 2 h of incubation, the samples were centrifuged for 10 min at 2000 g. The supernatant was diluted 2 times, and the absorbance at 546 nm was determined.

Statistical analysis

All results are expressed as mean $\pm$ SD. Differences between the treatments were tested with Student's t-test.

Results

In the viability assay with MG-63 cells VEP and VES induced cell death in a dose dependent way (Fig. 1). At a concentration of 40 μ M ($n = 5$) and 80 μ M ($n = 13$) the effect of VEP ($45 \pm 5\%$ and $85 \pm 7\%$ of cell death, respectively) was superior ($p < 0.001$ for both concentrations) to that of VES ($29 \pm 6\%$ and $70 \pm 7\%$ of cell death, respectively). The lowest concentration of 20 μ M did not result in a significant difference in cytotoxicity between VEP and VES ($16 \pm 3\%$ vs $14 \pm 2\%$ of cell death, respectively ($n = 3$)). As shown in Fig. 1 (insert), at a concentration of 80 μ M both VEP and VES hardly showed a conversion into VE ($1 \pm 0.4\%$ and $4.6 \pm 0.45\%$, respectively ($n = 3$)).

The cytotoxic effect of the VE esters was confirmed by their effect in the proliferation assay. The quantitative assay confirmed the qualitative impression that VEP is a potent antiproliferative agent. The antiproliferative effects of VEP, VES and VE were $75 \pm 2\%$ ($n = 5$), $41 \pm 5\%$ ($n = 5$) and $12 \pm 3\%$ ($n = 5$), respectively. A typical experiment of the qualitative antiproliferative effect is shown on the cover of this thesis; panel A is the control, panel B is VE, panel C is VES and panel D is VEP. The rank order of the potency of three compounds is VEP > VES > VE. The difference between these effects were all highly significant, i.e. $p < 0.0003$.

The apoptotic effect of VEP and VES was clearly observed in the MG-63 cells by DNA fragmentation and nuclear condensation. At a concentration of 80 μ M the percentage of

apoptotic cells was significantly higher after VEP ($64 \pm 11\%$, $n = 21$) than after VES ($46 \pm 9\%$, $n = 21$) with a p-value of 10^{-6} . VE had no apoptotic activity.

The membrane fluidity of erythrocytes, as measured five-fold by the ratio of the peak heights (h_0/h_{-1}), decreased significantly after treatment with $50 \mu\text{M}$ of VEP and VES ($h_0/h_{-1} = 8.1 \pm 0.7$ and 8.1 ± 0.3 , respectively) in comparison to the controls (7.2 ± 0.2) with p-values of 0.03 and 0.001, respectively. VE had no influence on the membrane fluidity ($h_0/h_{-1} = 7.36 \pm 0.13$). The decreased membrane fluidity by VEP and VES was also significantly changed in comparison to the effect of VE (p-values of 0.05 and 0.001, respectively).

VEP induced hemolysis of erythrocytes which was concentration and pH dependent. Hemolysis increased when the concentration of VEP was increased or the pH was lowered (Fig. 2).

Fig. 1. Cytotoxic effect of VES and VEP in MG-63 cells. Cells were incubated with different concentrations of VES and VEP. Percentages of cell death are shown. The insert shows the formation of VE from $80 \mu\text{M}$ VES and VEP in MG-63 cells after 2 h of incubation. Values are presented as mean $\pm$ S.D. * Indicates a significant difference between VEP versus VES ($p < 0.001$).

Fig. 2. Induction of hemolysis by VEP. Isolated erythrocytes were incubated for 2 h with different concentrations of VEP at pH 6.4 or pH 7.4 at 37 °C. The data are presented as mean $\pm$ S.D. of three separate experiments.

Discussion

Several studies have shown that VES induces growth inhibition and apoptotic activity on a variety of human and rodent cancer cell lines without affecting the proliferation of normal cells *in vitro* (4-11) as well as *in vivo* (12-15). Several authors have reported that VES might be a highly selective anticancer agent. It was suggested that VES is a promising anticancer drug or adjuvant, which is applicable in clinical practice. The goal of the present study is to determine whether VEP inhibits osteosarcoma cell proliferation and induces cell death and apoptosis. The effects of VEP are compared to those of VES. VEP induced cell death in a dose dependent manner and was more potent than VES. The conversion of VES or VEP into VE in MG-63 was very low, indicating that the induction of cell death by either VES or VEP is due to a direct effect of the parent compounds. In contrast to the stability of VEP in the osteosarcoma cell line, Nakayama et al. (18) recently reported that in cultured mouse skin VEP showed a massive conversion into VE by alkaline phosphatase. Furthermore, daily supplementation of VEP (30 mg/kg) in rats for 32 days increased levels of VEP (62%) as well as VE (40%) in the liver, indicating a possible conversion of VEP into VE (1). The low conversion of VEP in the cancer cell line MG-63 and the substantial conversion of VEP in normal cells, cultured mouse skin (18), and liver tissue (1) indicate that differences in hydrolysing enzymes, such as esterases and phosphatases exist between cancer and normal cells. This difference is used in the clinical application of amifostine as a protector of healthy tissues during chemo- and/or radiotherapy (19). Amifostine is a phosphorylated thiol and the removal of the phosphate group occurs at a higher rate in normal tissue com-

pared to cancer tissue (19). In this way, more of the protective thiol is formed in normal tissue than in tumour tissue, leading to protection of healthy tissues without comprising the antitumour effect of chemo-and/or radiotherapy (19).

Of the tested compounds, VEP appeared to be the most potent inhibitor of cell proliferation. VE has the lowest potency. The effect of VE is dependent on the form of VE used. The form of VE used in the present study, α -tocopherol, inhibits cell proliferation while a chemically closely related form of VE, β -tocopherol, is known to be ineffective (20). Esterification of the OH moiety of α -tocopherol by succinate or phosphate boosts the inhibition of MG-63 cell proliferation. Again VEP was the most potent compound tested.

VEP also appeared to be most potent in inducing DNA fragmentation and nucleus condensation. Birringer et al. (9) found that neither trolox succinate nor phytyl succinate caused apoptosis. Methylation of the free succinyl carboxyl group (giving VE methyl succinate) completely abolished the apoptotic activity of VES (9). This indicates that all parts of the molecule (i.e. the free succinate group, the chroman head and the phytyl tail) are essential for the induction of apoptosis by VES. Vitamin E glutarate has a very poor apoptotic activity compared to that of VES, vitamin E maleate and vitamin E fumarate. Apoptotic activity also differs between vitamin E oxalate, vitamin E malonate and VES. The relative potency of these VE esters correlates with the flexibility of the terminal carboxyl containing moiety (11); the longer the terminal dicarboxylic moiety, the more flexible this moiety and the lower the apoptotic effect. The phosphate group is not flexible and, based on analogy with the VE esters, this might be an important feature of VEP in its apoptotic activity.

In all the apoptotic vitamin E esters three structural domains can be recognized (Fig 3). Domain I is considered as functional domain which is the hydrophilic part of the detergent-like molecule of VES and VEP. Domain II is regarded as signalling domain, which is accountable for cell signalling activity of VE, VES and VEP. Domain III is an orientation domain, which mediates embedding of VES or VEP in the biomembranes.

The mechanism by which VES induces apoptosis is not fully understood. A combination of membrane destabilizing activity and dysregulation of signalling pathways may be involved. Destabilization of the cell membrane probably occurs due to the detergent like action of VES. VEP also has a detergent like activity (2).

The effect of VE, VEP and VES on membrane fluidity has been tested. Both VES and VEP decreased membrane fluidity of erythrocytes, whereas VE has no significant effect. Our recent study (2) showed that VES and VEP induced erythrocyte hemolysis. Previously, Neuzil et al. (8) reported that both α -tocopherol succinate and β -tocopherol succinate induced hemolysis to erythrocytes.

Fig. 3. Structures of VES and VEP showing 3 major domains. The domains are a functional domain (domain I), a signaling domain (domain II), and an orientation domain (domain III). For apoptotic activity, the three domains are required. VEP is the VE derivative that has the highest activity of the VE esters tested.

The detergent-like activity of VES increased with acidic pH (8). VES can exist in solution in two forms: the deprotonated charged species, and its protonated uncharged counterpart. The latter can enter cells by passive diffusion (8). Almost 99% of the total VES will be charged at neutral pH. The protonated uncharged form will increase 25-fold at pH 6.2 (8). The apoptotic activity of VES in Jurkat and MCF-7 cell lines was higher at acidic pH which may be due to the higher diffusion rate of protonated VES over cell membranes at acidic pH (8). The pH dependent effect might contribute to the selective toxicity of VES to cancer cells (7,13), since the pH of normal tissues is neutral compared to the acidic pH found in many tumours (21). Interestingly, we found that the toxicity of VEP on erythrocytes is also higher at acidic pH. Possibly, protonation of the phosphate group is involved, although the acidity (the pKa value) of VEP is not known. Thus, beside higher dephosphorylation in normal versus cancer tissue, also the more acidic pH in tumour cells may contribute to a higher toxicity of VEP in tumour cells.

VEP is an important constituent in our diet which has been overlooked in the past, because HPLC techniques for the measurement of VE did not detect VEP (1). Nevertheless, various prominent health effects of VEP have been reported. VEP has been promoted as a radioprotector. Felemovicius et al. (22) reported that a chronic oral supplementation of rats with VEP (5 mg/ml) significantly protected their intestine against acute irradiation. Pre-treatment of cultured mouse skin with a solution of 0.5% of VEP

(9.4 mM) substantially protected against UV-B triggered skin damage. Also a significant beneficial effect of VEP in combination with semen plasma protein was observed on sperm survival. These effects have been related to the ability of VEP to protect against lipid peroxidation, sunburn cell damage and DNA degradation (18). We have confirmed that VEP is indeed a potent inhibitor of lipid peroxidation (2).

Recently, Munteanu et al. (23) reported that a mixture of VEP (VEPm) of biological origin containing VEP (55.3%), di-VEP (30.6%), VE (5%), inorganic phosphate (2.6%), water (1.9%), oleic acid (1%) and impurities (3.57%), modulated cell proliferation and gene expression in rat aortic smooth muscle cells (RASMC) and human THP-1 monocytic leukaemia cells at a relatively low concentration. At higher concentrations, VEPm induced apoptosis in human THP-1 monocytic leukaemia cells. Munteanu et al. (23) focussed on a beneficial role of VEPm in atherosclerosis and inflammation. In our opinion, the apoptotic effect of VEPm on monocytic leukaemia cells might be of interest too.

We conclude that VEP induces cell death, inhibits cell proliferation and provokes apoptosis in the MG-63 cell line, probably due to a membrane destabilising activity by acting as a detergent. VEP fits in the structural requirements that have been described for the apoptotic activity of VE esters, i.e. the combination of three structural domains, a functional, a signalling and an orientation domain. Due to its potent biological activities and the presence of relatively high amounts in the diet and our body it is expected that VEP will play a role in health. Clinical studies are, however, needed to substantiate this claim.

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Chapter 4

The antioxidant activity of phloretin: the disclosure of a new antioxidant pharmacophore in flavonoids

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Abstract

Phloretin is a dihydrochalcone flavonoid that displays a potent antioxidant activity in peroxynitrite scavenging and the inhibition of lipid peroxidation. Comparison with structurally related compounds revealed that the antioxidant pharmacophore of phloretin is 2,6-dihydroxyacetophenone. The potent activity of 2,6-dihydroxyacetophenone is due to stabilization of its radical via tautomerisation. The antioxidant pharmacophore in the dihydrochalcone phloretin, i.e. the 2,6-dihydroxyacetophenone group, is different from the antioxidant pharmacophores previously reported in flavonoids.

1. Introduction

Generation of reactive oxygen species and reactive nitrogen species *in vivo* are involved in a wide range of human diseases, including cancer, cardiovascular, pulmonary and neurological diseases (1). Hence, agents with the ability to protect against these reactive species may be therapeutically useful. In line with this hypothesis is the widely accepted view that the positive health effects of flavonoids can be attributed to their antioxidant activity. Flavonoids are a group of more than 5000 different natural polyphenols substances. They are abundantly present in green vegetables, fruits, olive and soybean oils, red wine, chocolate, and teas (2). The flavonoids are classified into different categories. Within each category there is a variation in number and arrangement of hydroxyl moieties as well as sugar groups. Several antioxidant pharmacophores in the flavonoids have been described (3- 6).

Chalcones (1,3-diaryl-2-propen-1-ones) are flavonoids lacking a heterocyclic C ring. Also this category of flavonoids displays a broad spectrum of bioactivities such as anti-cancer, antifungal, antibacterial, antiviral and antiinflammatory properties (7). Dihydrochalcones which do not have α - β double bond comprise phloretin [β -(4-hydroxyphenyl)-1-(2,4,6-trihydroxypropiofenone) and its glucoside, phloridzin (phloretin 2- β -D-glucose) (Fig. 1). These compounds are abundantly present in apples (8). The goal of the present study is to examine the antioxidant activity of phloretin and to determine the pharmacophore responsible for antioxidant activity of phloretin.

2. Materials and Methods

2.1. Chemicals

Phloretin [β -(4-hydroxyphenyl)-1-(2,4,6-trihydroxypropiofenone)], phloridzin (phloretin-2- β -D-glucose), phloroglucinol (1,3,5-trihydroxybenzene), 2,4,6-trihydroxyacetophenone, 2,4-dihydroxyacetophenone, 2,6-dihydroxyacetophenone, 2-hydroxyacetophenone and KO_2 were obtained from Fluka. Resorcinol (1,3-dihydroxybenzene), phenol and dihydrorhodamine-123 (DHR-123) were obtained from Sigma. 2-Hydroxy-6-methoxyacetophenone was obtained from Aldrich. Nitrogen monoxide was obtained from AGA (Hamburg, Germany). All other chemicals were of the highest grade of purity.

2.2. Synthesis of peroxynitrite

Potassium oxoperoxynitrate (ONOOK) was produced from the reaction of solid KO_2 with NO-gas as described by Koppenol *et al.* (9). Briefly, the NO-gas slowly flowed over the mixture of KO_2 and quartz-sand, which was constantly stirred and kept on ice. The

mixture was poured into a cold potassium hydroxide solution. Addition of manganese dioxide removes the hydrogen peroxide that results from excess decomposition of potassium superoxide. The solution with peroxyxynitrite was obtained by filtering of the sand and manganese dioxide. The concentration of peroxyxynitrite was determined spectrophotometrically at 302 nm.

2.3. Peroxyxynitrite scavenging

Peroxyxynitrite scavenging was measured by the oxidation of DHR-123 as described by Kooy *et al.* (10). In short, the desired concentrations of the test compounds were added to one ml of 100 mM phosphate buffer at 37 °C. The final concentration of DHR-123 in phosphate buffer was 5 µM. During mixing the final concentration, 0.6 µM, of peroxyxynitrite was added. The fluorescent product, rhodamine-123, was measured by fluorescence detection with excitation and emission wavelengths of 500 and 536 nm, respectively. The effects are expressed as the concentration giving 50% inhibition of the oxidation of DHR (IC₅₀).

2.4. Isolation of rat liver microsomes

Male Lewis rats, 200-250 g, were killed by decapitation. Microsomes were prepared according to Haenen and Bast (11) with a slight modification. Livers were removed and homogenized (1:2 w/v) in ice cold phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA. The homogenate was centrifuged at 10,000 x g for 30 min at 4 °C. Subsequently, the supernatant was centrifuged at 65,000 x g for 60 min at 4 °C. The microsomal pellet was resuspended in the phosphate buffer (2 g liver/ml), corresponding with 1-1.3 mg protein/ml, and stored at - 80 °C. Before use the microsomes were thawed and washed twice, with ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing 150 mM KCl, followed by centrifugation at 65,000 x g for 40 min at 4 °C. Finally, the pellet was resuspended in 4 ml Tris-HCl buffer and then used. The protein concentration was measured according to Smith *et al.* (12).

2.5. Lipid peroxidation assay

At 37 °C, 250 µl of microsomes was added to 540 µl of Tris-HCl/KCl buffer. Ten µl of the test compounds with different concentration were added. Ascorbate solution (125 µl) was added giving a final concentration of 0.2 mM. The reaction was started by adding 75 µl of a freshly prepared ferrous chloride solution giving a final concentration of 10 µM. Lipid peroxidation was assayed by measuring thiobarbituric acid (TBA) reactive material. The incubation mixture (1 ml) was stopped by adding 2 ml of an ice-cold TBA-trichloroacetic acid-HCl-butyldihydroxytoluene (BHT) solution. The mixture was heated for 15 min at 90 °C and then centrifuged for 5 min. The absorbance was de-

terminated at 535 nm vs 600 nm. The TBA-trichloroacetic acid-HCl-BHT solution was prepared by dissolving 41.6 mg TBA/10 ml trichloroacetic acid (16.8 w/v in 0.125 N HCl). To 10 ml TBA-trichloroacetic acid-HCl 1 ml BHT (1.5 mg/ml ethanol) was added. The added chemicals did not interfere with the assay in the concentrations used.

3. Results

Phloretin is a potent antioxidant in peroxynitrite scavenging and the inhibition of lipid peroxidation. The concentration of phloretin needed to scavenge 50% of the peroxynitrite (IC_{50}) is 3.1 μ M and the concentration of phloretin needed to inhibit 50% of the lipid peroxidation is 24 μ M (Fig. 1). Occupation of 2-OH by glucose decreased the antioxidant activities of phloridzin 18 times in comparison to phloretin. The hydroxyl groups of sugar moiety have no role in the antioxidant activity of phloridzin, since the IC_{50} of glucose more than 1000 μ M for either peroxynitrite scavenging or inhibition of lipid peroxidation.

It has been shown that the antioxidant activity of flavonoids resides in the aromatic OH-groups (6). To determine the role of the different functional groups in phloretin, structurally related compounds were tested. Phenol is a very poor antioxidant. Introducing more OH groups in *meta* position, similar to the A-ring of phloretin, gives resorcinol or phloroglucinol. Resorcinol and phloroglucinol have a substantial higher antioxidant activity compared to phenol, however, it is still far less than that of phloretin. This points toward a substantial contribution of the carbonyl group in phloretin. However, 2-hydroxyacetophenone is a very poor antioxidant, its activity is even lower than that of phenol (Fig. 1). Remarkably, 2,4,6-trihydroxyacetophenone has an activity that is similar to that of phloretin. Surprisingly, the antioxidant activity of 2,6-dihydroxyacetophenone is comparable to that of 2,4,6-trihydroxyacetophenone, whereas 2,4-dihydroxyacetophenone is a very poor antioxidant (Fig. 2). 2-Hydroxy-6-methoxyacetophenone displayed virtually no antioxidant activity.

Fig 1. The antioxidant activity of dihydrochalcones (phloretin and phloridzin) and structurally related compounds in peroxynitrite scavenging and the inhibition of lipid peroxidation. The activity is expressed as the concentration of the compound that gives 50% scavenging of the peroxynitrite ($IC_{50} \text{ PON}$) and the concentration that is needed to inhibit 50% of the lipid peroxidation ($IC_{50} \text{ LPO}$). Values are presented as mean $\pm$ SD of at least three separate experiments.

Fig 2. The antioxidant activity of 2,4,6-trihydroxyacetophenone and structurally related compounds in peroxynitrite scavenging and the inhibition of lipid peroxidation. The activity is expressed as the concentration of the compound that gives 50% scavenging of the peroxynitrite (IC₅₀ PON) and the concentration that is needed to inhibit 50% of the lipid peroxidation (IC₅₀ LPO). Values are presented as mean ± SD of at least three separate experiments.

Discussion

Phloretin is a relatively potent antioxidant in peroxynitrite scavenging and inhibition of lipid peroxidation. The potent antioxidant activity of phloretin has also been found in other assays, e.g. hydroxyl radical scavenging (3, 13) and 1,1-diphenyl-2-picrylhydrazyl

radical scavenging (7). Comparison with structurally related compounds it was found that the activity of phloretin does not reside only in the three hydroxyl groups of ring A. Their activity is enhanced by the carbonyl group. Remarkably, introduction of a carbonyl group in phenol (giving 2-hydroxyacetophenone) reduces the antioxidant activity, whereas introduction of the same group in phloroglucinol (giving 2,4,6-trihydroxyacetophenone) increases the antioxidant activity. The activity of 2,4,6-trihydroxyacetophenone is comparable to that of phloretin.

Based on NMR data, it has been reported that in 2-hydroxyacetophenone the hydroxyl group forms an intramolecular hydrogen bridge with the adjacent double bound oxygen (14). This will drastically reduce radical scavenging by means of hydrogen donation, explaining the very poor antioxidant activity of 2-hydroxyacetophenone. In 2,4,6-trihydroxyacetophenone there is evidence that no intramolecular hydrogen bridge between the carbonyl group and one of the aromatic hydroxyl group is formed (14). Moreover, an intramolecular hydrogen bridge can only be formed with one of the aromatic hydroxyl groups, indicating that the carbonyl group cannot reduce the activity of all aromatic hydroxyl groups in 2,4,6-trihydroxyacetophenone. This can explain why the carbonyl group cannot effectively block the antioxidant activity of 2,4,6-trihydroxyacetophenone as in 2-hydroxyacetophenone.

Remarkable is also the difference in activity between 2,6-dihydroxyacetophenone, with an activity identical to 2,4,6-trihydroxyacetophenone, and 2,4-dihydroxyacetophenone, with virtually no antioxidant activity. This indicates that the effect of the carbonyl group is not due to electron donation via the aromatic ring, since this effect is comparable for the 4 and 6 position.

An explanation for the potent antioxidant activity of 2,6-dihydroxyacetophenone might be found in the possible stabilisation of the radical that is formed after hydrogen abstraction. As shown in figure 3, the free electron that is generated due to hydrogen abstraction of one of the hydroxyl groups can be delocalized over the three oxygen atoms present in the molecule. This involves a keto-enol transformation of the carbonyl group, and the transfer of an alpha hydrogen atom of the carbonyl group to the oxygen radical. In this way the unpaired electron is transferred to the carbonyl group. The unpaired electron can then be transferred to the other aromatic hydroxyl group by a hydrogen transfer. Such a stabilization of the radical is not possible in the radical of 2,4-dihydroxyacetophenone, which explains the poor antioxidant activity of 2,4-dihydroxyacetophenone.

Our explanation is confirmed by the poor antioxidant activity of 2-hydroxy-6-methoxyacetophenone (Fig. 2). The radical of this compound can also not be stabilized in the same way as 2,6-dihydroxyacetophenone. In phloridzin, one of two hydroxyl groups adjacent to the carbonyl group has been substituted by a sugar moiety.

Fig 3. The proposed mechanism for the antioxidant activity of 2,6-dihydroxyacetophenone.

Subsequently, also in this compound stabilization of its radical as in the radical of 2,6-dihydroxylacetophenone cannot be achieved. This explains the relatively poor antioxidant activity of phloridzin compared to phloretin. It has been reported that in another group of flavonoids, i.e. the flavonols, two different pharmacophores can be found, i.e. on the B-ring or the AC-ring (Fig. 4) (5, 6). In ring B this is the catechol moiety and the activity of one of the hydroxyl groups is enhanced by the electron donating effect of the other one. In the AC-ring the hydroxyl group at position 3 is the most reactive one and its activity is enhanced by the electron donating effect of the hydroxyl groups at position 5 and 7. In the dihydrochalcone phloretin another pharmacophore is present, i.e. the 2,6-dihydroxyacetophenone group. It is proposed that delocalisation of the unpaired electron in the radical that is obtained after hydrogen abstraction is essential for its antioxidant activity.

One of the pharmacophore in flavonols, i.e. the AC ring, has a strong chemical resemblance with the pharmacophore in phloretin, i.e. the 2,6-dihydroxyacetophenone group. In both pharmacophores there are two aromatic hydroxyl groups with a carbonyl group in between and an intramolecular hydrogen bridge can be formed with either hydroxyl group. Methylation of one of the aromatic hydroxyl groups in 2,6-dihydroxyacetophenone abolishes the activity of the compound. In contrast, 2,3',5,7-tetra-*O*-methyl-quercetine still has a potent antioxidant effect (6). This points toward a fundamental difference between both pharmacophores. In the AC ring an electronic ef-

fect is important, while in 2,6-dihydroxyacetophenone tautomerisation is proposed to play a pivotal role.

Fig 4. The antioxidant pharmacophores in flavonols. Two different pharmacophores have been proposed, i.e. the catechol group in ring B and the three hydroxyl groups in ring AC. The activity of the 3-OH group is enhanced by an electron donating effect of the hydroxyl groups at position 5 and 7.

This indicates that within the chemically heterogeneous group of flavonoids different pharmacophores with different characteristics can be found in the various categories.

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Chapter 5

Lipoic acid protects efficiently only against a specific form of peroxynitrite induced damage

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Abstract

The ability of the sulphur containing compounds glutathione (GSH), glutathione disulphide (GSSG), S-methylglutathione (GSMe), lipoic acid (LA) and dihydrolipoic acid (DHLA) to protect against hypochlorous acid (HOCl) mediated damage and peroxynitrite (ONOOH) induced damage has been compared. Protective activity was assessed in competition assays by monitoring several detectors, i.e. dihydrorhodamine-123 (DHR-123) oxidation, α_1 -antiproteinase (α_1 -AP) inactivation and glutathione-S-transferase P1-1 (GST-P1-1) inactivation. In addition, nitration of tyrosine was measured to assess protection of the sulphur containing compounds against ONOOH. For protection against HOCl, the efficacy of the antioxidant was controlled by the ratio of the reaction rate of the antioxidant and the detector molecule with the oxidant. The rank order of the activity of the antioxidants (GSH > DHLA $\approx$ LA $\approx$ GSMe > GSSG) appeared to be independent of the detector used. However, the rank order of the antioxidants against ONOOH induced damage is strongly dependent on the detector. LA was 40 times less active than GSH in the inhibition of ONOOH induced DHR-123 oxidation, whereas LA was 20 times more active than GSH in preventing the inhibition of GST-P1-1 by ONOOH. This points to different molecular mechanisms of ONOOH damage to DHR-123 compared to ONOOH damage to GST-P1-1. LA is a poor antioxidant in protecting against the form of ONOOH damage involved in DHR-123 oxidation. In the form of ONOOH toxicity involved in GST-P1-1 inhibition, LA is the most potent sulphur containing antioxidant in our series. It is proposed that an intermediate product in which both sulphur atoms of LA have reacted is involved in the reaction of ONOOH with LA. The high potency of LA to protect GST-P1-1 against ONOOH might be of therapeutic interest.

1. Introduction

Some sulphur containing compounds are efficient antioxidants and can protect against damage induced by peroxynitrite (ONOOH) (1-5) and hypochlorous acid (HOCl) (6,7). ONOOH and HOCl are highly effective antibacterial agents formed by neutrophils in innate immunity (8). Uncontrolled and excessive production of the reactive species, however, will cause collateral damage to surrounding tissue at the site of inflammation, e.g. LDL oxidation or DNA, RNA and protein damage (9-13). ONOOH is known to mediate both one- and two-electron oxidation reactions, whereas HOCl predominantly gives two-electron oxidation (14,15)

It has been found that lipoic acid (LA) is a very potent protector against ONOOH mediated damage (1-4). In contrast, preliminary studies in our laboratory indicated that LA is a very poor protector against ONOOH mediated oxidation of dihydrorhodamine-123 (DHR-123). This controversy might originate from differences in the procedures used to assess the antioxidant activity of LA. The activity of an antioxidant is usually determined by quantifying the ability of the antioxidant to prevent damage induced by a reactive species to a detector. Den Hartog *et al.* (7) emphasized that the efficacy of an antioxidant depends on the reaction rate of the oxidant with the detector molecule relative to the reaction rate of the oxidant with the antioxidant. This was illustrated by the difference in protecting activity against HOCl of several sulphur containing antioxidants using different detectors (7). The slower the reaction of the detector with the oxidant, the higher the apparent activity of a protector. The objective of the present study is to further examine the antioxidant activity of sulphur containing compounds in the presence of different detectors (protein and non-protein), and different oxidants (ONOOH and HOCl) in order to resolve the apparent discrepancy in the efficacy of LA in several antioxidant assays. The results show that, with HOCl, the rate of the reaction of HOCl with the oxidant relative to that of the detector is essential. With ONOOH, the molecular mechanism of damage and protection is decisive for the efficacy of the antioxidant.

2. Materials and Methods

2.1. Chemicals

Elastase, α_1 -antiproteinase (α_1 -AP), glutathione S-transferase P-1-1 (GST-P1-1), GSH, GSSG, GSMe, 1-chloro-2,4-dinitrobenzene (CDNB) L-tyrosine, 3-nitro-L-tyrosine, 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) and Sodium hypochlorite (NaOCl) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dihydrorhodamine-123 (DHR-123) and KO_2 were obtained from Fluka Chemie GmbH (Buchs, Switzerland). LA and β -LA were obtained from Asta Medica AG (Frankfurt, Germany). Nitrogen monoxide

was obtained from AGA (Hamburg, Germany). All other chemicals were of the highest grade of purity.

2.2. Synthesis of PON

Potassium oxoperoxonitrate was produced from the reaction of solid KO_2 with NO-gas as described by Koppenol *et al.* (16). Briefly, the NO-gas was slowly led over the mixture of KO_2 and quartz-sand, which was constantly stirred and kept on ice. The mixture was poured into a cold potassium hydroxide solution. Manganese dioxide was used to remove the hydrogen peroxide that results from decomposition of the excess of potassium superoxide. A solution with ONOOH was obtained by filtering off the sand and manganese dioxide. The concentration of ONOOH was determined spectrophotometrically at 302 nm.

2.3. Oxidation of DHR-123

The use of DHR-123 as a detector to monitor protection against ONOOH and HOCl induced oxidation was based on the procedure described by Kooy *et al.* (17). In short, 100 μl of the desired concentration of the test compound was added to 0.9 ml of 100 mM phosphate buffer at 37 °C. DHR-123 was added to give a final concentration of 5 μM . During vortexing 10 μl of ONOOH or HOCl was added giving a final concentration of 0.6 μM or 5 μM , respectively. The fluorescent product, rhodamine-123, was measured by fluorescence detection with excitation and emission wavelengths of 500 and 536 nm, respectively. The effects are expressed as the concentration giving 50% inhibition of the oxidation of DHR (IC_{50}). Control experiments showed that the test compounds in a concentration of 500 μM did not affect the fluorescence.

2.4. Elastase assay

Elastase activity was used as a detector according to the procedure described by Haenen and Bast (6), with minor modifications. The reagents were dissolved in potassium phosphate buffer (19 mM) pH 7.4, containing 140 mM NaCl. Twenty μg of α_1 -AP was pre-incubated at 37 °C for 5 min with the test compounds in the desired concentration. During vortexing, 10 μl of ONOOH was added. The final concentration of ONOOH was 150 μM . After 5 min incubation, 5 μg elastase was added. After 10 min incubation, 50 μl of a 10 mM solution of N-t-BOC-L-alanine p-nitrophenol ester in methanol was added. The increase in absorption at 410 nm was determined. Control experiments showed that the test compounds in a concentration of 100 μM did not affect the activity of control or ONOOH inactivated α_1 -AP.

2.5. *Gluthathione-S-transferase P1-1 assay*

GST-P1-1 (5 µg/ml) was mixed with the test compound in 100 mM potassium phosphate buffer (pH 6.5) and the mixture was incubated at 37 °C for 1 min. During vortexing ONOOH (10 µM, final concentration) was added. After 2 min incubation 1 mM of CDNB and 1 mM GSH were added. GST P1-1 activity was monitored spectrophotometrically by recording the increase in absorbance at 340 nm (18).

2.6. *Nitrotyrosine assay*

Under vortexing 10 µl of ONOOH solution (final concentration 150 µM) was added to a solution containing 100 µM L-tyrosine in 100 mM potassium phosphate buffer (pH 7.4) and the test compound in the desired concentration. This mixture was incubated for 5 min. Measurement of 3-nitrotyrosine was carried out by using HPLC. The eluent was 50 mM KH₂PO₄/K₂HPO₄ pH 7.0 with 5% acetonitrile (v/v). The column was a hypersil BDS-C18 column (150 mm x 4.6 mm) (Supelco Inc. Bellefonte, PA, USA). The UV detector was set at 278 nm.

2.7. *LA and GSH consumption by ONOOH and HOCl*

Hundred µl of the antioxidant (dissolved in 100 mM phosphate buffer) was added to 700 µl of 100 mM phosphate buffer at 37 °C giving a final concentration of 100 µM. During vortexing, 100 µl of the desired concentration of ONOOH or HOCl was added. GSH was determined spectrophotometrically by addition of 100 µl of 6 mM DTNB at 412 nm (19). LA was determined using HPLC and UV detection at 333 nm. A hypersil BDS-C18 column (150 mm x 4.6 mm) (Supelco Inc. Bellefonte, PA, USA) was used. The mobile phase was 10 mM phosphate solution in water (pH 2.7)/acetonitrile (65:35, v/v). The samples were acidified before HPLC analysis (pH 2.6-2.9). Determination of β-LA was performed on the same HPLC system at 230 nm. The mobile phase was 0.15% acetic acid in water/acetonitrile (75:25, v/v).

3. Results

Protection against HOCl was assessed in competition assays, using the detector molecules DHR-123, α₁-AP and GST-P1-1 (Table 1). The tested sulphur containing compounds all had a prominent protective effect against HOCl, except GSSG which only had a moderate protective effect against HOCl with DHR-123 as a detector molecule.

The protective effect of these sulphur containing compounds against ONOOH was also assessed in competition assays using the detector molecules DHR-123, α₁-AP, GST-P1-1 and tyrosine (Table 2). GSH efficiently protected these detector molecules against ONOOH, the IC₅₀ of GSH was practically independent of the detector. GSSG could not

protect any of the detector molecules used against ONOOH. Methylation of GSH (giving GSMe) abolished the ability to protect against oxidation of DHR-123 and nitration of tyrosine with ONOOH. GSMe still had a considerable protecting activity against ONOOH when α_1 -AP was used as a detector and to a lesser extent when GST P1-1 was used as a detector. Dihydrolipoic acid (DHLA), a dithiol, had a protective effect against ONOOH that was slightly lower than that of GSH when DHR-123 was used as detector. DHLA efficiently protected α_1 -AP or tyrosine against ONOOH mediated inhibition. Lipoic acid (LA), a compound that contains an intramolecular disulfide, was hardly effective in the prevention of DHR-123 oxidation by ONOOH, but it potently protected against ONOOH induced α_1 -AP damage and tyrosine nitration. Remarkably, both LA and DHLA have very potent protecting activity against ONOOH when GST-P1-1 was used as a detector. Their protective effect against ONOOH with this detector was approximately 20 times as potent than that of GSH.

Table 1. The protecting activity of several sulphur containing compounds against HOCl induced damage assessed using different detectors.

Compound	IC ₅₀ HOCl-DHR-123 (μ M)	IC ₅₀ HOCl- α_1 -AP (μ M)*	IC ₅₀ HOCl-GST-P1-1 (μ M)**
Reduced glutathione	1.3 $\pm$ 0.5	20	0.3
S-methylglutathione	1.5 $\pm$ 0.7	35	ND
Glutathione disulfide	9 $\pm$ 1	> 200	> 200
Lipoic acid	5 $\pm$ 3	30	1.2
Dihydrolipoic acid	3.3 $\pm$ 0.6	29	ND

Detectors used are DHR-123 oxidation, α_1 -AP inactivation, and GST-P1-1 inhibition. The activity is expressed as the IC₅₀ value, the concentration needed to reduce the HOCl-induced inhibition or oxidation by 50%. The lower the IC₅₀, the more potent the compound. Values are presented as mean $\pm$ S.D. of at least three separate experiments. ND, no data

* Data taken from Haenen and Bast (6).

** Data taken from den Hartog *et al.* (7).

Table 2. The protecting activity of several sulphur containing compounds against ONOOH mediated damage assessed with different detectors.

Compound	IC ₅₀ ONOOH-	IC ₅₀ ONOOH-	IC ₅₀ ONOOH-	IC ₅₀ ONOOH-
	DHR-123 (μM)	α ₁ -AP (μM)	GST-P1-1 (μM)	Tyrosine (μM)
Reduced glutathione	26 ± 9	38 ± 19	17 ± 1	20 ± 1
S-methylglutathione	> 1000	52 ± 6	104 ± 3	> 200
Glutathione disulfide	>1000	> 500	> 200	> 200
Lipoic acid	924 ± 66	17 ± 2	0.9 ± 0.1	24 ± 1
Dihydrolipoic acid	56 ± 9	17 ± 1	1.0 ± 0.1	23 ± 1

Detectors used are DHR-123 oxidation, α₁-AP inactivation and GST-P1-1 inactivation and tyrosine nitration. The activity is expressed as the IC₅₀ value, the concentration needed to reduce the ONOOH induced oxidation, inhibition or nitration by 50%. The lower the IC₅₀, the more potent the compound. Values are presented as mean ± SD of at least three separate experiments.

Fig. 1. LA or GSH concentration after the addition of a varying initial concentration of ONOOH (0–500 μM) to a solution containing 100 μM LA or 100 μM GSH. The formation of β-LA from LA was also determined. The symbols are as follows: closed circle, GSH; closed triangle, LA; and open triangle, β-LA. Based on the GSH consumption at a low concentration of ONOOH, the stoichiometry of the reaction is ≈1:1. Values are presented as mean ± S.D. of at least three separate experiments.

Fig. 2. LA or GSH concentration after addition of a varying initial concentration of HOCl (0–100 μM) to a solution containing 100 μM LA or 100 μM GSH. The formation of β -LA from LA was also determined. The symbols are as follows: closed circle, GSH; closed triangle, LA; and open triangle, β -LA. Based on the GSH or LA consumption, the stoichiometry of the reaction of GSH or LA with HOCl is 1:1. Values are presented as mean $\pm$ S.D. of at least three separate experiments.

The difference in protecting activity against ONOOH between GSH and LA prompted us to perform additional experiments. The consumption of GSH and LA by different concentrations of ONOOH was determined (Fig. 1). The stoichiometry of the initial reaction of GSH was approximately 1, i.e. 1 molecule of GSH reacts with 1 molecule of ONOOH. The apparent stoichiometry of LA was more than 1. With a five-fold excess of ONOOH over LA, less than half of the LA was consumed. The product formed was β -LA, the thiosulfinate of LA which has four possible isomers (4, 6).

The stoichiometry of the initial reaction of GSH with HOCl was comparable to that of LA, i.e. 1 molecule of the antioxidant reacts with 1 molecule of the oxidant (Fig. 2). The product formed by the reaction of LA with HOCl was β -LA (Fig. 2).

4. Discussion

Some sulphur containing compounds can form an effective line of defense against reactive oxygen and nitrogen species. The aim of the present study is to further examine the antioxidant activity of sulphur containing compounds in order to explain the difference in activity observed in some assays. The present study demonstrated differences in protecting activity of sulphur containing compounds against HOCl mediated oxidation of different detectors. This can be explained by the rate of the reaction of the sulphur antioxidant with HOCl relative to the rate of the reaction of the detector with HOCl. The rank order of the antioxidants is independent of the detector used. The ostensible activ-

ity of a poor antioxidant can be upgraded by using a slowly reacting detector. This is nicely shown by the relatively high potency of GSSG, a compound that reacts sluggishly with HOCl (6), to protect DHR-123 against HOCl mediated oxidation (Table 1) because DHR-123 reacts just as sluggishly with HOCl as GSSG. GSSG does not protect faster reacting detectors, e.g. α_1 -AP and GST-P1-1 against HOCl damage. Similarly, Den Hartog *et al.* (7) reported that, acetylcholinesterase is efficiently protected by GSSG against HOCl induced damage. This can also be explained by the relatively slow reaction of the target, acetylcholinesterase, with HOCl. Probably also other proclaimed antioxidant activities of GSSG, e.g. the ability of GSSG to protect against NO₂Cl induced DNA damage (20), are the result of a relatively slow reacting detector (DNA). *In vivo* NO₂Cl will probably react with other cellular targets than DNA. In such a case, the reported antioxidant effect of GSSG is of course physiologically irrelevant.

GSH protected all the tested detectors efficiently against ONOOH, whereas GSSG is not a protector against ONOOH (Table 2). GSMe did not protect DHR-123 and tyrosine, while it did protect the targets GST-P1-1 and α_1 -AP against ONOOH-induced inhibition. One of the vulnerable groups in α_1 -AP is methionine. Oxidation of methionine in α_1 -AP results in a loss of activity (21). Since in methionine and GSMe a chemically identical group is attacked by ONOOH, i.e. the S-methyl group (21), it was anticipated that α_1 -AP is protected by GSMe. The relatively high IC₅₀ of GSMe with DHR-123 and tyrosine can be explained by arguing that the reaction rate of these detectors with ONOOH is higher than that of GSMe, whereas the lower IC₅₀ of GSMe with α_1 -AP or GST-P1-1 indicates that the reaction rate of ONOOH with these detectors is comparable to the reaction rate of ONOOH with GSMe. This explanation is in line with the explanation described above for the difference in activity of an antioxidant towards HOCl in different assays. The efficacy of an antioxidant depends on the detector used.

The most striking result is the finding that the antioxidants LA and DHLA protect GST-P1-1 far more efficiently against ONOOH than GSH (Table 2). In contrast, LA provides a very poor protection against ONOOH induced DHR-123 oxidation (Table 2). GSSG was not able to protect the ONOOH mediated oxidation of DHR-123, α_1 -AP, GST-P1-1 or tyrosine (Table 2). Moreover, the rank order in potency of the antioxidants is not similar for the four detectors used (Table 2). This implies that a difference in protective activity of LA is not solely due to the difference in reactivity of the detectors. The low consumption of LA by ONOOH compared to GSH is peculiar (Fig. 1). At equimolar concentrations of LA and ONOOH (100 μ M) the consumption of LA was only 13%. The relatively low consumption of LA by ONOOH was also noted previously. Trujillo and Radi (4) reported that only 25% of LA was consumed when 5 mM of LA was mixed with 5 mM ONOOH. This denotes that the protection of GST-P1-1 against ONOOH by LA, that is superior to the protection offered by GSH, is not due to direct scavenging of ONOOH by LA. Apparently the mechanism of protection of GST-P1-1

against ONOOH by LA differs from the protection by GSH against ONOOH mediated DHR-123 oxidation. Also the difference in rank order of potency of LA and GSH obtained with the different detectors points to different mechanisms of damage and protection.

Tyrosine residues play a critical role in the GST inactivation by ONOOH (22). Previous studies (2,3) and the results presented in table 2 revealed that LA offered an efficient protection against ONOOH induced tyrosine nitration. The IC_{50} value of LA is 6 times lower than the initial concentration of ONOOH with tyrosine as a detector ($IC_{50} = 24 \mu M$, initial concentration of ONOOH = $150 \mu M$) and more than 10 times lower than GST-P1-1 ($IC_{50} = 0.9 \mu M$, initial concentration of ONOOH = $10 \mu M$). A similar difference was reported in the study of Whiteman *et al.* (2), the IC_{50} value of LA for the inhibition of tyrosine nitration was 10 times as low as the initial concentration of ONOOH. Theoretically, in a standard competition assay the minimal IC_{50} of the antioxidant is equal to half of the initial concentration of oxidant, i.e. when the antioxidant reacts much faster than the detector.

The explanations of Whiteman *et al.* (2) for the very low IC_{50} of LA compared to that of the initial concentration of ONOOH were that (i) one molecule of LA can scavenge several ONOOH molecules and (ii) LA can combine with reactive intermediates. The first explanation, *viz.* that LA scavenges several ONOOH molecules, does not seem probable. The oxidation products of LA that is formed by ONOOH is identical to that formed by HOCl (Fig. 1, 2), i.e. β -LA. This two electron oxidation product of LA is formed after a reaction of one LA with probably one ONOOH (4) or one HOCl (23). This makes it unlikely that LA reacts with several ONOOH molecules. The second explanation of Whiteman *et al.* (2), i.e. that LA can combine with the reactive intermediates of ONOOH seems more plausible. Whiteman *et al.* (2) stated that these intermediate products of ONOOH could be $NO_2^\bullet$ and NO_2^+ .

Interestingly, Nakagawa *et al.* (3) reported that LA is a selective inhibitor of tyrosine nitration by ONOOH, but LA does not inhibit ONOOH induced dityrosine formation from tyrosine. They argued that ONOOH has two reactive forms, i.e. a caged biradical $[ONO^\bullet \dots ^\bullet OH]$ and a caged bipolar $[ONO^+ \dots ^- OH]$ form. The caged biradical form of ONOOH yields dityrosine and the caged bipolar form of ONOOH yields nitrotyrosine. LA was suggested to protect against the bipolar form only, whereas thiol containing compounds such as GSH and DHLA protect against both the bipolar and the biradical form. Although this explanation is highly speculative, it nicely fits within our experimental data. The oxidation of DHR-123 could, just as the oxidation of tyrosine into dityrosine, be mediated by the biradical form of ONOOH and is therefore inhibited by sulfhydryl containing compounds and not by LA. Based on the prominent protective activity of LA, GST-P1-1 inactivation by ONOOH would be mediated by the bipolar form of ONOOH.

The major finding that is in conflict with this theory is the reported reaction of ONOOH by LA with a stoichiometry of 1:1 with a second order rate constant of $1400 \text{ M}^{-1} \text{ s}^{-1}$, which is almost identical to that of GSH ($1350 \text{ M}^{-1} \text{ s}^{-1}$) (4). Trujillo and Radi (4) determined this rate constant by monitoring ONOOH consumption in a stopped-flow spectrophotometer. In the same study the relatively low consumption of LA by ONOOH, identical to the observation depicted in figure 1, was also reported. This finding, i.e. the low consumption of LA in this study and study of Trujillo and Radi (4), is conflicting with the reported very high second order rate constant. The consumption of LA by increasing amounts of ONOOH should have been identical to that of GSH, as shown in Figure 2 for HOCl. As shown in Figure 1, this is clearly not the case for LA consumption by ONOOH. The low consumption of LA by ONOOH is better in line with the hypothesis that only a part of the ONOOH, i.e. the bipolar form, reacts with LA. It should be noted that Trujillo and Radi (4) calculated the apparent second order rate constant by dividing the observed rate of ONOOH consumption by the concentration of LA and the concentration of ONOOH. If only part of the ONOOH is able to react with LA, i.e. the bipolar form, the concentration of the bipolar form should have been used in the calculation instead of the total amount of ONOOH. Consequently, the apparent second order rate constant of the reaction of the bipolar form of ONOOH with LA would have been higher than the reported value of the rate of the second order reaction of ONOOH with LA. GSH reacts with both forms of ONOOH and therefore the second order rate constant of GSH cannot be adjusted in the same way as for LA. This would make the true second order rate constant of the reaction of the bipolar form of ONOOH with LA higher than that with GSH. This could explain the higher efficacy of LA compared to GSH in the protection of GST-P1-1.

Fig. 3. Proposed reaction of LA with ONOOH. ONOOH can be converted into a biradical $[\text{ONO}^\bullet \dots \bullet\text{OH}]$ or bipolar $[\text{ONO}^+ \dots \cdot\text{OH}]$ form. LA reacts with the bipolar form. One of the sulfur atoms of LA binds to the positively charged nitrogen. A double bond is created using one of the electron pairs of the second sulfur atom, and the negatively charged oxygen of the bipolar form of ONOOH binds to the second sulfur atom. The intermediate that is formed in this way decomposes to give β -LA and NO_2^- . $\text{R} = (\text{CH}_2)_4\text{COOH}$.

The question arises where the high protection of LA originates from. It is not the reactivity of the separate sulphur atoms, they are far less reactive than the sulphur in GSH. The most prominent feature of LA is that the sulphur atoms are neighboring in a five-member ring. The orientation of the sulphur atoms in the 1,2-dithiolane ring causes a partial overlap of the lone p_z electron pairs of the sulphur atoms. As a consequence the

p_z orbitals combine into binding and antibinding orbitals. The filled antibinding orbital facilitates the ability of LA to donate electrons or in other words this facilitates the ability of LA to reduce oxidants such as ONOOH and act as antioxidant. This the explanation for the reactivity of LA compared to a linear disulphide, such as GSSG, where an overlap of the p_z electron pair is partially absent (6).

In the reaction scheme proposed (Fig. 3), the antibinding electron pair binds one of the sulphur atoms to the positively charged nitrogen of the bipolar form of ONOOH. The binding electron that already has a partial double bond character (due to interchange with the free d-levels of the neighboring sulphur atoms) forms a double bond between the sulphur atoms. As a result, the sulphur atom that is not bound to the nitrogen becomes positively charged and the negatively charged oxygen of the bipolar form of ONOOH binds to this sulphur atom. The intermediate that is formed in this way decomposes to give β -LA and NO_2^- as shown in Figure 3. In this reaction scheme it is crucial that the p_z orbitals of the two sulphur atoms have been converted into a filled binding and antibinding orbital. Trujillo and Radi (4) have shown that the net reaction is that LA plus ONOOH are converted into β -LA and NO_2^- which is consistent with the proposed scheme.

In conclusion, the results obtained from this study underline that in the efficacy of a free radical scavenger, the reaction rate of the oxidant with the antioxidant relative to the reaction rate of the oxidant with the detector is a major determinant factor. This is illustrated by the difference in protection of the sulphur containing compounds against HOCl induced damage to different targets. The difference in protection against ONOOH of these sulphur containing antioxidants obtained with different targets illustrated that the molecular mechanisms of the damage inflicted by the reactive species and the molecular mechanism of protection by the antioxidant is of equal importance as the rate for the efficacy of the free radical scavenger. ONOOH damage proceeded via at least two mechanisms. LA does not protect against all forms of ONOOH damage. LA is a poor antioxidant in the form of ONOOH toxicity involved in DHR-123 oxidation. However, in the form of ONOOH toxicity involved in GST-P1-1 inactivation, LA is the most potent sulphur containing antioxidant in our series. It is proposed that an intermediate product in which both sulphur atoms of LA have reacted is involved in the reaction of ONOOH with LA. The high potency of LA to protect GST-P1-1 against ONOOH might be of interest for the therapeutic action of this drug.

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Chapter 6

Tetrahydrofolate and 5- methyltetrahydrofolate are folates with high antioxidant activity. Identification of the antioxidant pharmacophore

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Abstract

The presumed protective effect of folic acid on the pathogenesis of cardiovascular, hematological and neurological diseases and cancer has been associated with the antioxidant activity of folic acid. Peroxynitrite (PON) scavenging activity and inhibition of lipid peroxidation (LPO) of the physiological forms of folate and of structurally related compounds were tested. It was found that the fully reduced forms of folate, i.e. tetrahydrofolate (THF) and 5-methyltetrahydrofolate (5-MTHF), had the most prominent antioxidant activity. It appeared that their protection against LPO is less pronounced than their PON scavenging activity. The antioxidant activity of these forms of folic acid resides in the pterin core, the antioxidant pharmacophore is 4-hydroxy-2,5,6-triaminopyrimidine. It is suggested that an electron donating effect of the 5-amino group is of major importance for the antioxidant activity of 4-hydroxy-2,5,6-triaminopyrimidine. A similar electron donating effect is probably important for the antioxidant activity of THF and 5-MTHF.

1. Introduction.

Folates function as cofactors in the transfer and utilization of one carbon groups. This plays a key role in the biosynthesis of purines and pyrimidines and regeneration of methionine (1). Folic acid (FA) is made up of a pterin moiety (purine and pyrazine fused together) that is linked to the side chain containing *p*-aminobenzoic acid (pteroic acid) and glutamic acid (Fig. 1). FA exists in lactam and lactim tautomeric forms. The lactam form has a 4-OH moiety on the purine-type ring (Fig. 1). FA is *in vivo* reduced to dihydrofolate (DHF) and subsequently to tetrahydrofolate (THF). THF is enzymatically converted into 5-methyltetrahydrofolate (5-MTHF), the actual active form (1).

Epidemiological studies have shown that FA supplementation can significantly reduce the risk of cardiovascular and hematological diseases, (2,3) neurological and neuropsychiatric disorders, (4,5) neural tube defects (6) and cancer including cervical, lung, brain, pancreatic, colorectal and breast cancer (7,8). The production of reactive oxygen species, reactive chlorine species and reactive nitrogen species are implicated in these pathologies. Therefore, the antioxidant activity of FA has been suggested to be involved in the presumed effect of FA on health (9,10). There is no consensus about the free radical scavenging activity of FA. One study (10) suggested that the 4-OH group on the pterin ring has an important role. Another study (11) indicated that the side chain, i.e. N-(*p*-aminobenzoyl)-L-glutamic acid, is more important, since the products formed by nitration of FA were identified as 10-nitrofolate and 12-nitrofolate. The present study was designed to further examine the antioxidant activity of folic acid and its physiological metabolites and to determine which part of the molecule is important for this activity.

2. Materials and methods

2.1. Chemicals

Folic acid (FA), 7,8-dihydrofolate (DHF), 2,4-diamino-6-hydroxypyrimidine, 4-hydroxy-2,5,6-triaminopyrimidine and KO_2 were obtained from Fluka Chemie GmbH (Buchs, Switzerland). 2-Amino-4-hydroxypyrimidine, 5,6,7,8-tetrahydrofolate (THF), 5-methyl-5,6,7,8-tetrahydrofolate (5-MTHF), 5,6,7,8-tetrahydrobiopterin (BH_4), leucopterin and dihydrorhodamine-123 (DHR-123), 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of the highest grade of purity available.

2.2. Isolation of rat liver microsomes

Male Lewis rats, 200-250 g, were killed by decapitation. Microsomes were prepared according to Haenen and Bast (12) with a slight modification. Livers were removed and homogenized (1:2 w/v) in ice cold phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA. The homogenate was centrifuged at 10,000 *g* for 30 min at 4 °C. Subsequently, the supernatant was centrifuged at 65,000 *g* for 60 min at 4 °C. The microsomal pellet was resuspended in the phosphate buffer (2 g liver tissue/ml), corresponding with 1-1.3 mg protein/ml, and stored at -80 °C. Storage of microsomes at -80 °C has no effect on the oxidation state of microsomes in the *in vitro* assay. Before use the microsomes were thawed and diluted 5-fold with ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing 150 mM KCl and washed twice followed by centrifugation at 65,000 *g* for 40 min at 4 °C. Finally, the pellet was resuspended in 4 ml Tris-HCl buffer and then used.

2.3. Lipid peroxidation assay

2.3.1. Incubation conditions

Lipid peroxidation assay was carried out according to Haenen and Bast (12) with a minor modification. At 37 °C, 450 µl of Tris-HCl/KCl buffer was added to 250 µl of microsomes, the final concentration was 0.5 g liver tissue/ml. One hundred µl of a solution containing one of the test compounds were added to achieve the desired concentration. A solution of ascorbate (125 µl) was added giving a final concentration of 0.2 mM. The reaction was started by adding 75 µl of a freshly prepared ferrous sulfate solution giving a final concentration of 10 µM. After 20 min incubation, the reaction was stopped as described in spectral measurements. The effects are expressed as the concentration giving 50% inhibition of lipid peroxidation (LPO IC₅₀).

2.3.2. Spectral measurements.

Lipid peroxidation was assayed by measuring thiobarbituric acid (TBA) reactive material (12). The incubation mixture (1 ml) was stopped by adding 2 ml of an ice-cold TBA-trichloroacetic acid-HCl-butylhydroxytoluene (BHT) solution. The mixture was heated for 15 min at 90 °C and then centrifuged for 5 min. The absorbance was determined at 535 nm vs 600 nm. The TBA-trichloroacetic acid-HCl-BHT solution was prepared by dissolving 41.6 mg TBA/10 ml trichloroacetic acid (16.8 w/v in 0.125 N HCl). To 10 ml TBA-trichloroacetic acid-HCl 1 ml BHT (1.5 mg/ml ethanol) was added. The added chemicals did not interfere with the assay in the concentrations used.

2.4. Synthesis of peroxynitrite

Potassium oxoperoxynitrate (ONOOK) was produced from the reaction of solid KO_2 with NO-gas as described by Koppenol *et al.* (13). Briefly, the NO-gas slowly flowed over the mixture of KO_2 and quartz-sand, which was constantly stirred and kept on ice. The mixture was poured into a cold potassium hydroxide solution. Addition of manganese dioxide removes the hydrogen peroxide that results from excess decomposition of potassium superoxide. The solution with peroxynitrite was obtained by filtering off the sand and manganese dioxide. The concentration of peroxynitrite was determined spectrophotometrically at 302 nm.

2.5. Oxidation of DHR-123

Peroxynitrite scavenging was measured by the oxidation of DHR-123 as described by Kooy *et al.* (14). In short, 100 μl of the desired concentration of the test compound were added to 0.9 ml of 100 mM phosphate buffer at 37 °C. The final concentration of DHR-123 in phosphate buffer was 5 μM . During mixing 10 μl of peroxynitrite (in 0.01 M NaOH) was added giving the final concentration, 0.6 μM . The fluorescent product, rhodamine-123, was measured by fluorescence detection with excitation and emission wavelengths of 500 and 536 nm, respectively. The effects are expressed as the concentration giving 50% inhibition of the oxidation of DHR-123 (PON IC_{50}).

2.6. The Trolox Equivalent Antioxidant Capacity (TEAC) assay

The TEAC assay modified by Arts *et al.* (15) was used. The $\text{ABTS}^\bullet$ stock solution was prepared by dissolving 30 mg ABTS in 7.8 ml of 2.46 mM potassium peroxodisulfate. The $\text{ABTS}^\bullet$ stock solution was diluted with 100 mM phosphate buffer (pH 7.4) to give an absorbance at 734 nm between 0.68 and 0.73 at 37 °C. The concentration of $\text{ABTS}^\bullet$ was determined using a molar extinction coefficient of $1.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. For measuring the antioxidant capacity, a fixed concentration of the test compounds in this experiment, 5 μM , was mixed with the different concentrations (0 – 46 μM) of the $\text{ABTS}^\bullet$ solution. Absorbance was monitored at 734 nm after 6 min incubation at 37 °C. The total amount of $\text{ABTS}^\bullet$ scavenged by the antioxidant, i.e. “a” in the formula, was calculated using the exponential formula $y = a(1 - e^{(-bx)})$ (Sigma Plot, version 7). In this formula y is the reduction of the $\text{ABTS}^\bullet$ concentration after 6 minutes and x is the initial $\text{ABTS}^\bullet$ concentration. The TEAC value is determined using the equation $\text{TEAC}_{\text{test compound}} = a / (5 \times 1.9)$. In which 5 is the concentration of the test compound in μM used in the experiment and 1.9 is the average concentration of $\text{ABTS}^\bullet$ that is scavenged per μM trolox.

Fig. 1. The antioxidant activity of folic acid and its physiological metabolites measured by PON scavenging and inhibition of LPO. The activity is expressed as the concentration of the compound that gives 50% scavenging of peroxynitrite (IC_{50} PON) and the concentration that is needed to inhibit 50% of lipid peroxidation (IC_{50} LPO). FA is made up of two parts viz. a pterin moiety (A) and a N-(p-aminobenzoyl)-L-glutamic acid part (B). The numbering of the most important atoms is also shown in the chemical structure of FA. Values are presented as median $\pm$ half of the range of at least three separate experiments.

3. Results

Fig. 1 gives an overview of the antioxidant activities of FA and its physiological metabolites. The concentration of FA needed to prevent the PON mediated oxidation of DHR-123 by 50% (IC_{50} PON) is higher than 100 μM and the concentration of FA needed to inhibit LPO by 50% (IC_{50} LPO) is higher than 500 μM . Reduction of FA to DHF increased the PON scavenging activity (IC_{50} PON = 2.4 μM), whereas the concentration needed to inhibit LPO by 50% was still above 500 μM . Reduction of DHF to

THF resulted in a more than 3 fold increase in the ability to protect against LPO and also the ability to scavenge PON was increased. The PON scavenging activity of 5-MTHF compared to that of THF was higher (the IC_{50} values were $0.9 \mu M$ and $1.5 \mu M$, respectively) and the protective effect against LPO was lower (the IC_{50} values were above $500 \mu M$ and $189 \mu M$, respectively). The TEAC values of THF and 5-MTHF were equivalent to 1.8 ± 0.1 , and $0.7 \pm 0.1 \mu M$ trolox, respectively (Fig. 2).

Fig. 2. Determination of the TEAC of THF and 5-MTHF. The reaction of $5 \mu M$ THF (open circles) and $5 \mu M$ of 5-MTHF (closed circles) with various concentrations of $ABTS^{\bullet}$ was assessed. The concentration of $ABTS^{\bullet}$ that is consumed by the antioxidant in 6 minutes (y in the formula) is plotted against the initial concentration of $ABTS^{\bullet}$ (x in the formula). This could be fitted according to the exponential formula given in the figure. R is the correlation coefficient of the fit. The TEAC of THF (median $\pm$ half of range, $n = 3$) is 1.8 ± 0.1 and that of 5-MTHF is 0.7 ± 0.1 .

Fig. 3. The antioxidant activity of 4-hydroxy-2,5,6-triaminopyrimidine and structurally related compounds measured by PON scavenging and the inhibition of LPO. The activity is expressed as the concentration of the compound that gives 50% scavenging of peroxynitrite (IC_{50} PON) and the concentration that is needed to inhibit 50% of lipid peroxidation (IC_{50} LPO). Values are presented as median $\pm$ half of the range of at least three separate experiments.

FA can be split into two parts viz. a N-(*p*-aminobenzoyl)-L-glutamic acid part and a pterin moiety. The IC_{50} of N-(*p*-aminobenzoyl)-L-glutamic acid was higher than 500 μM for either PON scavenging or inhibition of LPO (data not shown). As shown in Fig-

ure 3, the antioxidant activity of tetrahydrobiopterin (BH_4) was comparable to that of THF (IC_{50} LPO = $86 \pm 1 \mu\text{M}$, IC_{50} PON = $0.5 \pm 0.2 \mu\text{M}$). The antioxidant activity of 4-hydroxy-2,5,6-triaminopyrimidine was also comparable to that of THF. Removing the 5 amino group or the 5 and the 6 amino group of 4-hydroxy-2,5,6-triaminopyrimidine drastically reduced the antioxidant activity. Pterin also displayed no antioxidant activity, whereas leucopterin has a moderate PON scavenging activity.

4. Discussion

An antioxidant effect of FA and its physiological metabolites might be involved in some of the positive health effects. The rationale for the present study is to determine the antioxidant activity of the various endogenous folates and to elucidate which moiety of the molecule determines this activity. FA has a poor antioxidant activity in PON scavenging and LPO inhibition. Its physiological metabolites are more active. Joshi *et al.* (10) reported that $500 \mu\text{M}$ of FA induced a 37% inhibition of LPO, which corresponds to the protection we found, i.e. IC_{50} LPO > $500 \mu\text{M}$. Based on this partial inhibition by a relatively high concentration, Joshi *et al.* (10) speculated on a physiological relevance of FA as an antioxidant. We think that the minor protective effect of FA on LPO is physiologically not relevant. The marginal antioxidant effect of FA is also seen from its poor PON scavenging activity.

Reduction of FA to DHF results in a more prominent PON scavenging activity than that of FA, whereas the IC_{50} for inhibition of LPO is still higher than $500 \mu\text{M}$. Further reduction to THF and conversion to 5-MTHF enhanced the PON scavenging activity even more. The fully reduced forms, i.e. THF and 5-MTHF were the most active compounds by inhibiting LPO. 5-MTHF had a relatively lower activity in the protection against LPO than THF ($500 \mu\text{M}$ of 5-MTHF induced 46% LPO inhibition), whereas the PON scavenging activity of 5-MTHF was superior to that of THF. Compared to other compounds, the protective effect of the folates against LPO seems less relevant than their PON scavenging activity. For example, the flavonoid quercetin is much more potent in inhibiting LPO (IC_{50} of quercetin = $9 \mu\text{M}$) (16) than the folates, whereas its PON scavenging activity is in the same order of magnitude (IC_{50} of quercetin = $0.53 \mu\text{M}$) (17)

To determine the pharmacophore which is responsible for the antioxidant activity of the reduced forms of folic acid, structurally related compounds were tested. The minor importance of the side chain for the antioxidant activity is demonstrated by (i) the absence of antioxidant activity of N-(*p*-aminobenzoyl)-L-glutamic acid, (ii) the large difference in activity of the various folates which all contain the same side chain and (iii) the antioxidant activity of BH_4 (that lacks the side chain) which is comparable to that of THF. Apparently, the antioxidant activity resides in the pterin moiety. An optimal antioxidant activity is achieved when the pterin ring is in its reduced form. By comparing structur-

ally related compounds (Fig 3), it was found that the antioxidant pharmacophore of the fully reduced form of folates, i.e. THF, is 4-hydroxy-2,5,6-triaminopyrimidine.

The potency of substituted phenols for PON scavenging and protection against LPO is governed by the electron donating effect of the substituent (16,17). The higher the electron donating effect, the higher the antioxidant activity. The electron donating effect of a substituent depends on the nature as well as the position of the substituent. For example, the activity of a strong electron donating group, such as the hydroxyl group, on the *ortho* and *para* position is much higher than that on the *meta* position. This can explain the potent antioxidant activity of catechol (hydroxyl group at the *ortho* position; IC_{50} LPO = 10 μ M, IC_{50} PON = 2.7 μ M) (16,17) and hydroquinone (hydroxyl group at the *para* position; IC_{50} LPO = 156 μ M, IC_{50} PON = 1.8 μ M) (16,17) compared to that of resorcinol (hydroxyl group at the *meta* position; IC_{50} LPO > 1000 μ M, IC_{50} PON = 42.5 μ M) (16,17). For comparison, for phenol the IC_{50} LPO is > 1000 μ M and the IC_{50} PON is 263 μ M (16,17). An amino group has an even higher electron donating effect than a hydroxyl group. For 2-aminophenol, the IC_{50} LPO is 5 ± 0.5 μ M and the IC_{50} PON is 0.6 ± 0.1 μ M. Similarly, the IC_{50} LPO of 4-aminophenol is 12 μ M and the IC_{50} PON is 0.9 μ M (16,17). In analogy with the difference in activity due to the position of the substituent given above, it is suggested that the 5 amino group of 4-hydroxy-2,5,6-triaminopyrimidine is of major importance. The location of this potent electron donating group at the *ortho* position of the 4 hydroxyl group will boost the activity of the aromatic hydroxyl group, and can explain the potent activity of 4-hydroxy-2,5,6-triaminopyrimidine to a great extent.

In leucopterin, the electron withdrawing effect of the carbonyl groups next to the 5 and 6 amino group will reduce the electron donating effect the 4-hydroxyl group receives. This can explain the lower antioxidant activity of leucopterin compared to 4-hydroxy-2,5,6-triaminopyrimidine and BH_4 .

A difference in electron donating effect may also explain the higher PON scavenging activity of 5-MTHF compared to THF. The methyl group at the 5 amino group in 5-MTHF will slightly enhance the electron donating effect of the 5-amino group. Based on the TEAC, the number of radicals that can be scavenged by 5-MTHF is lower than that of THF. Possibly, this can explain the low activity of 5-MTHF in the protection against LPO compared to that of THF. The actual antioxidant effect of folates *in vivo* might depend on both reduction by the enzyme 5,10-methylenetetrahydrofolate (MTHFR) and consumption which is related to vitamin B_{12} and homocysteine level.

In conclusion, FA is a poor antioxidant. The more the FA is reduced, the higher its antioxidant activity becomes: THF and 5-MTHF are the folates with the most prominent antioxidant activity. The antioxidant pharmacophore of THF and 5-MTHF, i.e. 4-hydroxy-2,5,6-triaminopyrimidine, resides in the pterin moiety. It is suggested that an electron donating effect of the 5 amino group is of major importance in the antioxidant

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activity of 4-hydroxy-2,5,6-triaminopyrimidine. A similar electron donating effect is probably involved in the antioxidant activity of THF and 5-MTHF.

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Chapter 7

Summary and conclusions

Summary

In this thesis, antioxidants were studied from a toxicological point of view. The fundament of toxicology is the risk-benefit analysis. Antioxidants are applied to counteract free radical damage, a process associated with numerous pathological conditions. A great deal of people takes antioxidant supplements in the belief that these compounds promote health. Antioxidants may, however, also have negative effects on health. The most striking example is the increase in the risk of getting lung cancer induced by a high β -carotene consumption in smokers. The positive as well as the negative health effects have to be assessed and related to each other to provide a rationale for the use of an antioxidant. The work performed in this thesis focuses on the beneficial effects of antioxidants and the need to understand the molecular mechanism of these effects.

In the general introduction it is discussed that antioxidants display very diverse biological effects. First of all, an antioxidant is often not a clearly defined compound. Of vitamin E, 40 different vitamers exist and over 5,000 different flavonoids have been identified. Secondly, antioxidants are converted in our body into several metabolites that also contribute to the biological effect of the parent compound. Additionally, antioxidants not only display various antioxidant activities, but they may also possess some prominent non-antioxidant effects. In the original research described in this thesis the molecular mechanisms of 4 antioxidants that were selected based on their toxicological relevance were explored, i.e. vitamin E phosphate, phloretin, lipoic acid and folic acid.

Vitamin E esters were regarded as precursors of vitamin E which only display an antioxidant activity after hydrolysis to vitamin E. In Chapter 2, however, it is described that vitamin E phosphate (VEP) has a potent antioxidant effect of its own without conversion into VE when tested as a protector against lipid peroxidation in non-heated and heated rat liver microsomes. Moreover, in contrast to VE, VEP is not consumed during this protection. Of the VE esters, VEP is most potent in inducing haemolysis of erythrocytes. EPR experiments using the spin label 16-doxylstearic acid showed that VEP reduces membrane fluidity, in contrast to VE. This indicates that VEP acts as a detergent and forms a barrier that might inhibit the transfer of radicals from one polyunsaturated fatty acid to another.

In analogy to VES, the detergent character of VEP suggests that it might be possible to use VEP as a novel apoptotic agent. In Chapter 3 we investigated the effect of VEP on the osteosarcoma cell line MG-63. VEP induced cell death, antiproliferative effects and DNA degradation to an even higher extent than vitamin E succinate (VES). An explanation for the more potent apoptotic effect of VEP compared to that of VES may be due to inflexibility of VEP. It is also suggested that the biological effect of VEP may be caused by a combination of membrane destabilisation and deregulation of signalling pathways. These activities of VEP are extra relevant since the recent discovery of VEP

in food and in our body where the levels of VEP sometimes equal or even surpass those of vitamin E itself.

The beneficial health effect of flavonoids has attracted a great deal of attention of the general public as well as of researchers in the field of nutrition. As elegantly shown in the recent thesis of Chantal Beerens-Heijnen (Maastricht University, 2004), the molecular mechanism of action of all 5,000 flavonoids is not identical. Two different nutriconophores could be identified within a subgroup of the flavonoids, the flavonols. A nutriconophore is defined as a specific structural element of a nutrient where the biochemical activity that is responsible for the health promoting effect of that compound resides. Within the dihydrochalcones, another subgroup of the flavonoids with phloretin as the most prominent compound, a third nutriconophore within the flavonoids was identified (Chapter 4). Phloretin was a potent antioxidant in peroxynitrite (ONOOH) scavenging and inhibited lipid peroxidation. Substitution of the 2-OH group by glucose giving phloridzin decreased the antioxidant activity 18 times in comparison to phloretin. Comparison with structurally related compounds revealed that the nutriconophore of phloretin is 2,6-dihydroxyacetophenone. The potent antioxidant activity of 2,6-dihydroxyacetophenone might be explained by tautomerisation which may stabilise its radical that is formed after hydrogen abstraction. Thus, the 2,6-dihydroxyacetophenone is a new nutriconophore which differs from those previously reported in flavonoids.

In Chapter 5, the antioxidant activity of lipoic acid (LA) was studied. It was found that, depending on the target that had to be protected, lipoic acid was either a poor or an extremely potent scavenger of ONOOH. LA is a poor antioxidant in protecting against dihydrorhodamine-123 oxidation by ONOOH. However, in protecting against glutathione-S-transferase P1-1 (GST-P1-1) inactivation by ONOOH, LA is the most potent sulphur-containing antioxidant in our series. The impact of this finding is that in one type of ONOOH-induced damage lipoic acid is the preferred compound for protection, while in the other type of damage by the same reactive species LA is of no value. A molecular mechanism explaining this discrepancy was given. In the dithiolane ring of LA the orientation of the two neighbouring sulphur atoms can explain the antioxidant potential of LA. It is proposed that both sulphur atoms play a crucial role in the reaction of a specific form of ONOOH with LA. The potency of LA to protect GST-P1-1 against ONOOH might be of therapeutic interest.

In the last experimental chapter (Chapter 6), the antioxidant effect of folic acid (FA) was studied. The metabolites of FA which contain the most prominent antioxidant activity, i.e. tetrahydrofolate (THF) and 5-methyltetrahydrofolate (5-MTHF), were established and the nutriconophore of these metabolites was identified. The antioxidant activity of these forms of FA resides in the pterin core, the nutriconophore is 4-hydroxy-2,5,6-triaminopyrimidine. It is suggested that an electron donating effect of the 5-amino group is of major importance for the antioxidant activity of 4-hydroxy-2,5,6-tria-

minopyrimidine. A similar electron donating effect is probably important for the antioxidant activity of THF and 5-MTHF.

Conclusions and implications

In the studies on the four selected antioxidants their nutricophores were identified i.e. 4-hydroxy-2,5,6-triaminopyrimidine in FA, the dithiolane ring in LA and 2,6-dihydroxyacetophenone in phloretin. In VEP the entire molecule contributes to its antioxidant activity. In a nutricophore the activity responsible for a health promoting effect of a specific nutrient resides. Knowledge on the nutricophore is essential for understanding the molecular mechanism of the health promoting effect.

For the impact of this knowledge, a comparison between drugs and diet can be made. The molecular mechanism of action of a compound provides the fundament for the desired health promoting effect and the rationale for the possible application of that compound, irrespective whether the compound is either a pharmacon or a specific nutrient. Identification of a nutricophore can also help to strengthen our therapeutic arsenal. It becomes possible to predict which ingredients of our diet are of potential interest and what kind of food should be consumed to increase the intake of these ingredients, when necessary. Alternatively, it can be used to determine which biosynthetic pathways in food crops might be altered by genetic engineering to increase the dietary content of these valuable compounds. In these strategies Paracelsus' rule should be kept in mind and the optimal dose should be determined first.

Knowledge on the nutricophore is also indispensable for designing even more potent compounds. The new nutricophore and mechanism found in the study on VEP may lead to the development of a new class of antioxidants for the protection against free radical mediated disorders. The same applies for the nutricophore discovered in phloretin, which greatly differs from those previously reported in flavonoids. The disclosure of several nutricophores within the flavonoids shows the diversity of the molecular mechanism of action within a group of compounds often incorrectly lumped together.

For the folates it was shown that the reduced metabolites display a superior antioxidant activity compared to the other forms of folate. This suggests that it is worthwhile to examine whether these reduced forms might be preferred instead of the oxidized form given at present as folate supplement.

The molecular mechanism disclosed for LA directly proves that antioxidant therapy has to be adjusted to the type of damage that occurs. LA only protects against a specific form of ONOOH damage. This underlines that in assessing the risk-benefit of applying antioxidants, the molecular mechanism has to be known.

Samenvatting

In deze dissertatie worden de toxicologische aspecten van een aantal actuele antioxidanten onderzocht. De basis van de toxicologie is de "risk-benefit" analyse. Antioxidanten worden toegepast om schade van vrije radicalen, die ten grondslag ligt aan diverse pathologische aandoeningen, te voorkomen. Veel mensen slikken antioxidanten omdat ze geloven dat dit hun gezondheid bevordert. Antioxidanten kunnen echter ook negatieve effecten hebben op de gezondheid. Een frappant voorbeeld is de toename in het risico om longkanker te krijgen als rokers β -caroteen gaan gebruiken. Zowel de positieve als de negatieve gezondheidseffecten moeten worden vastgesteld en met elkaar in verband worden gebracht om een rationale voor het gebruik van een antioxidant vast te stellen. De studies in deze dissertatie focuseren zich op de gunstige effecten van de antioxidanten en geven de noodzaak aan om de moleculaire mechanismen van deze effecten te leren kennen.

In de algemene introductie wordt uiteengezet dat antioxidanten zeer uiteenlopende biologische effecten kunnen hebben. Vaak is een antioxidant niet een eenduidig gedefinieerde verbinding. Zo bestaan van vitamine E wel 40 verschillende vitameren, terwijl meer dan 5000 verschillende flavonoiden zijn geïdentificeerd. Daar komt bij dat antioxidanten in ons lichaam worden omgezet tot metabolieten die op hun beurt kunnen bijdragen aan het biologische effect. Bovendien geven antioxidanten niet alleen verschillende antioxidant activiteiten, maar kunnen ze ook eigenschappen bezitten die niets te maken hebben met deze activiteit. In het onderzoek dat in deze dissertatie wordt beschreven, wordt het moleculaire mechanisme van een viertal toxicologisch relevante antioxidanten onderzocht. Het betreft: vitamine E fosfaat, phloretine, liponinezuur en foliumzuur.

Vitamine E esters zouden alleen een antioxidant activiteit verkrijgen na hydrolyse tot vitamine E. In Hoofdstuk 2 wordt echter beschreven dat vitamine E fosfaat (VEP) zelf, dus zonder omgezet te worden in vitamine E, ook een goed werkzame antioxidant is in de lipide peroxidatie assay met zowel verhitte als onverhitte lever microsomen van de rat. Bovendien werd geconstateerd dat VEP gedurende deze bescherming, in tegenstelling tot vitamine E zelf, niet werd verbruikt. Van de vitamine E esters veroorzaakte VEP de sterkste hemolyse van erythrocyten. EPR analyses waarbij 16-doxylstearinezuur als spinlabel werd gebruikt, lieten zien dat VEP, in tegenstelling tot vitamine E, de membraan vloeibaarheid vermindert. Dit wijst erop dat VEP in het membraan wordt opgenomen en daardoor een barrière vormt voor het transport van radicalen van het ene meervoudig onverzadigde vetzuur naar het andere.

Naar analogie van vitamine E succinaat (VES) suggereert het oppervlaktactieve karakter van VEP dat het een nieuw apoptotisch middel zou kunnen zijn. In Hoofdstuk 3 werd daarom het effect van VEP op de osteosarcoma cellijn MG-63 onderzocht. VEP in-

duceerde celdood, antiproliferatieve effecten en DNA degradatie die sterker was dan die met VES. Een verklaring voor het sterkere apoptotische effect van VEP t.o.v. dat van VES zou de starre structuur van het VEP kunnen zijn. Een andere suggestie voor het biologische effect van VEP is de combinatie van membraan destabilisatie en de ontregeling van de signaaltransductie. Deze effecten van VEP verdienen vooral aandacht, omdat VEP niet alleen in voedsel aanwezig blijkt te zijn, maar ook in ons lichaam met spiegels die soms gelijk zijn aan of zelfs hoger zijn dan die van vitamine E zelf.

Het gunstige effect van flavonoiden op de gezondheid heeft veel aandacht gevraagd van zowel de consument als onderzoekers op het gebied van voeding. Zoals Chantal Beereens-Heijnen elegant laat zien in haar recent verschenen dissertatie (Universiteit Maastricht, 2004) is het moleculaire mechanisme van de ca. 5000 flavonoiden niet identiek. Twee verschillende nutricosoren konden worden geïdentificeerd binnen de flavonolen, een subgroep van de flavonoiden. Een nutricosoor is gedefinieerd als een structuur element van een voedingsstof waar zich de biochemische activiteit bevindt, die verantwoordelijk is voor de bevordering van de gezondheid. Binnen de dihydrochalconen, een andere subgroep van de flavonoiden met phloretine als belangrijke representant, werd een derde nutricosoor in de flavonoiden geïdentificeerd (Hoofdstuk 4). Phloretine bleek een potente antioxidant bij het wegvangen van peroxynitriet en het beschermen tegen lipide peroxidatie. Substitutie van de 2-OH groep door glucose (waardoor de verbinding phloridzine ontstaat) verminderde de antioxidant activiteit met een factor 18 t.o.v. phloretine. Vergelijking met structureel verwante verbindingen wees erop dat de antioxidant pharmacosoor van phloretine het 2,6-dihydroxy-acetophenon is. De grote antioxidant activiteit van het 2,6-dihydroxyacetophenon kan worden verklaard door de tautomerisatie van het radicaal dat ontstaat na de eliminatie van waterstof. Het 2,6-dihydroxyacetophenon is dus een nieuwe antioxidant pharmacosoor die verschilt van de eerder ontdekte pharmacosoren in flavonoiden.

In Hoofdstuk 5 wordt de antioxidant activiteit van liponinezuur (LA) onderzocht. Afhankelijk van de target die beschermd moest worden, werd gevonden dat LA hetzij een slechte dan wel een zeer goede scavenger van peroxynitriet was. LA is een slechte antioxidant bij het beschermen tegen de vorm van peroxynitriet (ONOOH) schade bij dihydrorhodamine-123 oxidatie, maar bij de bescherming tegen de glutathion-S-transferase P1-1 (GST-P1-1) inactivatie door ONOOH was LA het meest actief van alle onderzochte zwavel-houdende antioxidanten. De betekenis van deze vinding is dat tegen het ene type schade dat door ONOOH wordt veroorzaakt LA kan beschermen, terwijl LA bij het andere type schade door ONOOH geen waarde heeft. Deze tegenstrijdigheid wordt verklaard met een moleculair mechanisme nl. dat de oriëntatie van de twee aan elkaar grenzende zwavel atomen het antioxidant profiel van LA bepaalt. We veronderstellen dat beide zwavel atomen van het LA een cruciale rol spelen

bij de reactie van een specifieke vorm van ONOOH met LA. De mogelijkheid van LA om GST-P1-1 te beschermen tegen ONOOH schade kan van therapeutisch belang zijn. In het laatste hoofdstuk (Hoofdstuk 6) werd het antioxidant effect van foliumzuur(FA) bestudeerd. De metaboliëten van FA die een prominente antioxidant activiteit bezaten werden geïdentificeerd. Dit waren tetrahydrofolaat (THF) en 5-methyltetrahydrofolaat (5-MTHF). Van deze verbindingen werd ook de nutricofoor geïdentificeerd, nl. 4-hydroxy-2,5,6-triaminopyrimidine. We veronderstellen dat het electronen donerende effect van de 5-amino groep van groot belang is voor de antioxidant werking van het 4-hydroxy-2,5,6-triaminopyrimidine. Een vergelijkbaar electronen donerend effect is waarschijnlijk belangrijk voor de antioxidant werking van THF en 5-MTHF.

الملخص

في هذه الرسالة تم دراسة مضادات الأكرية سدوية نظراً لسمية آخذهم في الاعتبار مبدأ علم السموم القاضى بالاعتماد تحديد نسبة الفائدة إلى الضرر. تستخدم مضادات الأكرية عادة لمنع التأثير الضار الناجم عن الجذور الحرة، حيث غالباً ما يرتبط هذا التأثير بحالات مرضية كثيرة. ولذلك تتناول شريحة كبيرة من الناس مضادات الأكرية ومن اعتقادهم أنها مفيدة للصحة. وغير مثال على ذلك زيادة نسبة حدوث سرطان الرئة عند المدخنين نتيجة للاستخدام الكبير للميثانكاربوسيم. إنه التأثيرات الإيجابية والسلبية لمضادات الأكرية يجب أخذها في الاعتبار عند إجراء تقييم منطقي لاستخدامها بنجاح. لقد هدفت هذه الرسالة إلى التركيز على التأثيرات السلبية لمضادات الأكرية وذلك فهم الآليات الجزيئية لهذه التأثيرات في المقدمة العامة ثم عرض التأثيرات البيولوجية كثيرة الشروع لمضادات الأكرية.

أولاً: غالباً ما يكون مضاد الأكرية مركب غير محدد قطعاً، فعلى سبيل المثال يوجد في نوعاً مختلفاً منه فيتاميسيد (VE)، وتم تحديد أكثر من ٥٠٠٠ مركبات صيدغية (فلافونويدات) مختلفة. ثانياً: إن مضادات الأكرية قد تتولد في الجسم إلى أليستات عديدة والتي بدورها تسهم في التأثير البيولوجي للمركب الأصلي. وعلاوة على ذلك فإن مضادات الأكرية قد لا تسمى فقط تأثيرات مضادة للأكرية متنوعة بل يمكن أن تمتلك بعض التأثيرات الجلبية الغير مرتبطة بالتأثير المضاد للأكرية. وصف البحث الرئيس في هذه الرسالة الآليات الجزيئية لآلية مضادات أكرية سد حيث صولها بتأثيرها السمية وهذه المضادات هي: فوسفات فيتاميسيد، إلفورنيم، هكس اللينوسيل وجيم الفوليل.

تعتبر استرات فيتاميسيد طلائع لفيتاميسيد، حيث تبدأ فاعلية مضادة للأكرية فقط عندما تتحول إلى فيتاميسيد. في الفصل الثاني من الرسالة وُصفت أن فوسفات فيتاميسيد (VEP) تمتلك بذاتها تأثيراً قوياً مضاداً للأكرية دونه تحولها إلى VE، وذلك عندما تم دراسة كاسي ضد عملية خوم الأكرية لديهم في ميكروزمات كبد الفئران الغير مسنة والمسخنة. علاوة على ذلك وعلى التقييم سد VE فإنه VEP لا يستهلك خلال عملية الطمارة هذه، ويعتبر VEP سد سيم اسعرات VE الأقوى في أحداث انحلال كريات الدم الحمراء. لقد أظهرت تجارب الـ EPR باستخدام حصص ١٦- دوكس سفيريك أن VEP يتفقد سد عرونة الأغشية الخلوية على عكس VE. وهذا يشير بدوره أن VEP يعمل كمنظف وليس كجهازاً بامكانه ربما تشييط انتقال الجذور سد حصص دهن معقد غير مشيع إلى آخر.

وبالقياس مع سكينيات فيتاميسيد (VES) فإن الخاصية التي يبدىها VEP كمنظف لتفترج استخدام VEP كعامل محدد لموت الخلية (أبوبتوس) جديد. وفي الفصل الثالث بحثنا تأثير VEP على خلايا السرغوم العظمى سد نوع ١٠ MG-63 حيث VEP عرأ

لقد ، تأثيرات مضادة للتآكل والى انخفاض الحامض النووي الديوكسي ريبوز DNA الى مستوى أعلى حتى ما أحدثته VES . إن التفسير لهذا التأثير اللايوتوتيك القوي لـ VEP مقارنة مع VES قلتم أنه يعزى الى عدم مرونة VEP . واقترح أيضاً أن التأثير البيولوجي لـ VEP يمكن أن يكون سبب كل من عدم استقرار الأغشية المخاطية واضطراب في تنظيم المراكز البينية . وهذه النشاطات الأخرى لـ VEP ذات صلة وأهمية كبرى خاصة بعد البكتريانات الأخيرة لتواجد VEP في الطعام وفي أجسامنا حيث أن مستويات VEP تعادل أحياناً أو حتى تفوق مستويات VE نفسه .

لقد جذب التأثير الضار الضيق للصبغيات النباتية انتباه الرأي العام والباحثين في مجال التغذية . لقد أظهرت الرسالة المميزة لشغال بيرلين - هيلمان (1) إلى أن اللثة الجزئية للعل ... ه صبغي غير متناهي . أعلم تقدير تزعيم تخليصهم من النوتريكوتور من ضمن زمرة معينة من الصبغيات ، الفلافونول . ويعرف النوتريكوتور على أنه عنصر بيئي خاص بالمادة الغذائية حيث أنه فاعلية الكيميائية الحيوية هي المسؤولة عن التأثير المفيد للصحة لهذه المادة . في الدى هيدروكسي تشاكولات - مجموعة تحمية أخرى من الصبغيات النباتية ، حيث أن الفلورينيم يشكّل المادة الأكثر سيطرة - تم تحديد نوتريكوتور ثالث مهم للصبغيات (الفصل الرابع) . يعتبر الفلورينيم مادة مضادة للأكسدة ذات فاعلية قوية كالمسكن للبروكسي شيت (ONOOH) وتثبيط عملية فرميد الأسة الدهم . إن استبدال مجموعة الـ 2-OH بالفلورين محطياً الفلورينيم أنتجت النشاط المضاد للأكسدة 18 مرة مقارنة بالفلورينيم . المقارنة مع مركبات وثيقة الصلة بنائياً بالفلورينيم . أظهرت أن النوتريكوتور الخاص بالفلورينيم هو 612- دى هيدروكسي أستوفينون . إن النشاط القوي المضاد للأكسدة الذي يتبعه 612- دى هيدروكسي أستوفينون يمكن تفسيرها بعملية الصنوية التي قد تقوم بتثبيت جندرها التي تتلوه بعد علاج انتزاع الهيدروكسي . لذلك يعتبر الـ 612- دى هيدروكسي أستوفينون مركباً نوتريكوتورياً جيداً يختلف عما تم تقريره سابقاً في الصبغيات النباتية .

في الفصل الخامس تم دراسة الفاعلية المضادة للأكسدة لمغنيسيوم الـ (LA) . وقد وجد أنه هناك التبريد يمكن أن يكونه كاساً قوياً أو ضعيفاً لـ ONOOH وذلك تبعاً للوقت والحرارة . فهو يعتبر مضاد أكسدة ضعيف عندما يتم بحمارة الدى هيدروكسي روبرامين - 122 مع الأكسدة بواسطة الـ ONOOH ، على الرغم أنه هناك التبريد مضاد الأكسدة الأكثر من الكفاءة مع سيم الأنواع التي تم دراستها هنا وذلك عندما قام بحمارة أنزيم جلوتاثيون - S - ترانسفيراز PI-1 (GST-PI-1) مع التثبيط بالـ ONOOH . إنه تأثير هذه الشائخ يمثل في أنه هناك التبريد يعتبر المادة النشطة في الحماية من الضرر الناتج مع الـ ONOOH أيضاً يعتبر عدم الضرر عند تثبيط في الحماية من ضرر آخر ناتج مع نفس الشائخ الأكسدة . ويمكن تفسير هذا الشافق مع معدل الآلية الجزئية لمغنيسيوم حيث لاحظ أنه الوضع الفراغي لذلك التبريد المتناهي في

المخلفات الأخرى المتبقية الثانية عليم أنه تعسر الفعالية الخاصة المضادة للأكسدة لهذا المحقق. ويصنف أنه كما ذكر في الأثرية لتعاضد دوراً حيوياً في تفاعل نوعي مع الـ ONOOH مع حمض الليبوسيل عليم فإن قدرة حمض الليبوسيل على حماية الـ GST-PH مع الـ ONOOH قد تكون ذات أهمية علمية.

في الفصل الثماني الأخير (الفصل السادس) تم دراسة التأثير المضاد للأكسدة لمحفز الفوليد (FA) حيث تم تحديد أليبيات حمض الفوليد التي تقع بالنشاطية الكبيرة المضادة للأكسدة وهي تراهدروبولات (THF) و ٥-ميتيل تراهدروبولات (5-MTHF). كما تم تحديد النورتيكوفور لهذه الأليبيات. إن الفعالية المضادة للأكسدة لهذه الأشكال تقع في ثلث البترسيم - والنورتيكوفور هو ٤-هيدروكس - ٦,٥٨٢ - تراي أسيتو بيراسيديم. واقترح أنه يكون التأثير المعطى للوكترون والتي تصنف به مجموعة ٥ - أسيتو يعبر دوراً هيكلياً كبيراً في تحديد الفعالية المضادة للأكسدة لـ ٤-هيدروكس - ٦,٥٨٢ - تراي أسيتو بيراسيديم. ومن المحتمل أنه يكون تأثير إعطاء اللوكترون مهماً كذلك في الفعالية المضادة للأكسدة لمركبات THF و 5-MTHF.

استنتاجات وتوصيات

لقد تم تحديد النورتيكوفورات الموجودة في مضادات الأكسدة الأربع التي تم اختبارها ودراستها. وهذه النورتيكوفورات هي ٤-هيدروكس - ٦,٥٨٢ - تراي أسيتو بيراسيديم في حمض الفوليد، والمخلفات الأخرى المتبقية الثانية في حمض الليبوسيل و ٦,٥٨٢ - تراي هيدروكس أسيتو فينول في الفلوريسيم. أما في VEP فإن الفعالية المضادة للأكسدة تعبر إلى الجزيء كله. إن معرفة النورتيكوفور ضرورية في فهم الآلية الجزيئية لتأثيرها على الصحة. ولهم تأثير هذه المعرفة فإنه عليم إجراء مقارنة بين الأدوية والغذاء. إن الآلية الجزيئية محل تركب ما عليم أنه تزودنا بعلوم أساسية للوصول إلى التأثير المرحلي والمفيد للصحة كما أن هذه الآلية الجزيئية عليم أنه تسهم في فهم مظهر الليكانية لتطعيم هذا المركب بجمع البقايا فيما إذا كان هذا المركب جزءاً من سلاسل (دوائ) أو مادة غذائية نوعية. كما أنه اعلم عليم تحديد النورتيكوفور في مضادات الأكسدة لتسهم في توعية وزيادة ترسانتنا العلمية. ولقد أصبح عليم أنه ستكون مكونات غذائية ذات الأهمية الفعالة ونوع الطعام الواجب استهلاكه لزيادة شمول هذه المكونات الغذائية الضرورية. بدلاً، فإنه تحديد ما هي النورتيكوفور عليم أنه تحديد المسارات التقليدية المطورة للمحاصيل الزراعية التي عليم تغييرها بوسائل الهندسة الوراثية للحصول على مردود عالٍ من هذه المواد القيمة. ومن هذه الدراسات تراكيبات يجب أن لا يضع عليم أنها قائمة بالأسس وتحدد الجرعة المثلى.

إنه تحديد مهم لنسب النوتركتونورات تعتبر أساسية في تصميم مركبات ذات قوة ومقاومة كبيرة. إنه مركبات النوتركتونورات الجديدة والمحددة في هذه الدراسة إضاهة إلى آلية VEP قد تعود إلى تطوير مجموعة جديدة من مضادات الآكسدة التي تحمي من حدوث الاضطرابات المرتبطة بالجذور الحرة، وهذا ينطبق أيضاً بدوره على النوتركتونورات المكتشفة في الفلورسنت والذئب. يختلف بشكل كبير عما تم دراسته سابقاً من الفلافونويدات. اكتشفنا العديد من النوتركتونورات في الصبغيات يظهر التنوع في الآلية الجزيئية لعملها ضمن مجموعة من المركبات عالمياً ما تكون محتملة معاً بشكل جاذب.

لقد أظهرت هذه الدراسة أن اللايضيئات المختزلة في الفولدرات. تتبني مغالطة مضادة للأكسدة هائلة مقارنة مع أشكال أخرى للفولدرات. وهذا يقتضي ضرورة أن يتم فحصها فيما إذا كانت الأشكال المختزلة مفضلة على الأشكال المؤكسدة المعطاة حالياً لفولدرات مضادة. إن الآلية الجزيئية لفرض الليوسيل المثار إليها تثبت بشكل مباشر أن المعالجة المضادة للأكسدة يجب تعديلها بحيث تتلائم مع فطر الضرر الحادث. إن فحص الليوسيل يجب فقط عند نمط نوعي مع الـ $ONOOH$ الحادث للضرر. وهذا بدوره يؤكد أنه عند تحديد مدى الفاعلية بالنسبة للضرر لاستخدام مضاد أكسدة ما، فإنه من اللاهية معرفة الآلية الجزيئية لهذا المركب.

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Curriculum Vitae

Bashir Mahmoud Rezk was born in 1972 on January 23, at El-Beheira, Damanhour, Egypt. In May 1995, he acquired his B.Sc. degree from the Faculty of Science, Cairo University. He received a scholarship for the Master program of the Academy of Scientific Research and Technology in the Pharmacology and Experimental Oncology Unit, National Cancer Insititute, Cancer Biology Department, Cairo University (1996-1999). He studied the effect of propionyl-L-carnitine on adriamycin-induced cardiotoxicity. This study was supervised by Assistant prof. dr. Mohamed M. Sayed-Ahmed, Prof. dr. Samia A. Shouman and prof. dr. Maher H. Khalifa. He successfully passed in the pre-master courses in physiology, Faculty of Science, Cairo University (October, 1997). In 1998, he was awarded the *Mawled Nabawy* Prize which he received from Mr President Mohamed Hosny Mubarak, President of the Arab Republic of Egypt. In 1999, he left his work as a journalist in the Egyptian Press after almost 2 years. In July 2000 he obtained his master degree in comparative physiology, Cairo University. From 2000 till 2001 he was responsible for the project of Experimental Therapy in the unit of Pharmacology and Experimental Oncology , National Cancer Insititute. In May 2001, he obtained a scholarship for a Ph.D. program in the field of human toxicology from the Foundation on Prevention and Treatment of Chronic Diseases, in the Department of Pharmacology and Toxicology, Faculty of Medicine, Maastricht University, The Netherlands. This work has resulted in the present Ph.D. thesis. The research described in the thesis was supervised by dr. Guido Haenen, prof. dr. Wim van der Vijgh and prof. dr. Aalt Bast.

المسيرة الذاتية

وُلِدَ لشفيق محمود زمره في الثالث والعشرين من شهر يناير لعام ٢١٩٧٢ بقرية هليل مركز دمنهور، البحيرة ، مصر . في مايو ٢١٩٩٥ حصل على درجة البكالوريوس في العلوم من كلية العلوم ، جامعة القاهرة . حصل على منحة أكاديمية البحث العلمي والتكنولوجيا لإكمال الماجستير لدراسة الماجستير بوحدة الفارماكولوجي والعلاج التجريبي للسرطان ، قسم بيولوجيا الأورام ، معهد الأورام جامعة القاهرة (١٩٩٦ - ٢١٩٩٩) . وقام بدراسة تأثير الهرمونات - ل - كارتينيم على التسمم القلبي لعقار الأدرينالين . وهذه الدراسة كانت بإشراف الدكتور محمد سعيد أحمد ، الأستاذة الدكتور - أ - عبد السميع سوامه والأستاذ الدكتور ماهر عصيم خليل . و اجتاز بنجاح المواد الدراسية للتمهيد ، الماجستير في الفسيولوجيا ، كلية العلوم ، جامعة القاهرة (أكتوبر ، ٢١٩٩٧) . في عام ٢١٩٩٨ حصل على جائزة المؤهل لدرجة الدكتوراه في استكمال قسم الرئيس محمد حسني مبارك رئيس جمهورية مصر العربية . في عام ٢١٩٩٩ ترك عمله كمحاضر بالجامعة المصرية بعد عامين تقريباً من العمل بها . في يوليو ٢٠٠٠ حصل على درجة الماجستير في الفسيولوجيا الحياتية ، جامعة القاهرة . من عام ٢٠٠٠ حتى عام ٢٠٠١ كان مسؤولاً عن مشروع العلاج التجريبي بوحدة الفارماكولوجي والعلاج التجريبي للسرطان ، معهد الأورام . في مايو ٢٠٠١ حصل على منحة للحصول على رسالة الدكتوراه في السموم من مؤسسة منغ وعلاج السموم الزمنية ببولندا . في قسم الفارماكولوجي والسموم ، كلية الطب ، جامعة ماستريخت ، وهذه الرسالة الحالية هي نتاج هذا العمل . البحث المنار اليه هذه الرسالة كان بإشراف الدكتور خيريه هاشم ، الأستاذ الدكتور وعيم فائز فايز والأستاذ الدكتور آلف ليست .