BHA and cancer risk : the role of prostaglandin H synthase in the carcinogenicity of butylated hydroxyanisole

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PROEFSCHRIFT

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Pauline Anne Elisabeth Louise Schilderman

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voor mijn ouders

Contents

Abbreviations

BHA nomenclature:

2(3)-BHA	2(3)-*tert*-butyl-4-hydroxyanisole
free-BHA	free (=unconjugated) BHA
total BHA	total (conjugated and unconjugated) BHA
4-BHA-OQ	4-tert-butyl-5-methoxy-1,2-benzoquinone
2(3)-BHA-OH	2(3)-*tert*-butyl-4,5-dihydroxyanisole
DBHA	3,5-di-*tert*-butyl-4-hydroxyanisole
free-TBHQ	free (=unconjugated) TBHQ
total TBHQ	total (conjugated and unconjugated) TBHQ
TBHQ	2-*tert*-butyl-1,4-hydroquinone
TBOQ	4-*tert*-butyl-5-methoxy-1,2-benzoquinone
TBQ	2-*tert*-butyl-1,4-paraquinone
TBSQ	*tert*-butyl-semiquinone

ADI	acceptable daily intake
BHT	3,5-di-*tert*-butyl-4-hydroxytoluene
BrdU	5-bromodeoxyuridine
CAS reg. No.	chemical abstracts service registry number
dG	2'-deoxyguanosine
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
DMSO	dimethylsulfoxide
DTPA	diethylenetriamine-pentaacetic acid
ECD	electrochemical detection
EDTA	ethylene diamine tetraacetic acid
EEC	Europian Economic Community
ESR	electron spin resonance spectroscopy
FAO	Food and Agriculture Organization
GRAS	Generally Recognized As Safe
GSH	gluthathione
GSSG	oxidized gluthatione
5-/11-/12-/15-HETE	5-/11-/12-/15-hydroxyeicosatetraenoic acid
HHT	12-hydroxy-5,8,10-heptadecatrinoic acid
9-/13-HODE	9-/13-hydroxyoctadecadienoic acid
HPLC	high-performance liquid chromatography
IARC	International Agency for Research on Cancer
JECFA	Joint Expert Committee on Food Additives
LI	labeling index
nd	not detectable
NOEL	No observed effect level

8-oxodG	7-hydro-8-oxo-2'-deoxyguanosine
$PGE_2(G_2/H_2)$	Prostaglandin E_2 (G_2/H_2)
PHA	phytohaemagglutinin;
PFC	pair-fed control
SCF	Scientific Committee for Food
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Tpot	potential doubling time
Ts	mean transit time through the S-phase of cells
SD	standard deviation
SOD	superoxide dismutase
ss	single-stranded
WHO	World Health Organization

Treatment of animals:

BA (AB):	BHA + acetylsalicylic acid
BE:	BHA + ethanol
BI:	BHA + indomethacin;
BF:	BHA + fibre
BW (CB/BC):	BHA only;
CA (AC):	acetylsalicylic acid controls
CI:	indomethacin controls;
CE:	ethanol controls
CF:	fibre controls
CW (CC):	controls

Chapter 1

Introduction

1.1 Food additives

An important goal for mankind has been and still is the attainment of a sufficient food supply to provide a healthy and productive life. Historically, the search for an adequate food supply has been the major expenditure of time and effort on both an individual and societal basis. Seasonal climatic conditions resulted in an abundance of food during the harvesting and hunting periods, but also in inadequate food supplies during the remaining period of the year, which limited the number of individuals that a particular territory could support. Therefore, techniques had to be developed to preserve the abundant food available at harvest time and the game collected during the hunting periods. Among the first substances added to food as preservatives were salt and smoke. Herbs and spices were used as flavouring agents, and natural colouring agents have been used for a long time to make food appear pleasant to the eye. The search for chemicals useful in increasing shelflife and palatability of preserved foods continues until today.
The industrial revolution in the latter half of the nineteenth century drew

large numbers of people to town, away from the country side where their food was grown. Concomitantly, the food industry expanded enormously. In addition, industrialized countries have undergone many lifestyle changes during the last several decades, such as the demand for "ready-to-eat" and snack foods, the interest in ethnic foods, the demand for a constant year-round supply of seasonal foods as well as the demand for stable and low food prices. All these demands increased the utilization of and need for food additives. Currently, there may be as many as 2800 substances used as food additives.

The increased use of various substances added for technological purposes, in combination with the public demand for an essentially risk-free food-supply, has intensified both the scientific and public debate over the safety of these additives. The development of toxicological methodology with increased sensitivity has further emphasized the concern on these substances. These factors have stressed the necessity for a scientific and reasonable approach to the regulation of these food additives. Decisions in this area have impact on both public health and economy.

The definition of food additive may be found in the legislation of the original US Food and Drug act of 1906, along with the Meat Inspection Act. The legislation has been expanded and amended several times. The current legal definition of food additive may be found in this legislation. The Federal Food, Drug and Cosmetic Act, as amended in October 1976, Sec. 201(s) states:

"The term "food additive" means any substance the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food (including any substance intended for use in producing, manufacturing, packing, processing, preparing, treating, packaging, transporting, or holding food; and including any source of radiation intended for any such use), if such substance is not generally recognized, among experts qualified by scientific training and experience to evaluate its safety, as having been adequately shown through scientific procedures or in the case of a substance used in food prior to January 1, 1958, through either scientific procedures or experience based on common use in food) to be safe under the conditions of its intended use; except that such term does not include: (1) a pesticide in or on a row agricultural commodity; or (2) a pesticide chemical to the extent that it is intended for use or is used in the production, or storage, or transportation of any raw agricultural commodity; or (3) a colour additive; or (4) any substance used in accordance with a sanction or approval granted prior to the enactment of this paragraph; or (5) a new animal drug."

The technical definition of food additives is generally broader than the legal definition and can be exemplified by the definition used by the Food Protection Committee of the Food and Nutrition Board of the National Academy of Science: " A substance or mixture of substances other than a basic food stuff which is present as a result of any aspect of production, processing, storage or packaging". Technically, food additives are divided into two major categories, based on the terminology used in the legal definition. Those substances that are

intentionally added to a food directly during production, etc., for a functional purpose are termed "direct or intentional food additives". The second category consists of the "indirect or non-intentional food additives" that are non-intentionally added to food, but result from either the environment of food production or processing and storage, such as a plasticizer that leaches from a package. The direct food additives are applied by the food industry for a variety of technical effects. The five main groups are: (1) Processing aids: these additives are intended to aid in the processing of foods during production and after purchase by the consumer and are exemplified by: anticaking agents, emulsifiers, various enzymes, flour-treating agents, etc.; (2) Texturing agents: which are provided in order to give specific foods a desirable consistency and texture: various enzymes, firming agents, stabilizers and thickener's, etc.; (3) Preservatives: these additives are used to decrease the rate of degradation of foods during packaging and storage: antioxidants, curing and pickling agents, antibacteriocides, etc. (4) Flavouring and appearance agents: these agents are applied either to enhance existing flavours or to add flavour to foods and to improve the appearance: flavour enhancers, flavouring agents, non-nutritive sweeteners etc.; (5) Nutritional supplements: these additives include the required nubtrients and are added either to replace those lost during processing or to supplement existing levels of nutrients; they may consist of varied analogs of macronutrients and micronutrients, including vitamins and trace minerals (1).

1.2 Lipid peroxidation

Lipid peroxidation has been broadly defined as "the oxidative deterioration of polyunsaturated lipids", i.e. lipids that contain more than two carbon-carbon double covalent bonds. Most foods contain some quantity of polyunsaturated fatty acids that can be oxidized through a variety of free radical mechanisms. The initiation of oxidation of lipids occurs under relatively mild conditions such as light, heat, traces of transition metals, the presence of oxygen, and/or by (metal-catalysed) decomposition of (hydro)-peroxides. The general process of lipid peroxidation can be envisioned as in the scheme below (2-6):

Initiation:	RH			(+ catalyst)	----->	R·	+	·H
	RH	+	O_2	(+ catalyst)	----->	R·	+	·OOH
Propagation:	R·	+	O_2		----->	ROO·		
	ROO·	+	RH		----->	ROOH	+	R·
Termination:	ROO·	+	ROO·		----->	ROOR	+	O_2
	ROO·	+	R·		----->	ROOR		
	R·	+	R·		----->	RR		

Initiation in a peroxidation sequence in a membrane or polyunsaturated fatty acid is due to the attack of any species that has sufficient reactivity to abstract a

hydrogen atom from a methylene group. This leaves behind an unpaired electron on the carbon. The carbon tends to be stabilized by molecular rearrangement to produce a conjugated diene, which subsequently easily reacts with an oxygen molecule to give a peroxy radical (ROO·). Peroxy radicals can abstract a hydrogen atom from another lipid molecule: this is called the propagation step of lipid peroxidation and so, once the process is initiated, it tends to continue. The peroxy radicals combines with the hydrogen atom that it abstracts, to give a lipid hydroperoxide (ROOH). The termination reaction is typified by the combination of two radicals to yield non-radical molecules. At physiological temperatures, lipid hydroperoxides are fairly stable molecules, but in the presence of transition-metal complexes, their decomposition is catalysed. Secondary products of lipid peroxidation resulting from the breakdown of lipid(hydro)peroxides include aldehydes, ketones, alcohols, esters and short-chain-hydrocarbons (malondialdehyde, pentane, etc), which impart unpleasant flavours and odours to rancid or reverted oils (2-6). The pattern of the products varies with the conditions of peroxidation, and aldehydes formed by peroxidizing fatty acids vary with the fatty acid undergoing peroxidation (5, 7). The decomposition of hydroperoxides formed from polyunsaturated fatty acids is likely to be the major cause of the cellular toxicity caused by lipid peroxidation. Cellular damage could arise in two ways. Fragmentation of the unsaturated fatty acid chains of phospholipid membrane components will cause disruption of plasma or subcellular membranes. In addition, or alternatively, cellular damage can be caused by decomposition of products of lipid peroxidation, which react at neutral pH with many biomolecules of the cells (7).

It is generally believed that peroxidation of unsaturated fatty acids is initiated by a free-radical mechanism (7). The radical involved is most commonly an oxygen free radical. The real nature of the 'active oxygen' species involved in lipid peroxidation is unknown, and whether there is one specific species or several is still an open question (7, 8). The hydroxyl radical, superoxide anion, singlet oxygen and the hydroperoxy radical have all been considered. In cells free radicals are generally produced by electron transfer reactions: these can be by non-enzymic autoxidation or by redox active enzymes (7, 9, 10). Superoxide anion, for example, is continually generated in all aerobic cells as an accidental by-product of cell metabolism, e.g., by electron leakage from the mitochondrial respiratory chain and other electron transfer centres. Spontaneous dismutation of superoxide anion or enzymic catalysis via superoxide dismutase, yields hydrogen peroxide, which can be a source of hydroxyl radicals in the presence of metal ions. The OH· radical is one of the most reactive free radical known; it is highly oxidizing and reacts with most organic molecules at near diffusion-controlled rates. If produced in a cellular environment it will react at or near its site of production. Superoxide anion, on the other hand, is much less reactive, is reductive in nature and probably does not cause any significant damage to macromolecules *per se*. It is also capable of diffusing away of its site of production. Radicals such as OH· and ROO· are reactive oxidizing species are capable of causing damage to lipids, proteins, carbohydrates and nucleic acids (9).

Under normal conditions, the deleterious effects of oxygen radicals are balanced by specific antioxidant defenses in tissues. However when the production of these toxic compounds increases or when the hosts oxidant defenses are impaired, excessive tissue injury results and physiological functions of cells and organs are altered (7, 9).

1.3 Consequences of lipid peroxidation

Lipid peroxidation in biological membranes is a very destructive process (4, 5, 9). The direct effects of lipid peroxidation on the cell membranes include changes in the biophysical properties of the membrane: fluidity is decreased due to loss of polyunsaturated fatty acids, electrical resistance decreases, protein mobility in the membrane is decreased and phospholipid exchange between halves of the bilayer is facilitated. Destruction of the membranes leads to the inactivation of membrane-bound enzymes and the loss of decompartmentalization that is essential in ordered cell metabolism. The indirect effects of lipid peroxidation can be attributed to the production of aldehydes (9).
The major constituents of biological membranes are lipid and protein, the amount of protein increasing with the number of functions the membrane performs. Lipid peroxidation can damage membrane proteins as well as the lipids. In most membranes, the propagation reactions of lipid peroxidation will not proceed very far before they meet a protein molecule, which can then be attacked and damaged. Aldehydes can react with -SH groups on proteins or attack amino groups on the protein molecule to form both cross links and also cross links between different protein molecules. Aggregation of membrane proteins have been observed. Enzymes that require $-NH_2$ or -SH groups for their activity are usually inhibited during lipid peroxidation. Products of lipid peroxidation with low molecular weight have been shown to inhibit protein synthesis. Several products of lipid peroxidation show prostaglandin like activity, probably due to the formation of cyclic endoperoxides. Peroxidation of liver or erythrocyte membranes is known to cause formation of high-molecular-weight protein aggregates within the membrane, probably due to radical processes. The surface receptor molecules that allow cells to respond to hormones can be inactivated during lipid peroxidation, as are enzymes such as glucose-6-phosphate or the Na^+K^+ATP-ase involved in maintenance of correct ion balances between cells. In general, the overall effects of lipid peroxidation are to decrease membrane fluidity, increase the leakiness of the membrane to substances that do not normally cross it (Ca^{2+} ions), and inactivate membrane-bound enzymes. Continued fragmentation of fatty acids side-chains to produce aldehydes and hydrocarbons such as pentane will eventually lead to complete loss of membrane integrity. For example, rupture of the membranes of lysosymes results in loss of hydrolytic enzymes in the rest of the cell which causes further damage. Lipid peroxidation of erythrocyte membranes causes them to lose their ability to change shape and squeeze through the smallest

capillaries, and it can eventually lead to haemolysis (4, 5).
The vitamin losses due to reaction with intermediate compounds involve vitamins A, C, D, E, K and folate. Vitamins A, C, E might also be considered antioxidants in the food system. Tocopherol is a potent electron donor and is easily degraded by active radicals into quinones and dimers. The quinones can react with protein residues and oxidized lipids (11, 12).
Lipid oxidation products react with proteins in several ways to cause cross-linking and/or chemical modification of side chains. The lipid peroxidation radicals can also serve as initiators of polymerization. Several studies observed substantial losses in amino acids when proteins were exposed to peroxidized lipids. Amino acids such as methionine, histidine, lysine and cystine, appear to be most vulnerable to damage (13). Lysine, par example, is lost typically by reaction with oxidation products such as aldehydes or ketones (14). Damage to protein quality may cause texture changes (e.g. foaming of egg whites)(6). Despite the modesty of modest losses in amino acid residues, there were significant losses in biological value and digestibility of the exposed proteins. This indicates that moderate levels of lipid oxidation can cause sufficient change to reduce the nutritional value of proteins in the system.
One of the most obvious impacts of lipid oxidation is flavour change. Taste of milk is particularly susceptible to oxidized flavours. Aldehydes formed as a result of lipid oxidation have flavour thresholds below 0.5 ppm in milk. In addition to their low thresholds lipid peroxidation products synergistically act at subthreshold concentrations. This indicates that even very small levels of lipid oxidation can dramatically affect the flavour of a product (6, 15).
Most food pigments, such as anthocyanins, carotenoids, chlorophyll or browning reaction products in baked goods, contain conjugated double bounds which can directly be oxidized or participate in free-radical chain reactions. Especially carotenoids are susceptible to oxidative changes leading to discolouration or bleaching. Carotenoids can be considered as natural antioxidants in food systems by being active radical scavengers (6, 16).
Oxidation of lipids initiates changes in the food system which influence various quality issues, such as nutritional quality, safety, colour, flavour and texture. Nutritional quality may be impaired by losses in essential fatty acids, essential amino acids and vitamins, while various degradation compounds associated with lipid oxidation may be acutely toxic or mutagenic or act as promotors (6, 15).

1.4. Antioxidant defenses

Fatty acid chain autoxidation may be terminated if the H atom required to form a hydroperoxide (see paragraph 1.2) is provided by a molecule other than a fatty acid. Compounds with this ability, the antioxidants, form stable structures and do not propagate the chain reaction (7).
Aerobic organisms have an array of antioxidant defenses to cope with the

threat of their self-generated and exogenous oxidative stress. Enzymatic defenses include gluthathione peroxidase, superoxide dismutase (SOD) and catalase. Non-enzymatic antioxidant defenses consist of ascorbate, urate, gluthathione (GSH) in the hydrophylic phase, and α–tocopherol and β–carotene in the lipid phase (10, 15, 17-20).

Gluthathione-peroxidase, a selenium-containing enzyme found in the cytosol and mitochondria of animal tissues, decomposes H_2O_2 to H_2O at the expense of reducing equivalents from GSH. Once GSH is oxidized to GSSG, GSSG is reduced by the action of the NADPH-dependent gluthathione reductase. Intracellular gluthathione is normally present in its reduced form. An increase in intracellular concentrations of hydrogen peroxide results in a drop of the GSH/GSSG ratio, which may serve to detect intracellular oxidative stress. Gluthathione-peroxidase appears to be the most important hydrogen peroxide detoxifying system at physiological conditions. The second enzymatic antioxidant defense is formed by superoxide dismutase. In mammalian cells mitochondrial Mn-SOD and a cytosolic Cu/Zn-SOD are found. SOD is capable of dismutating two molecules of $O_2^{-}\cdot$ to form hydrogen peroxide and oxygen about 10^4 times faster than spontaneous dismutation. The third enzymatic antioxidant defense is formed by catalase, a haeme-protein that decomposes hydrogen peroxide to O_2 and H_2O_2. Catalase is present in peroxisomes and several reports suggest that it could be also present in other cell compartments such as heart mitochondria. As a hydrogen peroxide-metabolizing system, catalase appeared to be more relevant at higher hydrogen peroxide concentrations, because of the absence of saturation kinetics for H_2O_2 (10, 15, 20).

α–Tocopherol is a key ·OH scavenger and a chain-breaking antioxidant in biological membranes. It reacts with lipid peroxy radicals or ·OH to form the long-living α-tocopherol radical, which can react with a second peroxyl radical to yield stable α-tocopherol-lipid adducts; it can react with a second α-tocopherol radical to yield a vitamin E dimer or it can be reduced by ascorbate which in turn is transformed into an ascorbyl radical. Ascorbate appears to help in maintaining normal α-tocopherol tissue levels. Ubiquinol-10 is as efficient as vitamin E in chain-breaking antioxidant reactions. Ubiquinol is present in different biomembranes but is especially concentrated in the inner mitochondrial membrane where it also functions as an electron carrier in the respiratory chain. The resulting ubisemiquinone cannot be reduced by ascorbate but it can be recycled by the electron transport chain. Uric acid, an endproduct of ureum metabolism in humans accumulates in extracellular compartments where it plays its antioxidant role. Uric acid is an inhibitor of xanthine oxidase activity. Circulating xanthine oxidase-derived active oxygen species can exert toxic effects by reactions with plasma components and with lining endothelium (15, 21).

The various defenses are complementary to each other because they metabolize or scavenge different radical species in different cellular compartments (5, 10, 15, 17-20).

Antioxidants may, based on their function be classified in two broad groups:

chain-breaking antioxidants and preventive and other secondary antioxidants which reduce the rate of chain initiation by a variety of mechanisms. In general, chain-breaking antioxidants break the autoxidation chain reaction by donating rapidly a hydrogen atom to a lipid radical to form a stable product and an antioxidant free radical, which is incapable of initiating or propagating the chain reaction. Preventive and secondary antioxidants retard autoxidation by mechanisms other than breaking the chain reaction. They function by a variety of mechanisms which include: -decomposition of peroxides or radicals (sulphur compounds, selenium, enzymic antioxidants, vitamins), -inactivation of catalytic metals (citric acid, phytic acid), -or synergism with other antioxidants (vitamin E, and ascorbic acid)(4, 8).

1.5 Prevention of lipid peroxidation by BHA

In order to prevent the oxidative damage of various food products, in addition to the occurring natural antioxidants different kinds of synthetic antioxidant compounds are applied in food industrial processes. Major chain breaking commercial antioxidants are phenolic compounds and include tocopherols, alkyl gallates, 2(3)-*tert*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) and 2-*tert*-butyl-(1,4)-hydroquinone (TBHQ). These substances are readily oxidized by means of atmospheric oxygen thus preventing the oxidative deterioration of food products (2, 3, 5). The most frequently used industrial antioxidants are BHT, BHA, ethoxyquin, propylgallate and a number of other compounds. These compounds, at least in small amounts, are therefore present in many commercial food products and thus constitute a fraction of human and animal diets (22). In order to be effective, antioxidants must be used in food material of good quality. They will not protect or mask fat or fatty food which has already deteriorated from abusive storage or which was prepared from damaged raw material (2, 3). Although animal fats respond well to BHA and BHT, these phenolics are almost ineffective with polyunsaturated vegetable oils. These oils however contain significant amounts of naturally occurring tocopherols and do not really require the addition of synthetic phenolics. Processors of animal fat may add an individual phenolic antioxidant to their product. More often however, they prefer combinations of two or more, usually in combination with the addition of citric acid as metal scavenger. Traces of metal salts (copper or iron) act as strong pro-oxidants. Since most fat contain some of these contaminants, it is necessary to complex the metals with citric acid or some other edible metal scavenger (2, 3, 5). Phenolic antioxidants (AH) interfere with the propagation step of the free radical chain reaction.

$$\begin{aligned} ROO\cdot + AH &\longrightarrow ROOH + A\cdot \\ RO\cdot + AH &\longrightarrow ROH + A\cdot \\ R\cdot + AH &\longrightarrow RH + A\cdot \end{aligned}$$

Among these reactions, scavenging of the carbon-centered radical R· is assumed to be less efficient than scavenging of the oxygen-centered radicals, especially of the peroxyl radical ROO·. By removal of ROO· the antioxidant has a chain-breaking action; this action is most powerful during the early induction period when peroxide accumulation proceeds slowly but will be less effective once considerable peroxide levels have been achieved. The antioxidants themselves are converted into resonance-stabilized phenoxyl radicals (A·), which is illustrated for 3-BHA in Figure 1-1. These phenoxyl radicals may undergo a variety of reactions. The radical can be further oxidized to yield a stable quinone, the parent quinone can be regenerated by reducing agents, dimerization can take place and the antioxidant radical can combine with lipid peroxyl radicals to yield various non-radical species (3, 23-25).

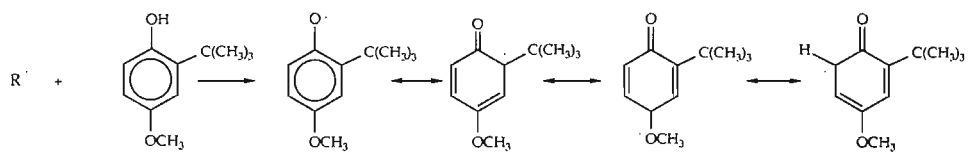
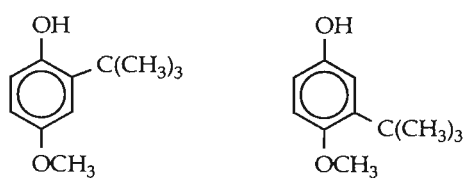


Figure 1-1: Resonance structures of the 3-BHA radical (the 2-BHA radical has comparable resonance structures).

1.6 General aspects of BHA

In this paragraph BHA is briefly reviewed with respect to chemical structure, nomenclature, physico-chemical data, use and regulatory status (2, 26-28).



3-BHA 2-BHA

Figure 1-2: Structures of 3-*tert*-butylhydroxyanisole (3-BHA) and 2-*tert*-butylhydroxyanisole (2-BHA).

* nomenclature:

Chemical Abstract Service Reg. No.	25013-16-5
names:	butylated hydroxyanisole
	(1,1-dimethylethyl)-4-methoxyphenol
	tert-butyl-4-methoxyphenol
	butyl hydroxyanisole
	tert-butyl-hydroxyanisole
	tert-butyl-para-hydroxyanisole
	tert-butyl-4-hydroxyanisole
	2(3)-*tert*-butyl-4-hydroxyanisole
	3(2)-*tert*-butyl-methoxyphenol
	E320
	and numerous trade names

BHA is a mixture of 2-*tert*-butyl-4-hydroxyanisole (<15%; CAS Reg. No.: 121-00-6) and 3-*tert*-butyl-4-hydroxyanisole (>85%; CAS Reg. No.: 88-32-4)

* Chemical and physical data:

molecular weight:	180.25 ($C_{11}H_{16}O_2$)
melting point:	48-55°C
boiling point:	264-270°C
state:	white or slightly yellow waxy solid
solubility:	practically insoluble in water
	soluble in many organic solvents

* Use: antioxidant in foods, cosmetics, food coating materials, waxes, vitamin A preparations, etc..

* Regulatory status: Generally Recognized As Safe (GRAS) status (US Food and Drug administration)(1984);
Temporary ADI of 30 mg BHA/adult in EEC countries (1978)(3)
Temporary ADI of 0-0.5 mg BHA/kg body weight set by WHO (1983)(29)
Permitted as a food additive in reportedly 50 countries (1984).

1.7 Toxicity of BHA

BHA does not seem to exert any overt toxicity in mammals. Apart from their carcinogenic and possible tumor promoting activity as well as modulation of toxicity of other chemicals discussed elsewhere in this introduction, the amounts consumed via food do not seem to have any adverse effect during chronic administration. However, high doses of BHA may increase the liver weight and lower the body weight of experimental animals.
Beagle dogs kept for six months on food containing up to 1% BHA showed no signs of toxicity, only dose-dependent retardation of growth and increased liver weights (30). In cynomolgus monkeys given up to 500 mg/kg body weight of BHA 5 times a week for 12 weeks, increased mitotic index of the oesophageal epithelium, increased liver size, but decreased hepatic monooxygenase activity was found (31). In pigs, fed a diet up to 400 mg/kg body weight BHA from mating until the 110th day of pregnancy, no effect on the reproduction was found nor an increase in defected fetuses. However, both the absolute and relative weights of liver and thyroid increased in a dose-dependent manner and the pigs on the highest dose of antioxidant showed lower weight gain increase (32). In weaning pups treated with BHA up to 250 mg/kg for 15 months, all organs examined appeared normal with the exception of liver injury in animals receiving the highest dose (33). However dogs, monkeys and pigs have a longer life span than rodents and therefore, in the mentioned studies potential carcinogenicity of BHA in animals without a forestomach has not been fully evaluated. In rodents, short-term administration of BHA resulted in increased relative liver weight and a decreased body weight gain, apart from increases in labeling indices in gastro-intestinal tract tissue and other histopathological changes as will be discussed in paragraphs 1.8 and 1.10.
Several reports observed increases in specific enzyme activities after short-term administration of BHA. Gluthathione-S-transferase was increased in rats (34, 35), mice (35-37) and hamsters (38), catechol-*O*-methyl-transferase in mice (38), epoxide hydrolase in rats (39), aniline hydroxylase in rats (39), UDP-glucuronosyl transferase in rats (40), mice (41) and murine liver (42), UDP-glucuronic acid in murine liver (42), NAD(P)H-quinone reductase in rats (43) and cytochrome p450 in mice and rats (44, 45). A full review on the biological and biochemical actions of BHA is however considered beyond the scope of this introduction. Numerous reviews have become available which cover this aspect sufficiently (3, 22, 26, 27, 29, 46-53).

1.8 BHA: carcinogenic and chemopreventive properties

Both the beneficial and adverse effects of BHA may depend upon a variety of factors. BHA may exert its action on the organism either via activation of detoxifying enzymes of the body, via interception of harmful free radicals, via inhibition of formation of ultimately carcinogenic metabolites and their

binding to DNA, and via modification of immune responses of the body. The mode of action of BHA is influenced by its own chemical structure, the strain of experimental animals, species, sex and age, the chemical structure of the carcinogen against which the antioxidant should protect the organism, the tissue upon which BHA is supposed to act, and the time of antioxidant administration in relation to the time of the carcinogenic insult (22, 54).

1.8.1 Modification of carcinogenesis by BHA

BHA suppressed the development of forestomach tumors in mice treated with benzo(a)pyrene or benzo(a)pyrene-7,8-dihydrodiol. The percentage of lymphomas in mice treated with benzo(a)pyrene-7,8-dihydrodiol and BHA was also significantly lowered (55). BHA reduced the incidence of mammary cancer in rats given 7,12-dimethyl-benz(a)anthracene (56). BHA inhibited the induction of hepatocellular neoplasms by aflatoxin B1 in rats (57). The incidence of colon cancer in mice induced by methylazoxy-methanol acetate was decreased by antioxidant treatment (58). The above and a number of other similarly conceived experiments proved the inhibitory effect of BHA upon the influence of certain pronounced carcinogens in certain tissues. BHA lowered in a dose-dependent manner the incidence of cancer as well as the number of tumors per animal. The mechanism of action is however not fully clear and may differ for different kind of carcinogens (22, 48, 54). The inhibitory effect of BHA on carcinogenesis may be related to their ability to prevent the *in vivo* activation of carcinogens to proximate or ultimate forms or to an action in increasing detoxification of the reactive intermediate, by inducing or decreasing relevant enzymes or by scavenging of active radical species (50).

1.8.2 Carcinogenicity of BHA

Next to its potential to modify chemical carcinogenesis, there has been provided sufficient evidence for the carcinogenicity of BHA in experimental animals (27). No antioxidant had been reported to be tumorigenic until 1983 when the synthetic antioxidant BHA appeared to be carcinogenic to rat forestomach epithelium (59).
Carcinogenic activity after prolonged exposure to doses of 1-2% in the diet resulted in squamous cell carcinomas in forestomach tissue of rats and hamsters (59-69). Time- and dose-dependent changes in the forestomach epithelium include hyperplasia, papillomas and carcinomas. Squamous cell carcinomas mainly appear in those regions where initially hyperplasia is most pronounced. Thus in the forestomach of rats treated with 2% BHA, tumors preferentially arise along the lesser curvature (101). These changes are accompanied by an increase in the labeling index (LI)(66, 67, 70-76). An increase in LI is therefore considered to represent an early event in the tumorigenic process in this organ. In rats, not only forestomach but also glandular stomach,

small intestine, colorectal tissues (77, 78) and the urinary bladder (79) are susceptible to the proliferation-enhancing effects of BHA. Moreover, adenomatous hyperplasia and adenomas were found in the lung of the Japanese house musk shrew (80). In animals with no forestomach, BHA administration by gavage also increased the mitotic index in the oesophagus of primates (81). BHA-feeding at subchronic levels induced proliferative and parakeratotic changes in the oesophagus of pigs (32, 82).
Several assays were used to test BHA for genotoxicity. For example, BHA was non-mutagenic in several tester strains in the Ames Salmonella/microsome test, with or without a liver S9-fraction (83-86). In the hepatocyte primary culture/DNA repair test, BHA did not induce DNA repair synthesis (83, 87). BHA was also non-mutagenic at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary cells in the presence of rat liver microsomes (83, 88, 89) and in Chinese hamster V79 cells in the presence of rat or hamster hepatocytes (90). Moreover, BHA did not induce sister chromatid exchange in Chinese hamster ovary cells (83, 90). In two studies on Drosophila no significant increase in sex-linked recessive lethal mutations was detected (91, 92). There is controversy concerning clastogenic effects of BHA. BHA was reported to be clastogenic to Chinese hamster ovary cells with metabolic activation (93) but no clastogenic effects were found in Chinese hamster CHO cells (94) or DON cells (95). Several studies revealed an absence of DNA-adducts in the stomach of exposed rats (96, 97). These negative results strongly indicate that BHA does not react with DNA. There is however evidence for protein binding (98, 99).

1.9 The importance of cellular proliferation induced by BHA

In view of its apparent non-mutagenicity BHA is considered to be a non-genotoxic carcinogen. Chemical carcinogens, known as non-genotoxic carcinogens, are defined as "those chemicals which fail either directly or indirectly to interact in a biologically significant manner with cellular DNA" (100-102).
Recently, 222 chemicals were tested for carcinogenicity in the U.S, 139 chemicals being recognized as carcinogens. The most interesting observation of this study was that while genotoxic carcinogens were able to exert their effects in most tissues, the non-genotoxic agents were apparently limited in their carcinogenic activity to 13 tissues out of the up to 40 tissues examined (100). Non-genotoxic carcinogens are therefore characterized by the induction of tumors at specific sites. The first criterion for significance in pre-tumor markers of a non-genotoxic carcinogen is therefore its presence in the part of the tissue from which tumors ultimately arise (100, 101, 103). Moreover, an excessive and often thresholded increase in cellular proliferation in the affected tissues appeared to be a consistent response to non-genotoxic carcinogens, which act either through cytotoxicity followed by regeneration or through some hormone-mimitic action (100, 101, 103, 104). This increased cellular proliferation may be mani-

fested either as a time-limited wave or it may be a more continuous stimulus as clearly demonstrated in the case of BHA (100, 105). Simple hyperplasia and papillomas induced by BHA appeared to be completely reversible after withdrawal of BHA, which indicates that simple hyperplasia and papillomas induced by the administration of BHA are not autonomously growing lesions but that continuous feeding of BHA is necessary for the increase in size and the progression to carcinoma (106). The importance of changes in the rate of cellular proliferation in tumor formation is confirmed in one other series of studies in which it was clearly demonstrated that dietary restriction or more specifically, restriction of the total calories in an animals diet inhibits cellular proliferation in each of the seven mouse tissues examined (107, 108). Moreover, dietary restriction inhibited the incidence of both naturally occurring and induced tumors in several organs of experimental animals (107, 108). Whether dietary restriction acts through caloric restriction, as it appears to do in the inhibition of carcinogenesis, or whether shortage of some other factor(s) is critical, remains to be solved. BHA fed 2% but not 0.5% in the diet led to papilloma or carcinoma of the forestomach in practically all rats treated for 2 years (59). The first observation of the short-term effect of 2% BHA on cellular proliferation revealed gross thickening of the squamous epithelium of the forestomach in the lesser curvature proximate to the glandular epithelium, which appeared to be due to an inflammatory hyperplastic lesion. Furthermore, the level of thymidine labelling in the lesser curvature was greatly increased as compared to the remainder of the forestomach epithelium (105). The results clearly showed that cells at and near the limiting ridge were considerably more sensitive to 2% BHA than cells in other parts of the forestomach epithelium. The greatly increased cellular replication occurred in the area of the forestomach from which the papillomas and carcinomas, as described by Ito et al (59), arose. This is in agreement with the first criterion for significance in pre-tumor marker of a non-genotoxic carcinogen, namely that it must be present in the part of the tissues from which tumors ultimately arise (59, 63, 64).

Since factors such as cellular proliferation seem to be crucial to tumor development in these cases, they may also prove to be valid in establishing thresholds or "no observed effect levels" (NOEL) for non-genotoxic carcinogens, since it implies that there may be dose levels to which test animals and possibly humans may be exposed without any adverse effect (100, 101, 109). Results gained at various sites in the rodent including the connective tissue, liver, bladder and forestomach show the existence of a threshold dose for various test materials/agents below which neither sustained tissue damage nor tumor induction occurs but above which level both effects are manifest (109). For BHA, a NOEL has been demonstrated at 0.25% in the diet (105, 110). The NOEL for BHA is particularly relevant since it fits well with the dose-effect curves of carcinomas and papillomas. Carcinomas and papillomas arise at dose-levels of 2% but not 0.5% in the diet (59).

Furthermore, lesion reversibility is a further possible criterion of an early non-genotoxic carcinogen marker, in contrast to those induced by genotoxic carcino-

gens which generally persist and develop into cancer (103). When animals, for example, treated with 2% BHA for 91 days were given a basal diet, the cell proliferation fell to control values in 7 days (110).
The strong similarity in the dose-response relationship for BHA in the induction of enhanced cell proliferation versus tumor formation, is demonstrated in a long-term carcinogenesis bioassay, a tumor promotion study, and a cell proliferation study in which cellular proliferation was measured by means of [^{3}H]-thymidine incorporation. All three studies were based on identical protocols and were performed in F344 rats. Both carcinomas and papillomas were induced in a range of experiments by 2% BHA but no carcinomas were found with 1% BHA and only hyperplastic lesions at 0.5% BHA. There appeared to be a close correspondence between the threshold doses for hyperplasia, increased thymidine labeling, and promoting effects in these studies: the sequence of effects are arranged according to dose in the following order: cell proliferation = promotion < papilloma < carcinoma (64, 111, 112). Established non-mutagenicity, lesion reversibility and the dependence of cell proliferation on the continuous presence of BHA in the diet, strongly indicate that BHA is a non-genotoxic carcinogen.
Forestomach neoplasia induced by the apparently non-genotoxic carcinogen BHA appears to arise as a result of sustained high levels of cellular proliferation (103). It must however not be assumed that increased cellular proliferation is the only significant mechanism in non-genotoxic carcinogenicity, nor that all non-genotoxic agents inducing proliferation in a particular tissue are necessarily going to prove to be carcinogens (100).

1.10 Histopathological characteristics of BHA-induced tumors

Histopathologically, lesions of the forestomach epithelium of rats and hamsters continuously fed BHA can be classified into hyperplasia, papilloma and carcinoma (59-69). In addition to these proliferative changes, infiltration of neutrophils, plasma cells, lymphocytes or eosinophils in the epithelial layer as well as in the submucosa, with or without necrosis or ulcer formation is usually observed in BHA-treated animals (105, 113). BHA induced both upward diffuse hyperplasia and downward basal cell hyperplasia (106, 114, 115). The response of the forestomach epithelial cells of rodents given BHA was very rapid. After only one week of exposure to 2% BHA in the diet of rats (113) or hamsters (72) epithelial damage, hyperkeratosis and hyperplasia occur and the labeling index increased within three days after treatment with BHA in hamsters (72). Although the initial proliferative response was very rapid, it takes a very long time before carcinomas develop (65). The BHA-induced rat or hamster squamous cell carcinomas were all of well-differentiated types. Squamous cell carcinomas possibly developed from fibro-epitheliomas, from papillomas or directly from the hyperplastic epithelium (50, 65). Cancer cells usually arose from the basal layer and invade downward into the muscular layer and further

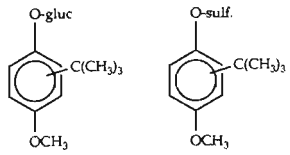
into adjacent tissues. If BHA-induced forestomach carcinogenesis was caused exclusively by irritation, it might be reversible at a particular early moment. Simple hyperplasia and papilloma in which the epithelium proliferates upward with and without basal cell proliferation appeared to be completely reversible (114). However, basal cell hyperplasia in which the epithelium proliferates downward, persisted long after cessation of BHA-administration (106, 114, 115). This indicates that BHA-induced papilloma may be caused by irritation and is reversible, but basal cell hyperplasia may have the potential to develop into carcinoma.

1.11 Possible mechanisms of action of BHA

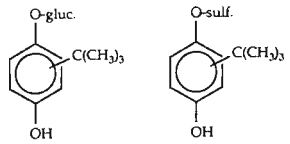
In contrast to the genotoxic carcinogens, whose reactive species interact with nucleic acids in their target organs with or without metabolic activation, the mechanism of action of the non-genotoxic carcinogen BHA is still unclear.

1.11.1 Tissue distribution and excretion of BHA

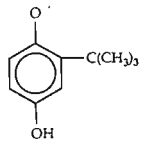
Most of the orally administered 3-BHA is absorbed and rapidly excreted in urine, faeces and expired air in rats (116-119), dogs (120, 121), humans (120, 122-124), and rabbits (125). There is however no evidence for accumulation of BHA in various tissues of the pig or pullet (126), rat (116), or dog (33, 127). Studies with radiocarbon-labelled BHA showed that essentially similar levels persisted in the forestomach, glandular stomach and oesophagus of rats, at various intervals after oral administration (117, 118). In the stomach of the dog, which does not have a forestomach, only a very small fraction of [methoxy-^{14}C]-labelled BHA remained 7 days after a single injection of labelled BHA (121). This indicates that tissue distribution of BHA may not be related to its carcinogenicity or toxicity in rat forestomach (118). The major metabolite appearing in the urine after administration of 3-BHA is the glucuronide, with a smaller amount of the TBHQ-sulphate, while unchanged 3-BHA and the 3-BHA-glucuronide were found in the faeces. The main metabolites of 2-BHA in urine were both the sulphate conjugates of 2-BHA and 4-*tert*-butyl-5-methoxy-1,2-benzoquinone (TBOQ) and the glucuronide of 2-BHA, while unchanged 2-BHA was found in the faeces (128). In dogs, 60% of orally administered BHA was excreted as unchanged BHA in the feces within 3 days, the remainder was found in the urine, largely as the sulphate-conjugates of BHA and TBHQ and some minor metabolites (121, 129). Relative high levels of BHA and relatively low amounts of unconjugated TBHQ in bile were reported in the rat, which indicates the possibility of enterohepatic circulation in this species (130).
In all species studied, minor metabolites of both 3-BHA and 2-BHA were identified in urine or feces.



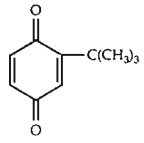
Conjugated-BHA



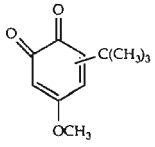
Conjugated-TBHQ



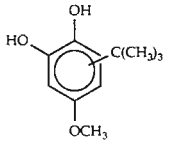
TBSQ



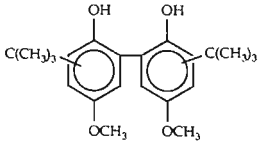
TBQ



BHA-OQ



BHA-OH



Di-BHA

(exact nature hitherto unidentified)

S-containing metabolites

Table 1-1: Metabolites of BHA identified *in vivo* and *in vitro*.

1.11.2 *In vitro* and *in vivo* metabolism of BHA

The principal metabolic pathway for BHA in all species studied is conjugation of the free hydroxyl group with both glucuronic acid and sulphate. In addition to these major biotransformation pathways, several other minor metabolites of BHA (Table 1-1) have been identified.
O-demethylation of BHA into TBHQ followed by conjugation with glucuronic acid (TBHQ-gluc) and sulphate (TBHQ-sulph) has been reported in several species, including man (118, 124, 128, 129, 131, 132). Minor metabolites in the urine or faeces of rats given 2-BHA or 3-BHA included *tert*-butylquinone (TBQ), TBHQ and its conjugates or 3(2)-*tert*-butyl-4,5-dihydroxyanisole (3(2)-BHA-OH)(133, 134). A very small amount of 3,3'(2,2')-dihydroxy-3,3'-di-*tert*-butyl-5,5'-dimethoxydiphenyl (di-3(2)-BHA) was detected in the faeces (128), in the plasma and intestine of rats (134). Other metabolites have been identified as *tert*-butylsemiquinone (TBSQ), 4-*tert*-butyl-5-methoxy-1,2-benzoquinone (4-BHA-OQ), di-BHA, and unknown sulphur-containing metabolites (133, 135).
These metabolites of oxidative metabolism have been found in *in vitro* systems. TBHQ, TBQ and BHA-OH are major metabolites of BHA in liver microsomes (98, 135, 136). Both 2-BHA and 3-BHA are easily metabolized to di-BHA by purified rat intestine peroxidase and horse radish peroxidase (137) and by rat liver microsomes (99, 135).

1.11.3 Prooxidant activity of BHA

In general, the ultimate reactive forms of carcinogens are electrophylic molecular species. BHA by itself however, is not an electrophile. It is possible that the carcinogenic effects of BHA are due to the conversion of the parent compound to more reactive metabolites (133). The major microsomal metabolites of BHA have been identified as TBHQ and BHA-OH both of which can oxidize into their corresponding quinones (135). In the chromosome aberration test, BHA appeared to be clastogenic to Chinese hamster ovary cells in the presence of a standard S9 mix or washed microsomes (93). The involvement of reactive oxygen species in the clastogenic effect of BHA was suggested by Cummings (1983)(136) and Rössing (1985)(138) who demonstrated that BHA increased the formation of hydrogen peroxide by microsomes.

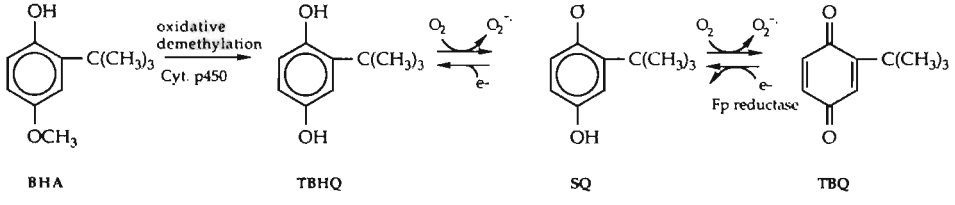


Figure 1-3: Oxidative demethylation of BHA by cytochrome p450 followed by the stepwise one-electron oxidation of TBHQ into its semiquinone radical and its corresponding quinone.

This effect appeared to be mediated by the interaction of the hydroquinone and quinone metabolites of BHA with NADPH-cytochrome c (p450) reductase.
Such an interaction occurs with many quinones which enter a redox cycle of NADPH-dependent one-electron reduction mediated by the reductase, followed by autoxidation of the semiquinone product, generating the quinone-metabolite and superoxide anion, as is shown in Figure 1-3.
Hydrogen peroxide is then formed by dismutation of superoxide and the quinone may again be reduced (136). The clastogenic effect of BHA in the presence of microsomes can therefore be ascribed to the quinone-metabolites of BHA which attack DNA via the formation of reactive oxygen species (93). In a two-stage transformation assay using BALB 3T3-cells the initiating activity of BHA and TBQ was tested; both BHA and TBQ showed initiating activity, the potency of TBQ was however 100 times higher than that of BHA (139). Since BALB/3T3-cells appear to be capable of metabolizing a variety of carcinogens, metabolites of BHA may act as initiators in this assay. TBQ might be one of the metabolites causing forestomach carcinogenesis in rodents given BHA, provided that TBQ is produced in the forestomach epithelium or its vicinity. Destaphney reported that *in vivo* TBQ is produced in the forestomach of rats given BHA (133). Saito (1989) reported that no DNA adducts were detected in the forestomach of rats given either a single or repeated oral administration of 3-BHA or TBQ (97). The initiating effect of BHA on transformation of BALB 3T3 cells might therefore be due to reactive oxygen species produced from active metabolites of BHA but not to covalent bonds between BHA-metabolites and DNA (139). Recently, it was shown that the semiquinone radical is formed from TBHQ and from TBQ in rat liver microsomes. In the presence of oxygen, the quinone and less extensively the hydroquinone induce excess production of superoxide in liver microsomes. It was concluded that autoxidation of the semiquinone formed from the quinone by microsomal enzyme activity is responsible for superoxide formation and that the hydroquinone enters the redoxcycling via the semiquinone radical. Moreover, the enzymic reduction of TBQ to yield TBSQ was dependent on the presence of NADPH and can be catalysed by a variety of flavoenzymes including NADPH-cytochrome p450-reductase (140, 141). TBSQ formation from quinones by microsomal enzymes in the absence of oxygen has been reported for a variety of quinones. The decay of TBSQ radicals at anaerobic conditions occurs slowly, while in the presence of oxygen the autoxidation of TBSQ readily takes place (140). It was concluded that with respect to superoxide formation TBQ is the ultimate reactive species derived in microsomes from BHA. Moreover, the induction of DNA single strand breaks in forestomach mucosa has been demonstrated using an alkaline elution assay; again, the DNA damaging capacity of TBQ appeared to be much stronger than that of TBHQ and BHA (142). Furthermore, it was demonstrated that BHA is capable of stimulating superoxide formation in liver microsomes from phenobarbital-pretreated rats. The hydroquinone metabolite is however much more active than BHA in stimulating superoxide production, whereas the paraquinone metabolite appeared to be the most potent activator. More-

over, the superoxide production of the hydroquinone in microsomes was accompanied by a high production of hydrogen peroxide and hydroxyl radicals (143).
In conclusion, the mechanism by which BHA exerts its carcinogenic action is still unknown; its carcinogenicity is thought to result from epigenetic effects. The results presented in this paragraph strongly suggest that the carcinogenic effects of BHA are due to the conversion of BHA to more reactive metabolites. The BHA-metabolites TBHQ and TBQ are relatively potent electrophiles, and can furthermore both generate active oxygen species as a result of redoxcycling via semiquinone radicals. Whereas the quinone-metabolite appeared to be the most potent activator of superoxide formation *in vitro*, the semiquinone formation and consequently superoxide formation appeared to be dependent on a flavoprotein catalysed or non-enzymatic reduction of TBSQ into TBQ. The hydroquinone-metabolite TBHQ can enter the redoxcycling after an one-electron oxidation into TBSQ. Hydroquinones however, tend to be relatively stable and are more easily conjugated than quinones. Therefore, the quinone-metabolite of BHA seems to be responsible for BHA-induced toxicity and carcinogenicity.

1.11.4 Effect of other chemicals on BHA-induced hyperplasia

Simultaneous administration of BHA and other chemicals can affect the hyperplastic changes induced by BHA.
Administration of BHA in combination with sodium L-ascorbate to male F344 rats, for instance, produced more severe hyperplastic changes in forestomach tissue than treatment with only BHA (144). Similarly, treatment with L-ascorbic acid enhanced BHA-induced forestomach epithelial proliferation (144). Recently, the enhancing effects of L-ascorbic acid on BHA forestomach carcinogenesis was confirmed in a long term feeding study. The enhancing effects of ascorbate may be attributed to its strong reducing capacity which can modulate the reduction of oxidized gluthathione to GSH, possibly resulting in elevated GSH levels (145). In the same study, the effects of BHA and other naturally occurring antioxidants, such as citric acid, benzoic acid and gallic acid, on the development of rat forestomach epithelial lesions were investigated. These three antioxidants appeared to have no effect on lesion development (145). Comparable results were found for other antioxidants such as butylated hydroxytoluene, ethoxyquin, propyl gallate and α-tocopherol (144); combined treatment with 1% GSH and 1% BHA for 1 week in rats pretreated with GSH for 1 week did not inhibit the resulting induction of forestomach hyperplasia by BHA (146). In contrast, the incidence of BHA-induced forestomach hyperplasia in rat was completely inhibited by concomitant treatment with 0.25% diethylmaleate, a well-known thiol depleter. This indicates that tissue gluthathione is directly involved in BHA-induced carcinogenesis (76). This was confirmed by DeStaphney, who reported that treatment with BHA resulted in a reduction in the amount of thiol groups (including GSH) in the forestomach epithelium (133).

Because during treatment with BHA an inflammatory reaction develops, the effects of anti-inflammatory agents on BHA-induced forestomach hyperplasia was examined in several studies. Indomethacin, 6-aminocaproic acid, dexamethazone and FOY305 were all simultaneously administered with BHA for 52 weeks, but none of them affected the incidence of BHA-induced forestomach hyperplasia (50, 76). However, treatment with acetylsalicylic acid, an inhibitor of prostaglandin H synthase, significantly inhibited proliferation in the forestomach epithelium of rats caused by 2% BHA (147).
If the metabolic activation of BHA is responsible for the induction of forestomach lesions, administration of drug metabolizing enzyme inducers might be expected to alter the process, depending on which enzymatic system is involved. The incidence of forestomach hyperplasia in rats treated with BHA and polychlorinated biphenyl was only slightly decreased and might have been due to decreased food intake (76). Similarly, phenobarbital had no effect on BHA-induced forestomach hyperplasia (76) or enhancement of cell proliferation (148). However, the oxidative demethylation of BHA into TBHQ is elevated after cytochrome p450 induction by phenobarbital.
These results indicate that it is unlikely that the proliferation-enhancing effects of BHA can be attributed to metabolic conversion of BHA into TBHQ by cytochrome-p450. Prostaglandin H synthase however might be involved in the carcinogenic effect of BHA, since inhibition of this enzyme resulted in a significant reduction of the proliferative effect of BHA in rat forestomach. A direct involvement of tissue gluthathione in BHA-induced forestomach carcinogenesis is also possible, since the reduction in the amount of thiol groups (including GSH) within forestomach epithelium might result in a decrease in the detoxification of BHA, thereby increasing the susceptibility of the target-tissue to interaction with carcinogens.

1.12 Aim of the thesis

Coadministration of acetylsalicylic acid, an inhibitor of prostaglandin H synthase, has been shown to result in a significant reduction of the proliferative effect of BHA in rat forestomach (147). Prostaglandin H synthase, an enzymatic system composed of both cyclooxygenase and peroxidase enzymes, occurs in many mammalian cells and is present at high levels in platelets, lungs, kidney and urinary bladder (149). The enzyme is membrane-bound and appears to be localized in the endoplasmatic reticulum and nuclear membranes (150). Prostaglandin H synthase is involved in the biosynthesis of prostaglandins, thromboxane and prostacycline, which occurs in two steps. The first step in the biosynthesis of these compounds is the oxygenation of a polyunsaturated fatty acid (e.g. arachidonic acid) into a hydroperoxy endoperoxide named prostaglandin G_2 (PGG_2) This reaction is catalysed by a haeme-containing oxygenase, called cyclooxygenase, that requires no external sources of electrons (150-153). The hydroperoxy group of PGG_2 is reduced to a hydroxy

group (prostaglandin H_2: PGH_2) by the peroxidase activity of prostaglandin H synthase that utilizes a wide variety of compounds to provide the required pair of electrons (150-152). The fate of PGH_2 is determined by a number of enzymes with relative activities varying from tissue to tissue (151). For example, thromboxane is the major product formed in platelets, whereas prostacycline predominates in aortic endothelial cells. Regardless of the fate of PGH_2, any tissue that synthesizes prostaglandins or thromboxanes contains prostaglandin H synthase for the formation of PGH_2. As a result, virtually all mammalian tissues display some prostaglandin H synthase activity (150, 153, 154). The substrate specificity for the cyclooxygenase reaction catalysed by prostaglandin H synthase allows only a small group of polyunsaturated fatty acids to be oxygenated, with arachidonic acid serving as the preferred substrate *in vivo* (154). In contrast prostaglandin H synthase peroxidase, like most peroxidases, is a promiscuous enzyme. Although PGG_2 is the primary hydroperoxide substrate, the peroxidase cycles equally well with simple lipid hydroperoxides, organic hydroperoxides, and even with hydrogen peroxide (154). Inhibition by acetylsalicylic acid or indomethacin of the synthesis of PGG_2 abolishes the ability of prostaglandin H synthase to cooxidize xenobiotics by the peroxidase moiety of prostaglandin H synthase (150). Cooxidations that occur are hydroperoxide dependent oxidation catalysed by the peroxidase that utilizes PGG_2 as a hydroperoxide substrate (150). It has been postulated that prostaglandin H synthase serves as an alternate enzyme for xenobiotic metabolism, particularly in tissues or cells with low monooxygenase activity. Consequently, the cooxidation of xenobiotics during prostaglandin biosynthesis might be a relevant alternate pathway in chemical carcinogenesis (150).

Free arachidonic acid or the products of its metabolism, commonly referred to as eicosanoids, are normally not found intra- or extracellularly in the absence of chemical, physical or hormonal stimulation (155). Arachidonic acid is usually bound to glycerol backbone of phospholipids. As such it is not a substrate for the metabolizing enzymes. The release of arachidonate is catalysed by phospholipase A_2 or diacylglycerol lipase following phospholipase C action (155). Several studies have shown that exposure of rats to strong irritants such as absolute ethanol, boiling water, 0.6M sodium hydroxide resulted in an enhancement of prostaglandin production (156). The increased synthesis of prostaglandins may represent a physiological, natural defense mechanism that may be necessary to maintain cellular integrity of the gastro-intestinal mucosa (156). Products of prostaglandin H synthase-mediated metabolism appeared to be involved in inflammatory processes, wound repair and proliferative skin diseases (157). Prostaglandins of the E-type have often been associated with the regulation of cell proliferation. *In vitro*, E-type prostaglandins have been found to modulate human keratinocyte proliferation (157). Others have reported that addition of arachidonic acid metabolites to various cell cultures stimulates cell proliferation (158). In mouse skin *in vivo*, PGEs turned out to be mediators of the hyperplastic transformation in chemically induced two-step carcinogenesis (159). Many malignant tumors tend to have a high rate of cell turnover and

produce large amounts of prostaglandins (158). However, it has also been reported that endogenously produced PGEs can inhibit cell proliferation in rat colon by enhancement of cAMP levels (158, 160). Several studies have shown that coadministration of acetylsalicylic acid or indomethacin, two inhibitors of prostaglandin H synthase activity, with certain tumor promotors or chemical carcinogens resulted in an inhibition of tumor promotion and tumor development, respectively (161-163). For example, simultaneous treatment of indomethacin and the tumor promotor 12-*O*-tetradecanoylphorbol-13-acetate (TPA) resulted in a significant decrease in PGE_2 release, and concomitantly in a reduction of TPA-induced epidermal DNA synthesis in mouse skin (157). Moreover, among the pleiotropic effects of TPA and other phorbol esters, stimulation of arachidonic acid release and its metabolism, and oxygen radical production are common features which can be negatively influenced by either inhibitors of phospholipase A_2, cyclooxygenase and lipoxygenase pathways in the arachidonic acid cascade or by antioxidants such as SOD (164). In case of phenobarbital, a well known tumor promotor in rodents, phenobarbital treatment is also associated with an increase in prostaglandin E_2 and F_2 in rat liver *in vivo* (164). Arachidonic acid release and oxygen radical production appeared to be interrelated (164). Other studies have shown that prostaglandins can alter the proliferative activity of normal epithelium (160).

BHA has strong irritating properties. Moreover, inhibition of prostaglandin H synthase has been shown to result in a significant reduction of the proliferative effect of BHA (147). Metabolites of arachidonic acid may therefore be involved in the mechanism of BHA-induced enhancement of cell proliferation. BHA may cause an enhanced phospholipid turnover, especially a release of arachidonic acid catalysed by phospholipase A_2. An increase in arachidonic acid metabolism could ultimately result in a disturbance of gastro-intestinal tissue homeostasis i.e. the steady state between cell gain and cell loss, possibly resulting in hyperplasia. This hyperplastic transformation may be characterized by inflammation, stimulation of the arachidonic acid-cascade, desensitization of antiproliferative signals such as catecholamines, induction of ornithine decarboxylase, and interruption of intercellular communication (159, 165). The inflammatory reaction may be accompanied by enhanced amounts of reactieve oxygen intermediates produced by polymorphonuclear cells (166). Furthermore, arachidonic acid itself has been shown to stimulate superoxide anion radical production *in vitro* in inflammatory cells (167). This suggests that arachidonic acid itself or metabolites formed as a result of BHA treatment might also be involved in the process of BHA-induced carcinogenesis through enhanced production of oxygen radicals. In addition, BHA is a stimulator of the formation of reactive oxygen species (93, 136, 138-141, 143). We therefore hypothesize that products of prostaglandin H synthase-mediated metabolism, in particular the production of prostaglandin E_2, might be involved in the mechanism of BHA-induced enhancement of cell proliferation, as is shown in Figure 1-4.

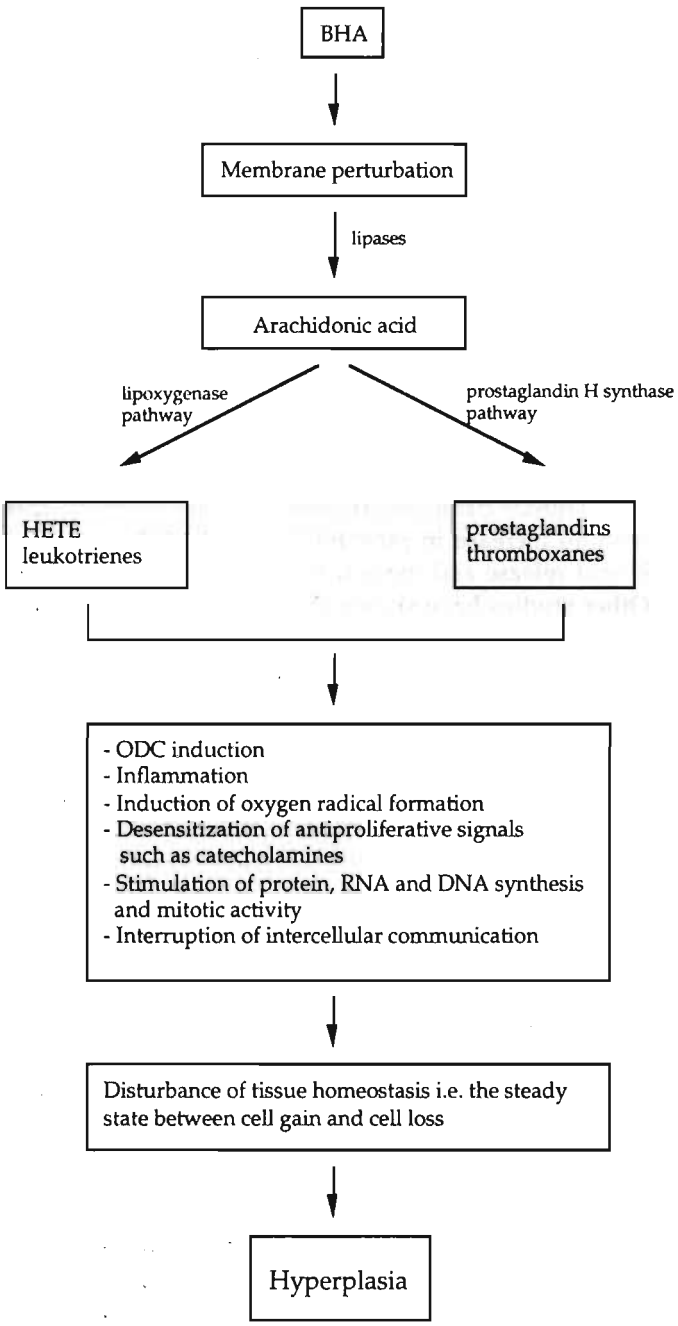


Figure 1-4 : Proposed mechanism of action of BHA

1.13 Outline of the thesis

In this thesis several experiments are described intended to unravel the unknown mechanism of the carcinogenicity of BHA.
In Chapter 2 the effect of oral administration of BHA on arachidonic acid and linoleic acid metabolism in correlation with changes in gastro-intestinal cell kinetics were determined. Proliferative effects of BHA were regarded as markers of the carcinogenic potential of BHA and were determined by assessing the labeling index and other cell kinetic parameters by flow cytometry and immunocytochemistry following two weeks of administration of 1.5% BHA in the diet of male Wistar rats. The involvement of metabolites of arachidonic acid in the mechanism of the proliferative responses of BHA in rat forestomach, glandular stomach and colon/rectum were investigated by coadministration of two inhibitors of prostaglandin H synthase: acetylsalicylic acid and indomethacin.
In Chapter 3 studies were performed in order to determine whether prostaglandin H synthase is involved in the metabolic activation of BHA by converting the cytochrome p450-generated hydroquinone to the corresponding quinone. Therefore, the metabolism of BHA, in particular its prostaglandin H synthase-mediated oxidation of TBHQ into TBQ, with and without inhibition of prostaglandin H synthase, was monitored in rats fed 1.5% BHA in the diet for two weeks. The ability of the hydroquinone to induced the formation of oxygen radicals in presence and absence of both lipoxygenase and prostaglandin H synthase was tested by means of electron spin resonance analysis. The peroxidative transformation of TBHQ by lipoxygenase and prostaglandin H synthase was also assayed spectrophotometrically and quantitated by HPLC.
In Chapter 4 we determined the role of prostaglandin H synthase-mediated metabolism in the induction of oxidative DNA damage by BHA metabolites. Two test systems were used to determine the ability of BHA, TBHQ and TBQ to induce oxidative DNA damage namely: biological inactivation of single-stranded bacteriophage ϕX-174 DNA and the induction of 7-hydro-8-oxo-2'-deoxyguanosine in deoxyguanosine *in vitro* in presence and absence of peroxidases.
In Chapter 5 we determined the metabolic activation of BHA by prostaglandin H synthase on the nature and time-dependency of early lesions in rat forestomach, glandular stomach and colon/rectum in combination with 8-oxodeoxyguanosine formation in the epithelial cells of glandular stomach and colon/rectum as well as in the liver
In Chapter 6 we investigated the carcinogenic potential of BHA and its primary metabolites TBHQ and TBQ to induce oxidative DNA damage and cell proliferation in human lymphocytes, cultured *in vitro*.
In Chapter 7 the possible interaction of BHA with other food constituents, namely ethanol and dietary fibre, on gastro-intestinal cell proliferation and other cell kinetics was studied in rats fed 1.5 % BHA in the diet and/or 10 % ethanol in the drinking water and/or diet supplemented with 20% dietary fibre.

In Chapter 8 the outcome of the studies is discussed and suggestions for further research are given.

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Chapter 2

Effects of butylated hydroxyanisole on arachidonic acid and linoleic acid metabolism in relation to gastro-intestinal cell proliferation in the rat.

P.A.E.L. Schilderman, W. Engels[1], J.J.M. Wenders, B. Schutte[2], F. Ten Hoor[3] and J.C.S. Kleinjans.

Departments of Health Risk Analysis and Toxicology, [2]Internal Medicine, and [3]Human Biology, [1]Cardiovascular Research Institute, University of Limburg, P.O.Box 616, 6200 MD Maastricht, The Netherlands.

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Abstract

In order to determine the effect of oral administration of 2(3)-*tert*-butyl-4-hydroxyanisole (BHA; dose-level: 1.5% BHA of the diet) on arachidonic acid and linoleic acid metabolism in correlation with changes in gastro-intestinal cell kinetics, we coadministered two inhibitors of prostaglandin H synthase, acetylsalicylic acid and indomethacin, to rats.
Co-administration of acetylsalicylic acid acid (0.2%) and indomethacin (0.002%) in the drinking water, resulted in a significant reduction of the BHA-induced enhancement of cell proliferation in forestomach and glandular stomach. Acetylsalicylic acid completely counteracted the effect of BHA on labeling indices in colon/rectum whereas indomethacin exhibited no effect in this organ. Both inhibitors had no direct effect on cell kinetics in the control groups. Acetylsalicylic acid, and to a lesser degree indomethacin, inhibited PGE_2-release in all tissues examined. Whereas acetylsalicylic acid did inhibit lipoxygenase-mediated metabolism of arachidonic acid in forestomach tissue, acetylsalicylic acid did not affect the release of arachidonic acid- and linoleic acid-derived hydroxy fatty acids in glandular stomach and colon/rectum. Indomethacin did not affect lipoxygenase production. BHA, however appeared to be a strong inhibitor of both routes of arachidonic acid metabolism. While acetylsalicylic acid nor indomethacin affected linoleic acid metabolism, BHA inhibited both prostaglandin H synthase-mediated and lipoxygenase-mediated metabolism of arachidonic acid and linoleic acid. A causal role of arachidonic acid or linoleic acid metabolites in the process of cell proliferation enhancement induced by BHA, can therefore be excluded. Prostaglandin H synthase may however be involved in BHA activation by converting the hydroquinone metabolite of BHA

to the corresponding quinone by redoxcycling, which is probably accompanied by reactive intermediates production.

Introduction

The synthetic phenolic antioxidant butylated hydroxyanisole (BHA) is widely used as a food preservative. It has been included at low concentrations in the human diet for many years, without evidence of adverse effects (1). This antioxidant is however carcinogenic to F344 rat and Syrian Golden hamster forestomach epithelium (2, 3). After prolonged exposure to doses of 1 à 2% in the diet, squamous cell carcinomas appear mainly in those regions where hyperplasia initially is most pronounced. Thus, in the forestomach of rats treated with 2% BHA, tumors preferentially arise along the lesser curvature (4). This is generally accompanied by an increase in forestomach labeling index (LI). An increase in LI is therefore considered to represent an early event in the tumorigenic process in this organ. In rodents however, also other tissues appear to be target organs for cell growth-enhancing effects of BHA (5-7). In non-rodents, BHA administration by gavage also increased the mitotic index at the distal part of the oesophagus of primates by 40% (8). Furthermore, BHA-feeding at subchronic levels induced proliferative and parakeratotic changes in the oesophagus of pigs (9). The mechanism by which BHA exerts its carcinogenic action, is unknown; its carcinogenicity is thought to result from epigenetic effects (10). Although BHA has been tested for DNA-interactivity in various *in vitro* systems, it does not show genotoxic activity in most assays (11-15), except for the induction of chromosomal aberrations in cultured Chinese hamster ovary cells (16). However, BHA appears to have tumor initiating activity on mouse skin *in vivo* (17).

The fact that the dose of 2% BHA in the diet of rats and hamsters which caused carcinomas, is far above the estimated daily intake by man (18), does not necessarily exclude the possibility that BHA may play a role in the development of neoplasia in man. This is supported by data which indicate that BHA is a modulator of chemically induced mutagenesis and carcinogenesis (12, 19-25). Elucidation of the mechanism of action of BHA is therefore relevant for risk assessment in man.

Co-administration of acetylsalicylic acid, an inhibitor of prostaglandin H synthase, has been shown to result in a significant reduction of the proliferative effect of the phenolic antioxidant in rat forestomach (26). Metabolites of arachidonic acid may therefore be involved in the mechanism of BHA-induced enhancement of cell proliferation in rat gastro-intestinal tract tissues. Correspondingly, BHA may cause an enhanced phospholipid turnover, especially a release of arachidonic acid catalyzed by phospholipase A_2. An enhancement of the arachidonic acid metabolism could ultimately result in a disturbance of gastro-intestinal tissue homeostasis i.e. the steady state between cell gain and cell loss, possibly resulting in hyperplasia. This hyperplastic transformation

may be characterized by inflammation, stimulation of the arachidonic acid-cascade, induction of ornithine decarboxylase, desensitization for antiproliferative signals such as catecholamines, and interruption of intercellular communication (27, 28). An inflammatory response may be accompanied by enhanced amounts of reactive oxygen intermediates produced by polymorphonuclear cells (29). In addition, arachidonic acid has been shown to be a stimulator of superoxide anion radical production *in vitro* in inflammatory cells (30). This suggests that arachidonic acid itself or metabolites of arachidonic acid formed as a result of BHA treatment might also be involved in the process of BHA-induced carcinogenesis through enhanced production of oxygen radicals.
We therefore hypothesized that BHA increases arachidonic acid release and metabolism, in particular the production of PGE_2, which may ultimately result in an increase in cell proliferation. Arachidonic acid, but also linoleic acid, is a substrate for both the prostaglandin H synthase and lipoxygenase pathway. Inhibition of the prostaglandin H synthase-mediated pathway could therefore result in an increased significance of the lipoxygenase pathway. Earlier reports indicate that lipoxygenase products may play a role in inflammation (28, 31-34). In the present study, we therefore determined the influence of BHA on both arachidonic acid and linoleic acid metabolism in gastro-intestinal tract tissues in comparison with effects on cell kinetics in these target organs.
We also evaluated effects of two inhibitors of prostaglandin synthase: acetylsalicylic acid and indomethacin. We determined the following prostaglandin H synthase-mediated metabolites of arachidonic acid: PGE_2, 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 11-hydroxyeicosatetraenoic acid (11-HETE), and of lipoxygenase-mediated metabolism: 5-, 12-, and 15-hydroxyeicosatetraenoic acid (5-HETE, 12-HETE and 15-HETE). From the possible range of linoleic acid metabolites we determined 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE), since the formation of 9-HODE is clearly dependent on prostaglandin H synthase activity, while lipoxygenases are responsible for the formation of 13-HODE (35-38).

Materials and Methods

Materials

BHA (food grade BHA, purity >99%, 97.5% 3-BHA) was obtained from J. Dekker Co., (Wormerveer, The Netherlands). PGE_2 was purchased from Cayman (Ann Arbor, MI, USA) and 16,16-dimethyl PGE_2 was obtained from Sigma (St Louis, USA). Hexane, chloroform, acetonitrile and methanol were HPLC grade and were obtained from Rathburn (Walkerburn, U.K.), 5-bromodeoxyuridine (BrdU) from Serva (Heidelberg, Germany), the monoclonal antibodies rabbit anti-mouse IgG-FITC, peroxidase-conjugated rabbit anti mouse IgG (F313, resp. F114) from Dakopatts, (Denmark) and propidium iodide from Calbiochem. (Behring diagnostics, San Diego, CA, USA). Tetrahydrofuran was purchased from FSA lab. suppl. (Loughboruogh, U.K.) and diethylether from BDH (Poole,

England). KOH (suprapur) and silicagel 60 (70-230 mesh) were purchased from Merck (Darmstadt, FRG). Water was purified by means of a milli-Q water purification system. Analytical reagent-grade chemicals were used in all other instances.

Animals and maintenance
A total of 36 male, 5 week old Wistar rats (Winkelmann, Borchen, Germany) (weighing 97 ± 6 g; Mean ± SD) were used. They were housed individually in metabolic cages in an air-conditioned room, maintained at 21-22°C and 50-55% relative humidity with a 12-hour light-dark cycle. The animals were randomly allocated to 6 groups comprising 6 animals each. During 7 days of acclimatization the animals had free access to powdered standard lab chow (diet no. SRM-A; Hope Farms, Woerden, The Netherlands) and water.

Test procedure
At 6 weeks of age, rats were given an experimental diet consisting of powdered laboratory chow supplemented with 0 (controls) or 1.5% BHA, during a period of 14 days. The dietary level of 1.5% BHA was chosen on the basis of previous experiments indicating a submaximal response of forestomach LI at this food concentration level (39). Rats fed a diet supplemented with BHA, reduce their food consumption immediately, due to nonpalatability of the food. Since a reduced dietary intake may interfere with BHA-induced alterations in cell kinetic parameters, we used pair-fed control groups (PFC). PFC groups were given a control diet restricted to the mean daily food intake (g/day) of rats in the corresponding groups (39). The BHA-fed rats had free access to the food.
One PFC and one 1.5% BHA received 0.2% acetylsalicylic acid in the drinking water (26, 40). These two groups of rats were encoded CA and BA, respectively. Two similarly fed groups received drinking water supplemented with 0.002% indomethacin (41)(CI and BI, respectively). Another PFC group and a 1.5% BHA fed received drinking water only (CW and BW, respectively). Both acetylsalicylic acid and indomethacin were added to the drinking water, together with ethanol (for dissolving indomethacin) and sucrose carrier (5 g/l). The final concentration of ethanol in the drinking water was 1%. The vehicle-treated control rats received drinking water with ethanol and sucrose carrier only. Drinking water was available to all rats *ad libitum*. Fresh drinking water was provided at three-day intervals. Water and food intake were recorded daily in order to calculate the average daily intake of BHA, acetylsalicylic acid, resp. indomethacin. Body weights were determined three times per week.

Assessment of cell kinetic parameters in gastro-intestinal tract tissues.
On the 14^{th} day of the experimental diet, all rats were injected ip with 1 ml of 7.5 mg BrdU/ml in phosphate-buffered saline (pH 7.4). After 4 hours, the animals were sacrificed under ether anaesthesia by exsanguination via the aorta. In order to avoid inter-group differences in the period of last food consumption, food was not withdrawn before killing. The gastro-intestinal

tract tissues (forestomach, glandular stomach and colon/rectum) were dissected, cut into tiny pieces, partly fixed in ethanol for determination of cell kinetic parameters and partly stored at -80°C for determination of prostaglandin, respectively hydroxy fatty acid contents. Preparation of the forestomach and glandular stomach for analysis of cell kinetic parameters by flow cytometry was carried out as described previously (39, 42). In short: four samples per tissue per rat were taken at random, cut into smaller pieces, treated with pepsin and subsequently incubated with HCl for DNA denaturation.The released nuclei were incubated with anti-BrdU-antibody (clone II B 5) and additionally with fluorescein-conjugated rabbit anti-mouse IgG-FITC. Total DNA was stained with propidium iodide. Double stained cells were analyzed on a FACS IV cell sorter (Becton and Dickinson, CA, USA). The amount of bound anti-BrdU-antibody was measured as log green fluorescence, whereas the red fluorescence was measured and recorded as the amount of bound propidium iodide. The LI is expressed as the percentage of BrdU-positive cells. The LI detection limit is about 0.5%. Besides the LI, the mean transit time (Ts) and potential doubling time (Tpot) were determined. Cell kinetic parameters were calculated as described by Begg et al. (1985)(43).
Because the LI in colon/rectum cannot be analyzed adequately by flow cytometry, randomized samples from colorectal tissue were embedded in paraffin for microscopic evaluation as described previously (7). In short: tissue sections were mounted on glass slides. For detection of BrdU-containing cells in these sections, an indirect enzyme-labeled antibody technique was applied using anti-BrdU-antibody (clone II B 5) and peroxidase-conjugated rabbit antimouse IgG. The peroxidase-containing antibody sites were visualized by means of diaminobenzidine. The sections were counterstained with haematoxiline. Slides were encoded and epithelial cells were scored by light microscopy for immunoreactivity. Slides were scored by two independent observers, the inter-observer variation was 9 $\pm$ 7% (mean $\pm$ SD), which is in agreement with earlier results (7). For each animal, 20 crypts were counted. Discrimination between the proliferating and non-proliferating compartment was obtained by making cross sectional slides of the crypts. The LI is expressed as the percentage of positively BrdU-stained cells. The number of cells per crypt cross section were also counted in order to exclude false changes in the overall labeling of the total crypt cell population possibly due to effects on the compartimental sizes.

Extraction of prostaglandins and hydroxy fatty acids from gastro-intestinal tract tissues

Extractions prior to HPLC analysis of prostaglandin and hydroxy fatty acid production were carried out according to methods of Kivits and Nugteren, with minor modifications (44). In short: two samples per tissue were cut into small pieces, suspended in HBSS and after ultrasonic treatment incubated for 45 min at 37 °C. Samples were acidified with acetic acid to pH 4-5 and 100 ng 16,16-dimethyl PGE_2 and 107 ng C20-2 15-OH (a generous gift of dr. W.Engels, Cardiovascular Research Institute, University of Limburg, Maastricht, The

Netherlands) were added as internal standards. Samples were extracted twice with methanol/chloroform (2:5; v/v) and the combined extracts were dried under nitrogen. The residue was dissolved in diethylether/acetic acid (100:0.5; v/v) and fractionated by silica gel chromatography. The hydroxy fatty acid fraction was eluted with diethyl ether/hexane/acetic acid (60: 40:0.5; v/v/v). The prostaglandin fraction was subsequently eluted with diethylether/ methanol/ acetic acid (90:10:0.5; v/v/v). The hydroxy fatty acid fractions were evaporated, dissolved in 100 µl mobile phase and analyzed by reversed phase HPLC. Recovery of fatty acids after this purification procedure was 70 ± 10% (n=216). The prostaglandin fractions were dried and the residue was treated with KOH for 1h at room temperature, followed by neutralization with KH_2PO_4 to pH 5.5. Through this alkali treatment, PGE_2 which poorly absorbs in the ultra-violet region, is converted into prostaglandin B_2, which has a high ultra-violet absorption at 278 nm. After extraction with diethylether, the samples were evaporated to dryness, dissolved in the mobile phase (100 µl) and analyzed by reversed phase HPLC at 278 nm. The overall recovery of the prostaglandins after the whole procedure was 60 ± 9% (n=216).

Reversed phase HPLC

Reversed phase HPLC was performed with a Kratos spectroflow 400 pump and a Kratos spectroflow 783 programmable absorbance detector. A Chromspher C18 column (200 mm x 3 mm I.D., 5 µm particles)(Chrompack, Middelburg, The Netherlands) was used in conjunction with a Chrompack guard column (75 mm x 2.1 mm I.D) filled with pellicular RP-18 material. The mobile phase of the prostaglandin assay was acetonitrile/methanol/H_2O/acetic acid (30:30:40: 0.05; v/v/v/v). Aliquots of 20 µl were analyzed by reversed phase HPLC at 278 nm, the flow rate was set at 0.7 ml/min. Analyses were executed at room temperature. The mobile phase for the hydroxy fatty acid assay consisted of tetrahydrofuran/acetonitrile/H_2O/acetic acid (15:30:55:0.05; v/v/v/v). Samples of 20 µl were analyzed at 235 nm, the flow rate was set at 0.8 ml/min. Analyses were performed at 40 °C. The limit of detection was about 1 ng absolute for both the prostaglandin and the hydroxy fatty acid assay.
Prostaglandin and hydroxy fatty acid release was expressed as ng/mg protein. Protein content of gastro-intestinal tissue samples was determined by the method of BioRad using bovine serum albumin as standard.

Statistics

Results are expressed as mean ± standard deviation of the mean. Statistical evaluation of differences between several groups with respect to BHA-intake via food, acetylsalicylic acid respectively indomethacin intake via drinking water, as well as mean food intake and mean body weight over the overall period of 14 days, was performed by means of one-way analysis of variance. In all other instances student's *t*-test for unpaired values was applied to evaluate the statistical significance of differences between experimental and respective control groups; $p<0.05$ is considered significant.

Results

Rats fed a diet supplemented with 1.5% BHA, immediately reduced their food intake. After 4-5 days their food consumption remained more or less constant. There were no significant differences in mean food intake over the overall period of 14 days, between the experimental and respective control groups, within the BHA-fed groups (BA resp BI vs BW) or within the control groups (CA resp. CI vs CW). There appeared to be no significant differences in averaged daily BHA-intake between the three BHA-fed groups (BW: 1.31 ± 0.07 g BHA/kg/day; BI: 1.27 ± 0.11 g BHA/kg/day; BA: 1.32 ± 0.05 g BHA/kg/day), nor in acetylsalicylic acid-intake in acetylsalicylic acid-consuming groups (BA: 292 ± 19 mg acetylsalicylic acid/kg/day; CA: 266 ± 24 mg acetylsalicylic acid/kg/day) nor indomethacin-intake in indomethacin-administered groups (BI: 3.61 ± 0.55 mg indomethacin/kg/day; CI: 3.74 ± 0.66 indomethacin/kg/day). One-way analyses of variance furthermore indicated that there were no significant differences in the calculated cumulative doses of BHA, acetylsalicylic acid respectively indomethacin. The food intake in all groups was reflected by the body weight throughout the experiment. After the 14 days experimental period there were no significant differences in mean body weight between the experimental and respective PFC groups, between the BHA-fed groups (BA resp BI vs BW) or between the control groups (CA resp CI vs CW)(data not shown).
LIs in randomized samples of forestomach, glandular stomach and colorectal tissue are presented in Table 2-1. In all of the tissues examined, BHA induced a significant increase in LI compared with the appropriate PFC-group. The increase in colorectal LI was clearly observable as increased immunoreactive staining in the proliferative compartment of the colonic crypts. There were no significant differences in colon crypt height between the six groups of rats. In both forestomach and glandular stomach, the increases in LIs, as a result of short-term BHA-administration, were paralleled by decreases in Tpot. Ts was not altered. Acetylsalicylic acid, and to a lesser degree indomethacin, counteracts the BHA-induced enhancement of cell proliferation in forestomach and glandular stomach. Moreover, in colorectal tissue acetylsalicylic acid completely inhibits the increase in cell proliferation induced by BHA. Indomethacin induced a significant reduction of BHA-induced increase in cell proliferation in forestomach and glandular stomach but not in colon/rectum. Acetylsalicylic acid or indomethacin alone did not affect BrdU-incorporation in these organs, since there were no differences in LI between the control groups (CA vs CW; CI vs CW).
Figures 2-1[a], 2-2[a] and 2-3[a] show the release of prostaglandin H synthase and lipoxygenase metabolites of arachidonic acid from resp. forestomach, glandular stomach and colorectal tissue. In the vehicle-treated control rats (CW), the PGE_2 release from forestomach, glandular stomach and colon/rectum was respectively 4.7 ± 1.5, 6.8 ± 4.3 and 11.3 ± 4.5 ng/mg protein. 14 Day treatment with acetylsalicylic acid (CA) significantly reduced PGE_2 release by about 100% in forestomach, 97% in glandular stomach and 100% in colon/rectum. The

Table 2-1: Labeling indices in forestomach-, glandular stomach- and colorectal-tissue.

Tissue	Labeling Index (% BrdU positive cells) BW group	BI group	BA group	CW group	CI group	CA group
Forestomach	14.6 ± 0.9[a]	12.4 ± 0.6[a/b]	10.9 ± 1.2[a/b]	8.0 ± 1.4	8.6 ± 1.8	8.3 ± 1.3
Glandular stomach	4.6 ± 0.6[a]	3.8 ± 0.3[a/b]	3.6 ± 0.6[b]	3.0 ± 0.2	2.9 ± 0.3	3.0 ± 0.5
Colon/rectum	15.2 ± 1.5[a]	14.4 ± 1.3[a]	10.7 ± 2.4[b]	10.6 ± 2.0	9.3 ± 0.8	10.5 ± 0.5

The labeling indices in forestomach and glandular stomach were determined by flow cytometry, the LI in colorectal tissue was determined by immunocytochemistry. Values marked with superscripts differ significantly (student's-*t*-test) from those of the corresponding PFC-group (a: $p<0.01$), values of the BA- resp. BI-group from those of the BW-group (b: $p<0.01$).
BW: BHA only; BI: BHA + indomethacin; BA: BHA + acetylsalicylic acid; CW: controls; CI: indomethacin controls; CA: acetylsalicylic controls.

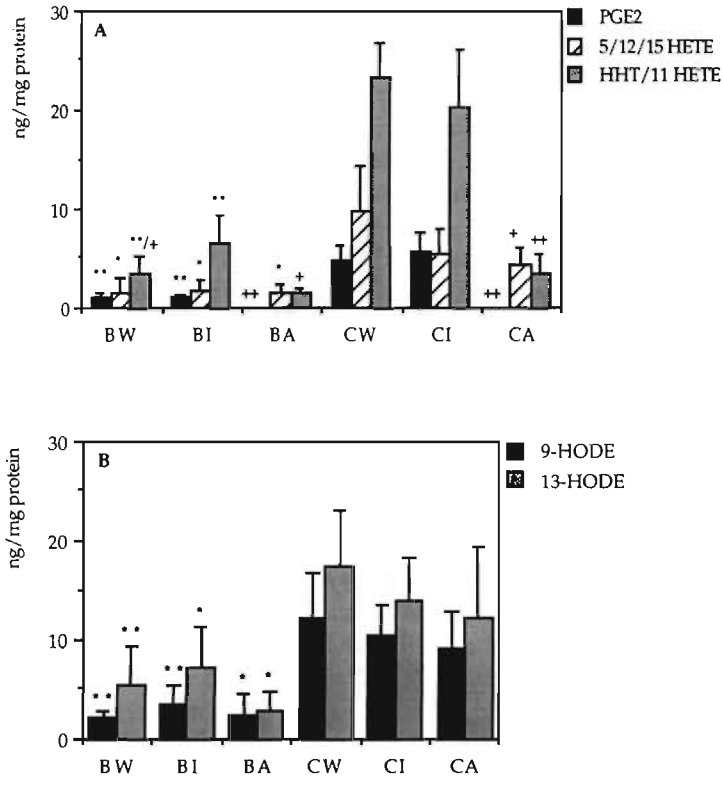

Figure (2-1[a]): Release of arachidonic acid metabolites from forestomach tissue during 45 min. at 37°C. **(Figure 2-1[b])** Release of linoleic acid metabolites from forestomach tissue during 45 min. at 37°C. Values marked with superscripts differ significantly (student's-*t*-test) from the corresponding control-group (**: $p<0.001$; *: $p<0.05$), values of the BA- resp. BI-group from those of the BW-group and values of the control-groups (CA resp. CI) from those of the CW-group (++: $p<0.001$; +: $p<0.05$).
BW: BHA only; BA: BHA + acetylsalicylic acid; BI: BHA + indomethacin; CW: controls; CA: acetylsalicylic acid controls; CI: indomethacin controls.

effects of 14-day treatment with indomethacin (CI) were less dramatic and resulted in a reduction of PGE_2 release of 53% in glandular stomach and 57% in colorectal tissue. Single BHA-treatment for 14 days however also reduced the PGE_2-release significantly in forestomach, glandular stomach and colorectal tissue by resp. 77%, 57% and 35%.
Simultaneous treatment of BHA and acetylsalicylic acid as compared to its appropriate control group (BA vs CA) resulted in all three organs in a complete inhibition of PGE_2 release. Treatment with BHA in combination with indomethacin caused a significant reduction in PGE_2 release in forestomach

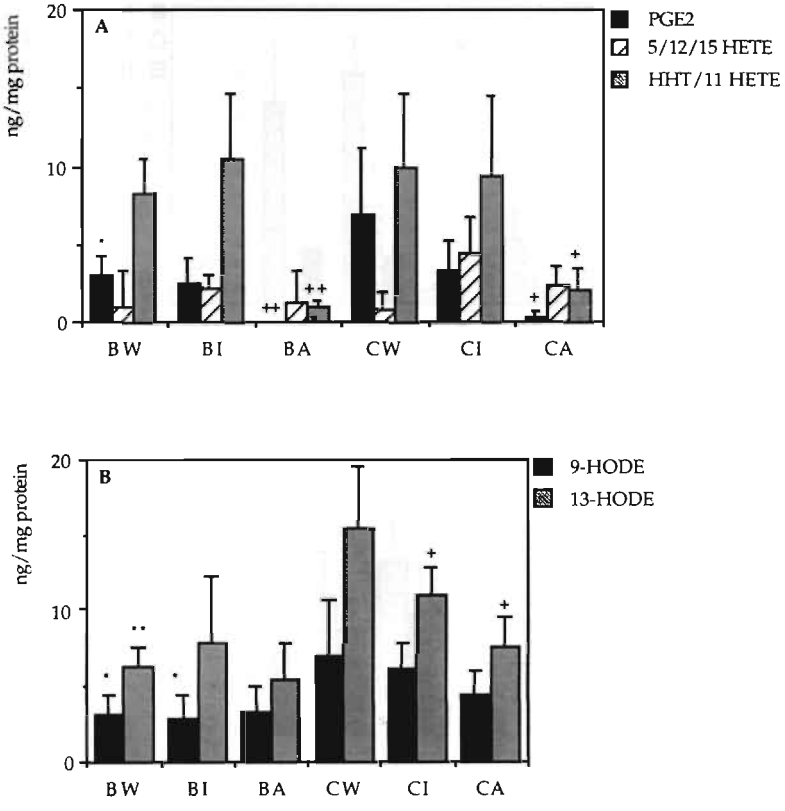

Figure (2-2[a]): Release of metabolites of arachidonic acid from glandular stomach tissue during 45 min. at 37°C. **(Figure 2-2[b])** Release of metabolites of linoleic acid metabolism from glandular stomach tissue during 45 min. at 37°C. Values marked with superscripts differ significantly (student's-*t*-test) from the corresponding control-group (**: $p<0.001$; *: $p<0.05$), values of the BA- resp. BI-group from those of the BW-group and values of the control-groups (CA resp. CI) from those of the CW-group ($^{++}$: $p<0.001$; $^{+}$: $p<0.05$).
BW: BHA only; BA: BHA + acetylsalicylic acid; BI: BHA + indomethacin; CW: controls; CA: acetylsalicylic acid controls; CI: indomethacin controls.

tissue of 82% and in glandular stomach of 21% whereas in colorectal tissue no difference in PGE_2 production was measured.
A similar pattern for the production of the hydroxy fatty acids HHT and 11-HETE, also metabolites of prostaglandin H synthase-mediated metabolism of arachidonic acid, was observed. In the CW group the release of HHT/11-HETE in forestomach, glandular stomach and colon/rectum was resp. 23.2 $\pm$ 3.4, 10.0 $\pm$ 4.7 and 19.5 $\pm$ 4.4 ng/mg protein. Two weeks of single BHA-treatment inhibited the release of these hydroxy fatty acids by 86% in forestomach, and

18% in glandular stomach, while the effect in colon/rectum tissue was negligible. Single acetylsalicylic acid treatment however inhibited the release of HHT/11-HETE in all tissues examined while indomethacin did not affect the production of these hydroxy fatty acids. The effect of simultaneous treatment of BHA and acetylsalicylic acid or indomethacin was comparable with the effects of single acetylsalicylic acid respectively indomethacin treatment. The major hydroxy fatty acids of lipoxygenase-mediated metabolism of arachidonic acid are 5-/12- and 15-HETE. Acetylsalicylic acid treatment for 14 days resulted in a decrease of 5-/12- and 15-HETE release from forestomach tissue, but had no effect on the release of these HETEs measured in glandular stomach and colorectal tissue. Treatment with indomethacin did not affect the release of these HETEs in the examined gastro-intestinal tract tissues. However BHA treatment, with or without acetylsalicylic acid or indomethacin administration, resulted in a strong inhibition of 5-/12- and 15-HETE release in rat forestomach tissue, while the effect of BHA in glandular stomach and colon/rectum was negligible.

Figures 2-1[b], 2-2[b] and 2-3[b] show the release release of metabolites of linoleic acid metabolism in forestomach, glandular stomach and colon/rectum, respectively. In the vehicle-treated PFC rats (CW) the release of 9-HODE from forestomach, glandular stomach and colorectal tissue was 12.1 $\pm$ 4.4, 7.0 $\pm$ 3.7 respectively 5.0 $\pm$ 2.2 ng/mg protein. Two weeks treatment with acetylsalicylic acid or indomethacin had no effect on 9-HODE release in the tissues examined. BHA administration however, reduced the 9-HODE release by 83% in forestomach, 55% in glandular stomach ($p<0.05$) and only 6% in colon/rectum. Simultaneous administration of BHA with acetylsalicylic acid or indomethacin as compared to its appropriate PFC groups, resulted in a significant reduction of the 9-HODE release in all three organs. The release of 13-HODE from forestomach, glandular stomach and colon/rectum, in the vehicle-treated PFC rats was 17.8 $\pm$ 5.5, 15.5 $\pm$ 4.2, respectively 9.5 $\pm$ 1.6 ng/mg protein. Two weeks treatment with acetylsalicylic acid or indomethacin did not affect 13-HODE release in these organs. BHA-treatment however, inhibited the 13-HODE release by 70% in forestomach tissue and by 60% in glandular stomach tissue whereas no effect was found in colorectal tissue. Simultaneous treatment of BHA with acetylsalicylic acid or indomethacin resulted in a significant inhibition of 13-HODE release in all tissues examined.

In summary, LI in rat forestomach, glandular stomach and colon/rectum were significantly enhanced in the BHA-fed groups as compared to their appropriate control groups. Co-administration of acetylsalicylic acid resulted in a significant reduction of BHA-induced increase of cell proliferation in all tissues examined. Indomethacin induced a reduction of the proliferation enhancing effect of BHA in forestomach- and glandular stomach-tissue. Both acetylsalicylic acid and indomethacin had no effect on cell kinetics in the control groups. Acetylsalicylic acid, and to a lesser degree indomethacin, inhibited PGE_2-production in all tissues examined. While indomethacin did not affect the release of arachidonic acid-derived hydroxy fatty acids in forestomach, glandular stomach

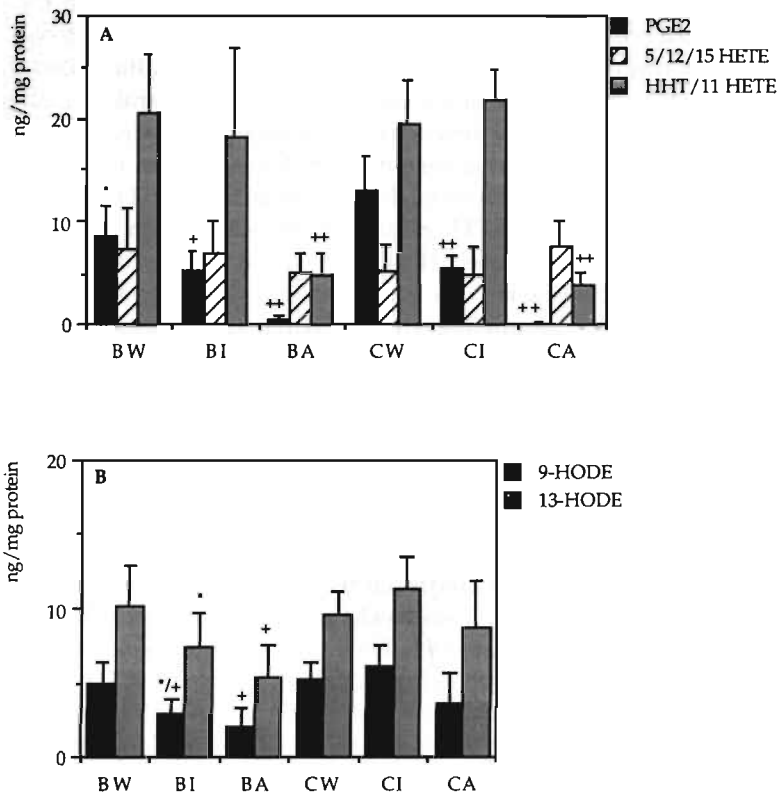

Figure (2-3[a]): Release of metabolites of arachidonic acid from colorectal tissue during 45 min. at 37°C. **(Figure 2-3[b])** Release of metabolites of linoleic acid from colorectal tissue during 45 min. at 37°C. Values marked with superscripts differ significantly (student's-*t*-test) from the corresponding control-group (**: $p<0.001$; *: $p<0.05$), values of the BA- resp. BI-group from those of the BW-group and values of the control-groups (CA resp. CI) from those of the CW-group (++: $p<0.001$; +: $p<0.05$).
BW: BHA only; BA: BHA + acetylsalicylic acid; BI: BHA + indomethacin; CW: controls; CA: acetylsalicylic acid controls; CI: indomethacin controls.

and colon/rectum, acetylsalicylic acid did inhibit lipoxygenase-mediated metabolism of arachidonic acid in forestomach-tissue. Both acetylsalicylic acid and indomethacin did not affect linoleic acid metabolism. BHA inhibited both prostaglandin H synthase-mediated and lipoxygenase-mediated metabolism of arachidonic acid and linoleic acid in all gastro-intestinal organs examined.

Discussion

BHA is a rodent forestomach carcinogen (2, 3, 45-47) and enhances cell proliferation in other tissues of the gastro-intestinal tract (6, 7) as well as in non-intestinal organs (5, 6). Furthermore, BHA is a potent modulator of chemically induced mutagenesis and carcinogenesis (12, 19-25). The mechanism by which BHA exerts carcinogenic effects on the forestomach epithelium, is still matter of dispute. The range of effects in forestomach epithelium includes lesions, inflammation, hyperplasia, papillomas and ultimately carcinomas, depending on duration of BHA-administration and dose level (48).

Data on LIs in the present study confirm our previous results (7, 49) that in rats, several tissues from the gastro-intestinal tract (forestomach, glandular stomach and colon/rectum) are susceptible to the proliferation enhancing potential of BHA. The LIs in all examined tissues (forestomach, glandular stomach and colon/rectum) as compared with the corresponding PFC groups, were significantly enhanced after 14 days of administration of 1.5% BHA in the diet. The increase in LI was accompanied by a decrease in Tpot while Ts was not altered, which indicates that BHA increases the growth fraction of cells and not the individual cell duplication rate.

Simultaneous administration of acetylsalicylic acid, an inhibitor of prostaglandin H synthase, resulted in a significant inhibition of the proliferative effect induced by BHA (26). We therefore hypothesized that products of prostaglandin H synthase-mediated metabolism, which are known to stimulate cell proliferation, might be involved in the mechanism of BHA-induced enhancement of cell proliferation.

Products of prostaglandin H synthase-mediated metabolism of arachidonic acid are prostaglandins, leukotrienes and hydroxy fatty acids. These metabolites have been shown to be involved within inflammatory processes, wound repair and proliferative skin diseases (31). Prostaglandins of the E-type have often been associated with the regulation of cell proliferation. *In vitro*, E-type prostaglandins have been found to modulate human keratinocyte proliferation (31). In mouse skin *in vivo*, PGEs turned out to be mediators of the hyperplastic transformation in chemically induced two-step carcinogenesis (28). Others have reported that addition of arachidonic acid metabolites to various cell cultures stimulates cell proliferation (50). Moreover, many malignant tumors that tend to have a high rate of cell turnover, produce large amounts of prostaglandins (50). However, it has also been reported that endogenously produced PGEs can inhibit cell proliferation in rat colon by enhancement of cAMP-levels (51, 52).

Acetylsalicylic acid and indomethacin are both inhibitors of prostaglandin H synthase. Coadministration of acetylsalicylic acid or indomethacin with certain tumor promotors or chemical carcinogens resulted in an inhibition of respectively tumor promotion and tumor development (53-55). For instance, simultaneous administration of indomethacin and the tumor promotor 12-*O*-tetradecanoylphorbol-13-acetate (TPA) resulted in a decrease in PGE_2-release, and concomitantly in a reduction of TPA-induced epidermal DNA-synthesis in

mouse skin (31).
In the present study, the effect of acetylsalicylic acid and indomethacin on BHA-induced alterations in cell kinetic parameters in relation to PGE_2-release was evaluated in rats. In forestomach and glandular stomach, acetylsalicylic acid and indomethacin inhibited the BHA-induced enhancement of cell proliferation. Acetylsalicylic acid completely counteracted the effect of BHA on LI in colon/rectum whereas indomethacin exhibited no effect in this organ. Administration of acetylsalicylic acid or indomethacin without BHA did not affect cell proliferation in the tissues examined. Both acetylsalicylic acid and indomethacin inhibited PGE_2 release in the BHA-fed groups and in the PFC groups. Since, BHA itself appeared to be a potent inhibitor of PGE_2-production in these organs, it is concluded that enhancement of cell proliferation induced by BHA in forestomach, glandular stomach and colon/rectum is not attributable to an increase of PGE_2-release in these organs. Similar results were found for the two major hydroxy fatty acids formed by prostaglandin H synthase-mediated metabolism of arachidonic acid. The fact that at the chosen drinking water concentrations acetylsalicylic acid appears to inhibit prostaglandin H synthase more than indomethacin, is possibly related to the insolubility and instability of indomethacin in water which may lead to insufficient tissue levels to gain effective synthase inhibition.
It has been argued that hyperplasia, as observed in rodent forestomach after long-term BHA ingestion, may be a reaction to initial BHA-induced epithelial irritation and inflammation. Nera et al (56) observed a localized thickening of the forestomach epithelium of male F344 rats along with acute inflammatory cell infiltration in the underlying lamina propria and submucosa after 9 days treatment of 2% BHA. After 27 days of BHA treatment, the thickening of the squamous mucosal epithelium had spread widely over the forestomach mucosa, while there were no longer inflammatory cell infiltrates observed in the underlying lamina propria or submucosa. Prostaglandins can also modulate the release of gastric mucus. Inhibition of prostaglandin synthesis by BHA could therefore result in a reduction of the normal amount of protective gastric mucus. Recent results reported by others (57) however indicated a significant increase in mucus secretion in gastro-intestinal tract tissues of BHA-treated rats. It seems therefore unlikely that BHA causes ulceration and hyperplasia in forestomach tissue as a result of inhibiting either mucus synthesis or secretion (57). If BHA would induce an increase in gastro-intestinal cell proliferation via an initial inflammatory response, tissue PGE_2 release is more likely to be increased, in contrast with reductions as found in this study. An initial transient increase in PGE_2 release can however not be excluded.
Arachidonic acid is a substrate for both the prostaglandin H synthase and lipoxygenase pathways. Inhibition of the prostaglandin H synthase-mediated pathway could therefore result in an increased significance of the lipoxygenase pathway. Acetylsalicylic acid exerted an additional inhibitory action on 5-/12-/15-HETE release in forestomach tissue. Indomethacin had no effect on lipoxygenase-mediated metabolism of arachidonic acid. This suggests that in general

inhibition of one pathway of arachidonic acid metabolism does not affect metabolic rate of the other pathway. BHA appeared to be an inhibitor of lipoxygenase-mediated metabolism of arachidonic acid in forestomach tissue. While acetylsalicylic acid nor indomethacin affected linoleic acid metabolism by either the prostaglandin H synthase and the lipoxygenase pathway, BHA treatment resulted in a significant inhibition of HODEs release by both pathways. Again, this was most pronounced in the upper gastro-intestinal tract tissues, whereas in colorectal tissue the effect was negligible. Forestomach tissue is exposed to higher concentrations of BHA after oral intake than colorectal tissue where BHA which remains after resorption (58), is diluted by faecal bulk. This suggests a dose-response relationship for the inhibitory action of BHA on both linoleic acid and arachidonic acid metabolism. It is concluded that the increase in cell proliferation induced by BHA in all the organs examined, is not attributable to an increase in linoleic acid- or arachidonic acid-derived hydroxy fatty acids.

Nevertheless, BHA enhanced cell proliferation in rat gastro-intestinal tract and coadministration of prostaglandin synthase inhibitors significantly reduced the effects of BHA on LIs. Earlier reports indicate that the reactivity of BHA itself is probably not high enough to damage cellular molecules. 3-BHA is thought to become carcinogenic after being metabolized to more reactive compounds (59). Of the possible BHA-metabolites, *tert*-butyl(1,4)hydroquinone and *tert*-butyl paraquinone are most likely to represent the active compounds which are capable of attacking biomacromolecules (59-62). BHA has previously been reported to be metabolized by peroxidases resulting in the formation of dimeric products (63). It is possible that also the *O*-demethylation product of BHA, *tert*-butylhydroquinone, can be metabolized by peroxidases into its corresponding quinone. Since high concentrations of peroxidases are found *in vivo*, the peroxidative activation of BHA-metabolites could be relevant for the toxicity and carcinogenicity of this agent (64). Prostaglandin H synthase occurs in many mammalian cells and is present at high levels in platelets, lungs, kidney and urinary bladder (65). Prostaglandin H synthase is composed of both cyclooxygenase and peroxidase enzymes. It is therefore possible that the peroxidase enzyme of prostaglandin H synthase is involved in the metabolic activation of BHA by converting the hydroquinone metabolite to the corresponding quinone by redoxcycling. This would also explain why oral intake of BHA decreases gastro-intestinal tissue release of prostaglandin species. This reduction in metabolites of arachidonic acid- and linoleic acid-metabolism could be due to a competition between arachidonic acid and/or linoleic acid and BHA for prostaglandin H synthase.

In summary, products of prostaglandin H synthase-mediated metabolism of arachidonic acid and linoleic acid do probably not contribute to the induction of proliferative changes in rat gastro-intestinal tract tissues by BHA. Since BHA inhibits both prostaglandin and hydroxy fatty acid release in all organs examined, it seems unlikely that an inflammatory action is directly responsible for enhancement of cell proliferation induced by BHA. Cooxidation by prostaglandin H synthase of the BHA metabolite *tert*-butylhydroquinone into *tert*-butyl-

quinone yielding active oxygen species might be an important process in toxic or carcinogenic responses induced by this antioxidant. Further research on the mechanism underlying the carcinogenicity of BHA will have to focus on these aspects.

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Chapter 3

Oxygen radical formation during prostaglandin H synthase-mediated biotransformation of butylated hydroxyanisole.

P.A.E.L. Schilderman, J.M.S. van Maanen, E.J. Smeets, F. ten Hoor[1], and J.C.S. Kleinjans

Departments of Health Risk Analysis and Toxicology, and [1]Human Biology, University of Limburg, PO box 616, 6200 MD Maastricht, The Netherlands.

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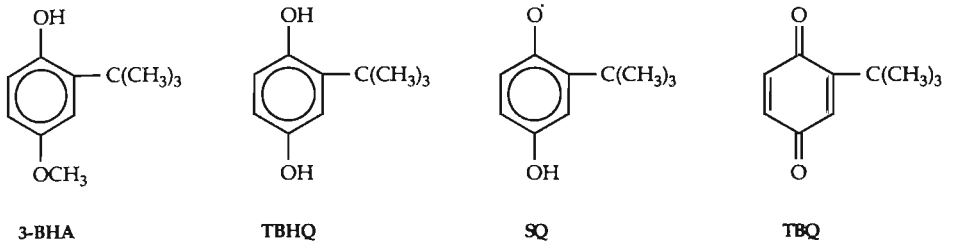
Abstract

The dominant metabolic pathway of the presumably carcinogenic food antioxidant 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) includes *O*-demethylation to 2-*tert*-butyl(1,4) hydroquinone (TBHQ) and subsequent peroxidation to 2-*tert*-butyl(1,4)paraquinone (TBQ). In order to determine the ability of TBHQ to induce the formation of oxygen radicals, electron spin resonance (ESR) measurements were performed in presence and absence of peroxidases. ESR analyses showed that prostaglandin H synthase administration resulted in a substantially accelerated metabolism of TBHQ into TBQ, which is accompanied by formation of superoxide anion, hydroxyl radical and hydrogen peroxide. Spectrophotometric measurements revealed that prostaglandin H synthase and lipoxygenase are both capable of converting TBHQ into TBQ. In order to determine the effect of prostaglandin H synthase on BHA (dose-level: 1.5% BHA of the diet) metabolism *in vivo*, we coadministered two inhibitors of prostaglandin H synthase acetylsalicylic acid and indomethacin, with BHA to rats. Coadministration of acetylsalicylic acid (0.2%) in the drinking water resulted in a significant increase of urinary TBHQ excretion. Both acetylsalicylic acid and indomethacin (dose-level: 0.002% in the drinking water) induced a significant decrease in TBQ excretion into urine. Cooxidation by prostaglandin H synthase of the BHA-metabolite TBHQ into TBQ, yielding reactive oxygen species might therefore be responsible for the carcinogenic and toxic responses elicited by this antioxidant.

Introduction

Butylated hydroxyanisole (BHA), a synthetic phenolic antioxidant, is widely used as a food preservative without evidence of adverse effects (1). However, in 1983, this antioxidant appeared to be carcinogenic to rat forestomach epithelium (2). Carcinogenic activity after prolonged exposure to doses of 1-2% in the diet resulted in squamous cell carcinomas in forestomach tissue of rats and hamsters (3, 4). Dose-dependent changes in rodent forestomach (hyperplasia, papillomas and carcinomas) appeared accompanied by an increase in cellular proliferation rate monitored as ^{3}H- or BrdU-labeling index. More recently, it was demonstrated that in rodents other tissues also present a target for these cell-growth enhancing effects of BHA (5-7). In non-rodents, BHA induced proliferation enhancing effects in the oesophagus of pigs (8) and primates (9). Because BHA did not show genotoxic activity in most tests for mutagenicity (10-14), its carcinogenicity is thought to result from epigenetic effects (15).
Although the toxic and carcinogenic effects of BHA are well described, little is known about the exact mechanism by which this antioxidant causes toxicity or carcinogenicity. In general, the ultimate reactive forms of carcinogens are electrophilic species. BHA by itself however, is not an electrophile. It is possible that the carcinogenic effects of BHA are due to the conversion of the parent compound to more reactive metabolites (16). Studies on the metabolism of BHA *in vivo* and *in vitro* have revealed that the main metabolic transformations are dimerization, conjugations and *O*-demethylation (16). Demethylation of BHA by cytochrome p450 has demonstrated to yield the 2-*tert*-butyl (1,4)hydroquinone (TBHQ) *in vivo* in dogs (17), in rats and in man (18) and *in vitro* in rat liver microsomes (19). Furthermore, oxidative demethylation of BHA into the hydroquinone-derivative is elevated after cytochrome p450 induction by phenobarbital, which however, did not affect forestomach labeling index in rat (20). It is therefore unlikely that the proliferation enhancing effects of BHA are attributable to this p450 mediated biotransformation pathway. Earlier reports indicate that BHA can also be metabolized by peroxidases resulting in the formation of dimeric products (19). It is furthermore possible that also the *O*-demethylation product of BHA, TBHQ, can be metabolized by peroxidases into its corresponding 2-*tert*-butyl(1,4)paraquinone (TBQ). Hydroquinones, as well as quinones, are relatively potent electrophiles and can furthermore both generate active oxygen species as a result of redoxcycling via semiquinone radicals (21, 22). Recently, it was demonstrated that BHA is capable of stimulating superoxide formation in liver microsomes from phenobarbital-pretreated rats. The hydroquinone metabolite is however much more active than BHA in stimulating superoxide production, whereas the paraquinone metabolite is the most potent activator of superoxide formation. Moreover, the superoxide production of the hydroquinone in microsomes was accompanied by a high production of hydrogen peroxide and hydroxyl radicals (22). Recently, it was shown that the DNA-damaging capacity of TBQ was much higher than

that of TBHQ and BHA in forestomach epithelium of male F344 rats after oral administration of 3-BHA and its metabolites (23). Furthermore, a considerable binding of one or more metabolites to protein was demonstrated, which indicates that metabolites of BHA, TBHQ and TBQ have the capacity to combine with macromolecules. It is therefore most likely that of the known BHA-metabolites, TBHQ and TBQ are the active compounds which are capable of attacking macromolecules (16), probably due to the generation of active oxygen species.



Scheme 3-1. Structures of 3-*tert*-butyl-4-hydroxyanisole (BHA); 2-*tert*-butyl(1,4)hydroquinone (TBHQ), 2-*tert*-butylsemiquinone (SQ), 2-*tert*-butyl(1,4)paraquinone (TBQ).

Recently, we have demonstrated that coadministration of acetylsalicylic acid and indomethacin, both inhibitors of prostaglandin H synthase, resulted in a significant reduction of the proliferative effect of BHA in rat gastro-intestinal tract tissues (24). Prostaglandin H synthase, an enzymatic system composed of both cyclooxygenase and peroxidase enzymes, occurs in many mammalian cells and is present at high levels in platelets, lungs, kidney and urinary bladder (25). It is possible that prostaglandin H synthase is involved in the metabolic activation of BHA by converting the cytochrome p450-generated hydroquinone metabolite to the corresponding quinone by redoxcycling, thereby yielding active oxygen species. This might be an important process in toxic or carcinogenic responses induced by this antioxidant. Therefore, in the present study, the ability of TBHQ to induce the formation of oxygen radicals in presence and absence of both prostaglandin H synthase and lipoxygenase was tested by means of electron spin resonance (ESR) analysis. The peroxidative transformation of TBHQ by prostaglandin H synthase and lipoxygenase was also assayed spectrophotometrically and quantitated by HPLC. Administration of acetylsalicylic acid resp. indomethacin simultaneously with BHA in the diet of rats, may decrease the metabolism of TBHQ to TBQ *in vivo*. Therefore, the metabolism of BHA, in particular its prostaglandin H synthase-mediated oxidation of TBHQ into TBQ, with and without inhibition of prostaglandin H synthase, was monitored in rats fed 1.5% BHA in the diet for two weeks.

Materials and Methods

Materials

BHA (food grade BHA, purity > 99%, 97.5% 3-BHA) was obtained from J. Dekker Co. (Wormerveer; the Netherlands). TBHQ (purity > 97%), 3,5-di-*tert*-butyl-4-hydroxyanisole (DBHA)(purity > 97%), *tert*-butyl alcohol and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were purchased from Aldrich Chemical Co. (Brussels, Belgium). Lipoxygenase (type V)(specific activity: 646600 units/mg), chromium trioxide, diethylenetriamine-pentaacetic acid (DTPA), limpet acetone powder (type 1) and superoxide dismutase (SOD) from Sigma (St Louis, MO), diethylether from BDH (Poole, UK), and prostaglandin H synthase (specific activity: 42600 Units/mg) from Cayman (Ann Arbor, MI). Methanol was HPLC grade and was obtained from Rathburn (Walkerburn, UK). Water was purified using a milli-Q water purification system. Analytical reagent-grade chemicals were used in all other instances. 2-*tert*-butyl(1,4)paraquinone was synthesized by oxidizing 2-*tert*(1,4)butylhydroquinone (10 mmol) with chromium trioxide (60 mmol) in pyridine as described by Ratcliffe and Rodehorst (1970). The resulting *tert*-butylquinone was characterized by its visible and ultraviolet spectrum. The UV spectrum displayed an absorbance with a maximum at 250 nm. Furthermore, the mass spectrum of TBQ appeared to have a fragmentation pattern consisting of a parent peak (m/e 164), loss of two protons from *tert*-butylhydroquinone (m/e 166), loss of CH_3 (m/e 149), and loss of CO (m/e 121). This fragmentation pattern is consistent with 1,4-quinones, as reported previously (27).

Electron spin resonance spectroscopy (ESR)

ESR spectra were recorded at room temperature on a Bruker ESP-300 with an ESP 1600 data processor equiped with an ER 4102 standard rectangular cavity. Conditions of ESR spectrometry for measurement were as follows: modulation amplitude: 0.990 G; receiver gain: $2 \cdot 10^5$; time constant: 40.96 ms; scan time: 20.97 s; power: 25 mW; magnetic field: 3480 ± 40 G. Number of scans is 10. All incubations were performed at 37°C during 15 min, prior to ESR measurement. The incubation mixtures contained 1 mM TBHQ, in a total volume of 0.5 ml 0.01 M phosphate-buffered saline (PBS: pH 7.3). Radical formation as a result of peroxidation of TBHQ by prostaglandin H synthase (200 units) and lipoxygenase (350 units) were performed in presence and absence of arachidonic acid (0.2 mM) and linoleic acid (0.2 mM) as cofactors respectively. All incubations using prostaglandin H synthase contained 1 µM haematine as additional cofactor whereas incubations with lipoxygenase contained 1 nM hydrogen peroxide. DMPO was used as a spin trap for both hydroxyl and superoxide anion radicals. In order to remove background ESR signals DMPO solutions were purified as described previously (28). Since DMPO can react with both superoxide and hydroxyl radicals SOD (200 units) was added in order to investigate the role of superoxide. To determine the influence of hydroxyl radicals incubation mix-

tures were supplemented with *tert*-butyl alcohol (100 mM). Incubations with catalase (1000 units) were performed to investigate the role of hydrogen peroxide in radical formation. DTPA was used as an iron chelator. Since a concentration of 0.1 mM TBHQ appeared to be too low to detect spin adduct formation, the concentration of TBHQ used for ESR measurements was increased to 1 mM. Furthermore, the measurements on TBHQ and TBQ were performed both in 1% DMSO solutions and in saturated solutions in order to avoid the influence of DMSO on hydroxyl radical trapping.

Peroxidase assay
Peroxidase activated transformation of TBHQ to TBQ was assayed spectrophotometrically. Consecutive scans were performed by a UV/visible rapid scanning spectrophotometer (Beckman, DU-64). All incubations were performed in 1 ml 0.01M PBS (pH 7.3) containing 0.1 mM TBHQ. Concentrations of enzymes and cofactors as well as incubation conditions are comparable to those applied in the ESR analysis. TBHQ was dissolved in DMSO; final concentration: 1%. The reactions were initiated by the addition of TBHQ and were monitored by the decrease of absorbance at 290 nm and increase of absorbance at 250 nm due to oxidation of TBHQ into TBQ. Reactions were terminated by the addition of 0.5 ml 5% trichloro-acetic acid. Production formation was subsequently analyzed by HPLC with diethylether extraction as described below.

Animals, maintenance and test procedure.
Male Wistar rats (Winkelmann, Borchen, FRG), five weeks old (body weight: 97 ± 4 g; mean ± SD), were housed individually in metabolic cages in an air-conditioned room at 21-22°C and 50-55% humidity with a 12 h light-dark cycle. Rats were randomly divided into three groups of six animals each. At 6 weeks of age, rats were given an experimental diet containing 1.5% BHA during a period of 14 days. One 1.5% BHA group received 0.2% acetylsalicylic acid (encoded: BA) in the drinking water, another BHA-fed group received 0.002% indomethacin (BI) in the drinking water while a similar fed group received drinking water only (BW). The drinking water also consisted ethanol (for dissolving indomethacin; final concentration: 1%) and sucrose carrier (5 g/l). Food and drinking water were available ad libitum. Fresh drinking water was provided at three day intervals. Body weights were determined three times per week whereas food and drinking water intake were recorded daily.

Extraction of BHA, TBHQ and TBQ from urine samples
Urine was centrifuged for 30 min at 2000 g. 0.5 ml urine was diluted to 2.0 ml with 1M sodium acetate buffer pH 4.8 and 100 µl methanol, containing 250 µg/ml DBHA, was added as an internal standard. In order to deconjugate the glucuronic acid and sulphate conjugates of BHA, TBHQ and TBQ 200 µl limpet acetone powder (200 mg/ml) was added. The tubes were incubated overnight at 37°C. On the next day, samples were extracted twice with 5 ml diethylether for

15 min, and centrifuged at 700 g for 20 min. The organic phases were combined and 1 ml methanol was added. The ether fraction was subsequently evaporated under vacuum until 0.45 ml methanol was left, in order to prevent votality of TBQ. The residue was diluted to 1 ml with ammonium formiate buffer pH 3.5 and stored at -20°C until HPLC analysis. For the determination of free BHA (i.e. unconjugated BHA), free TBHQ and free TBQ, the procedure was the same except for the addition of limpet acetone powder and the overnight incubation step.

High Performance Liquid Chromatography (HPLC)
Reversed phase HPLC was performed with a Gynkotek High Precision Pump 480 (Separations, Leiden, The Netherlands) and a Kratos spectroflow 783 programmable absorbance detector. A chromspher C18 column (200 mm x 3 mm I.D., 5 µm particles) (Chrompack, Middelburg, The Netherlands) was used in conjunction with a chrompack guard column (75 mm x 2.1 I.D) filled with pellicular RP-18 material. The mobile phase consisted of methanol and 0.1 M formiate buffer, pH 3.5. A linear gradient was used from 0 till 30 min (40 to 85 % methanol, 15 % per min). The retention times of the products were as follows: TBHQ; 8.5 min, TBQ; 14.0 min, BHA; 18.6 min and DBHA at 29.0 min. TBHQ, BHA and the internal standard DBHA were monitored at 290 nm; TBQ at 250 nm. The limit of detection was 1 ng absolute for both BHA and TBHQ and 0.5 ng for TBQ. The recovery of BHA was 88.2 ± 0.5%; TBHQ: 90.4 ± 3.2% and TBQ: 78.1 ± 2.9%.
Excretion of BHA and its metabolites is expressed as percentage of the amount of BHA ingested, calculated on basis of food (and consequently BHA) consumption during the period of urine collection.

Results

ESR measurements
In Figure 3-1, ESR spectra of incubations of saturated solutions of TBHQ in absence and presence of DMPO were analysed for the presence of semiquinone (SQ) and DMPO-OH signals. Incubation of TBHQ only resulted in the semiquinone signal; A_{H1}=4.52 G, A_{H2}=1.94 G (fig. 3-1b). Due to the presence of the *tert*-butyl group, the three protons in the *tert*-butyl(1,4)semiquinone ring are non-equivalent. The presence of the double doublet suggests that the free electron is mainly present on the oxygen atom meta to the *tert*-butyl-group, which is in line with the inductive effect of the *tert*-butyl group. The double doublet is obtained by splitting from the two non-equivalent β-protons. A further hyperfine splitting of 0.97 G is also present in two of the four lines, and is due to the presence of the γ-hydrogen opposite to the *tert*-butyl group. Addition of DMPO in saturated solutions of TBHQ resulted in a mixed signal of DMPO-OH and SQ-signal (Fig. 3-1c). Since integration of a mixture of ESR

signals appeared not to be accurate, alterations in signal intensity are calculated from signal-to-noise ratios. The standard DMPO-OH signal (A_N=A_H=14.8 G) was obtained after incubation of DMPO with hydrogen peroxide (Fig. 3-1a). Addition of *tert*-butyl alcohol increased the intensity of the SQ-signal by 20% while the DMPO-OH signal intensity was reduced by 22% (Fig. 3-1d). The intensity of the SQ-signals was not influenced by addition of catalase; the DMPO-OH signal however was reduced by 68% (Fig. 3-1e). Furthermore, the DMPO-OH signal in TBHQ incubations was increased by 10% by addition of SOD, whereas the SQ-signal was decreased by 10% (Fig. 3-1f). Addition of DTPA to TBHQ incubations resulted in a complete disappearance of the SQ signal as analysed by ESR (spectrum not shown).

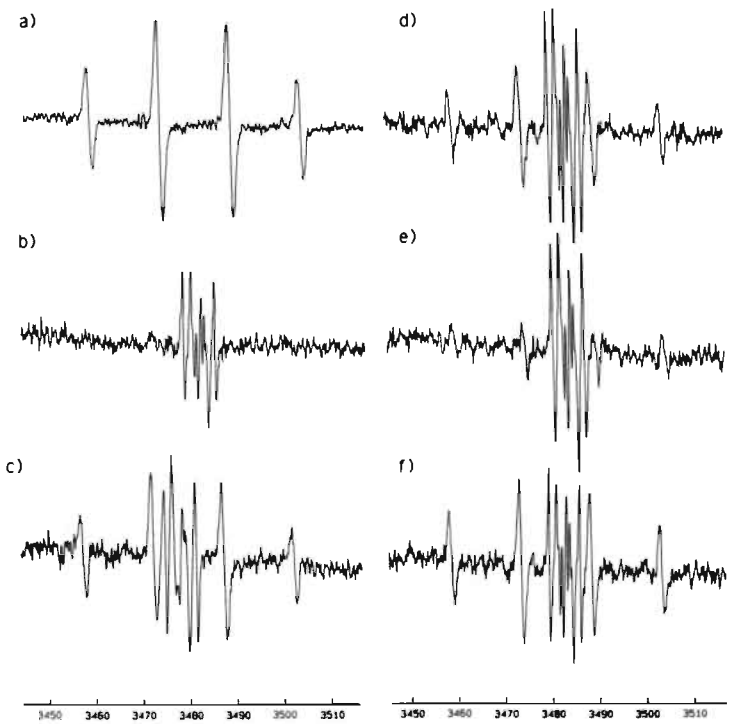


Figure 3-1. ESR signals of incubations of a): 0.1 M H_2O_2/10 mM DMPO, DMPO-OH: A_N=A_H 14.8 G; b): 1 mM TBHQ, SQ: A_{H1}=4.52 G, A_{H2}=1.94 G; c): 1 mM TBHQ/ 10 mM DMPO; d): as incubation (c) with 100 mM *tert*-butyl alcohol; e): as incubation (c) with 1000 units catalase; f): as incubation (c) with 120 units SOD. Instrumental conditions are as indicated in the methods section.

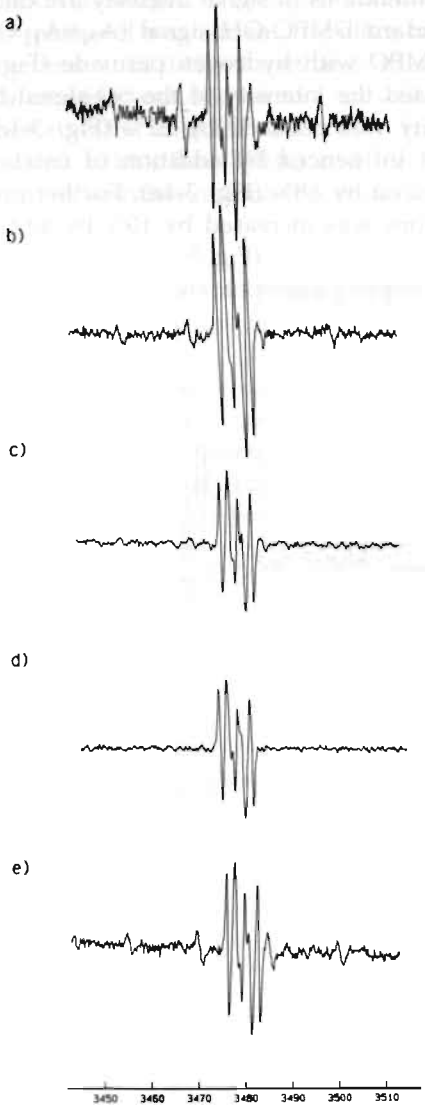


Figure 3-2. ESR signals of incubations of a): 1 mM TBHQ /10 mM DMPO; b): 1 mM TBHQ/ 200 units prostaglandin H synthase/ 0.2 mM arachidonic acid/ 1μM haematine/ 10 mM DMPO; c): as incubation (b) with 100 mM *tert*-butyl alcohol; d): as incubation (b) with 1000 units catalase; e): as incubation (b) with 120 units SOD. Instrumental conditions are as indicated in the methods section.

TBHQ incubation with prostaglandin H synthase and arachidonic acid (Fig. 3-2b) resulted in a threefold increase of the SQ signal as compared to the control incubation without enzyme and cofactor, as is shown in Figure 3-2a. The

intensity of the DMPO-OH signal was comparable. The intensity of the SQ signal was not influenced by adding *tert*-butyl alcohol; the DMPO-OH signal was however decreased by 15% (Fig. 3-2c). Addition of catalase resulted in a small increase in intensity of the SQ signal but diminished the DMPO-OH signal to background intensity (Fig. 3-2d). The ESR signal of SQ after incubation of TBHQ with prostaglandin H synthase and arachidonic acid resulted in a two-fold instead of a three-fold increase after addition of SOD while the DMPO-OH signal increased by 30% (Fig. 3-2e).

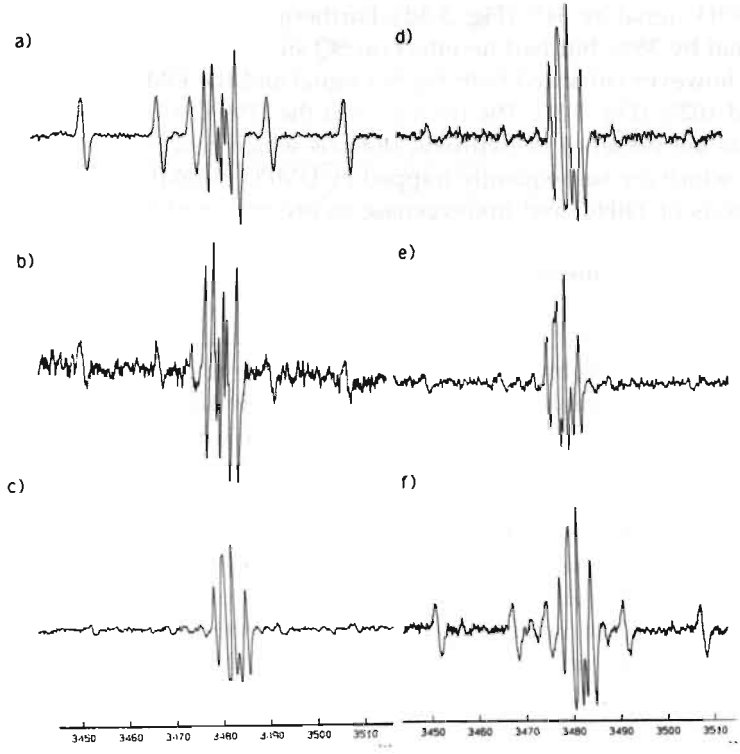


Figure 3-3. ESR signals of incubations of a): 1 mM TBHQ /10 mM DMPO /10% DMSO; b) 1 mM TBHQ/ 10 mM DMPO/ 1% DMSO, c): 1 mM TBHQ/ 200 units prostaglandin H synthase /1 μM haematine /10 mM DMPO /1% DMSO; d): as incubation (c) with 100 mM *tert*-butyl alcohol; e): as incubation (c) with 1000 units catalase; f): as incubation (c) with 120 units SOD. Instrumental conditions are as indicated in the methods section.

All ESR measurements were also performed with 1% DMSO-TBHQ solutions; the effects of scavengers on signal intensity were comparable. Higher amounts of DMSO did however affect the signal intensity of SQ. A hundred-fold increase of SQ was seen in 30% DMSO-solution as compared to an 1% DMSO solution.
In incubations in the presence of DMSO, the DMPO-OH signal was not observed; however a signal with the characteristics of the DMPO-CH_3 adduct did appear (Fig. 3-3a)(29). TBHQ incubations, in the presence of 1% DMSO, with prostaglandin H synthase in absence of arachidonic acid caused a stimulation of SQ formation of 200% (Fig. 3-3c) as compared to the control incubation without enzyme (Fig. 3-3b); the DMPO-OH signal intensity was not affected. Addition of *tert*-butyl alcohol increased the SQ signal intensity by 43% and decreased the DMPO-OH signal by 54% (Fig. 3-3d). Furthermore, catalase reduced the DMPO-OH signal by 35%, but had no effect on SQ signal intensity (Fig. 3-3e). Addition of SOD however enhanced both the SQ signal and the DMPO-OH signal by resp. 24% and 102% (Fig. 3-3f). The formation of the DMPO-CH_3 adduct gives further proof for the presence of hydroxyl radicals which react with DMPO to methyl radicals which are subsequently trapped by DMPO to DMPO-CH_3 (30).
Incubations of TBHQ and lipoxygenase in presence and absence of linoleic acid did not result in an enhancement of SQ formation or DMPO-OH signal formation (spectra not shown).

Peroxidase assay
Metabolism of TBHQ by prostaglandin H synthase and lipoxygenase in presence and absence of arachidonic acid respectively linoleic acid was assayed spectrophotometrically by a decrease of absorbance at 290 nm and an increase of absorbance at 250 nm due to oxidation of TBHQ into TBQ. Product formation was quantitated by HPLC. Effect of arachidonic acid on prostaglandin H synthase-dependent metabolism of TBHQ is shown in Figure 3-4a/b (spectra not shown). In the presence of prostaglandin H synthase and arachidonic acid, approximately 38% of the amount of TBHQ was metabolized after 60 minutes of incubation, while in absence of arachidonic acid only 10% was converted into TBQ. The rate of autoxidation of TBHQ in absence of enzyme is minimal when compared to TBHQ metabolism in the presence of enzyme (2% in 60 min).
Spectral analysis of lipoxygenase-dependent metabolism of TBHQ into TBQ, with linoleic acid as cofactor, is shown in Figure 3-5. TBHQ (0.1 mM) had an optical density of 0.350 at its absorbance maximum of 290 nm. In the presence of lipoxygenase and linoleic acid, the optical density of TBHQ decreased to 0.280 after 60 min of incubation while an additional peak appeared with an absorbance maximum at 250 nm which increased with time. The optical density of TBQ increased time-dependently from 0.060 till 0.310. Subsequent HPLC analysis revealed that 26% of the original amount of TBHQ was converted into TBQ. Oxidation of TBHQ by lipoxygenase in absence of linoleic acid resulted in a 10% conversion of TBHQ in TBQ (spectra not shown). When the concen-

tration of hydrogen peroxide was increased to 0.1 mM in incubations with lipoxygenase, the metabolism of TBHQ increased by respectively 24 and 21% in presence and absence of linoleic acid respectively.

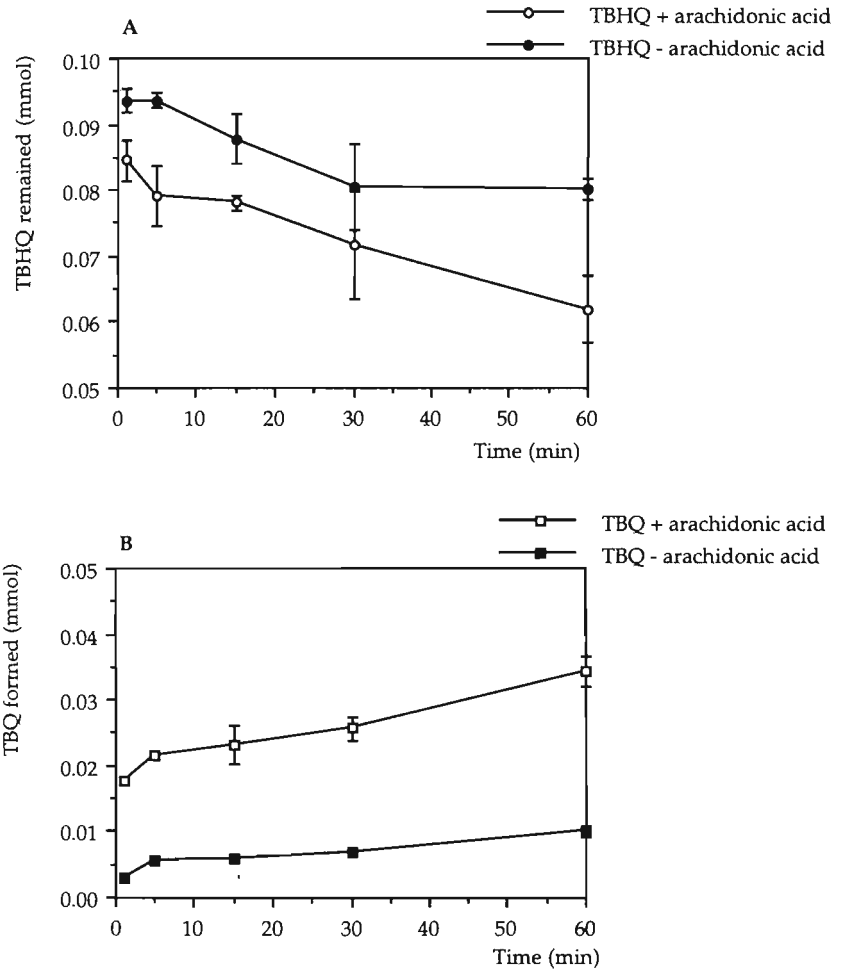


Figure 3-4a/b. Effect of arachidonic acid on the prostaglandin H synthase-dependent metabolism of TBHQ (Fig. 3-4a); formation of TBQ (Fig. 3-4b). Incubation conditions were as indicated in the methods section. Each point represents the mean and SD of two incubations.

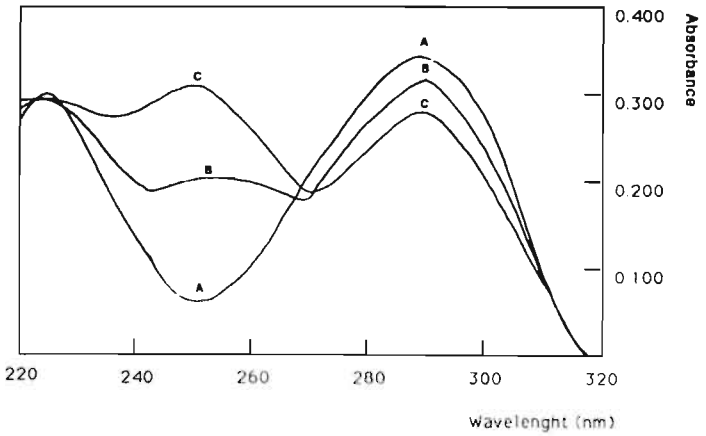


Figure 3-5. Spectral properties of TBHQ (absorption maximum at 290 nm) and its oxidation product TBQ (250 nm) after lipoxygenase/linoleic acid-dependent metabolism, A: t=5 min; B: t=30 min; C: t=60 min. Incubation conditions were as indicated in the methods section. Consecutive scans were performed at the times indicated using a UV/visible rapid scanning spectrophotometer.

BHA metabolism in vivo

Immediately after the onset of BHA administration, rats decreased their food consumption considerably. After 4-5 days, their food intake remained more or less constant. There appeared to be no significant differences in food intake between the three groups during the experimental period. The food intake in all groups was reflected by the body weight throughout the experiment. There were no significant differences in averaged daily BHA-intake between the three BHA-fed groups (BA: 1.32 ± 0.05 g BHA/kg/day; BI: 1.27 ± 0.11 g BHA/kg/day; BW: 1.31 ± 0.07 g BHA/kg/day)(24). The averaged daily acetylsalicylic acid-intake in acetylsalicylic acid-consuming group was 292 ± 19 mg acetylsalicylic acid/kg/day while indomethacin-intake in indomethacin-administered group was 3.61 ± 0.55 mg indomethacin/kg/day.

Data on excretion into urine of BHA, its demethylated metabolites TBHQ and TBQ, either as free-BHA, free-TBHQ and free-TBQ or conjugated with glucuronic acid or sulphate are shown in Table 3-1. There appeared to be no differences in the levels of total-BHA in the urine between the BA resp. BI and BW treated rats. Excretion of total-BHA into urine accounted for 42-45% of the ingested amount of BHA (i.e. 0.57 ± 0.02 g/kg/day), only 11 to 31 mg/kg/day (0.81-2.39%) being detected as free BHA. The amount of free-BHA was significantly lower in the BA- vs the BW-group. TBHQ was mainly excreted in its conjugated form. Averaged urinary excretion of total-TBHQ during the experimental period was enhanced in the BA-rats as compared to the BW-group (0.88 ± 0.44 resp., 0.59 ± 0.16% of the ingested amount of BHA). The amount of free-TBHQ in urine was significantly lower in the BA and BI group as compared to the control group

(BW) (0.014 ± 0.007 resp. 0.015 ± 0.005 vs 0.037 ± 0.015%). The effect of 14-day treatment with acetylsalicylic acid resp. indomethacin resulted in a significant decrease of total-TBQ excretion in urine (BA: 0.67 ± 0.19; BI: 0.78 ± 0.28; BW:1.66 ± 0.96% of the amount of BHA ingested during the period of urine collection). TBQ was also mainly conjugated with glucuronic acid or sulphate.

Table 3-1: Excretion of BHA and metabolites into urine

Treatment	BW: BHA only	BI: BHA + indomethacin	BA: BHA + acetylsalicylic acid
free BHA	2.392 ± 0.763	1.912 ± 1.001	0.801 ± 0.196[a]
total BHA	44.61 ± 5.02	44.10 ± 8.46	41.93 ± 8.11
free TBHQ	0.037 ± 0.015	0.015 ± 0.005[a]	0.014 ± 0.007[a]
total TBHQ	0.589 ± 0.164	0.489 ± 0.160	0.878 ± 0.435
free TBQ	0.002 ± 0.001	0.001 ± 0.0003	0.0004 ± 0.0002[a]
total TBQ	1.679 ± 0.955	0.781 ± 0.280[a]	0.672 ± 0.189[a]

Excretion of BHA, TBHQ and TBQ in the urine determined by HPLC. Values are expressed as the amount of BHA ingested during the period of urine collection. Values marked with superscripts differ significantly (Students-*t*-test) from the BW group (BI cq BA versus BW; a: $p<0.05$). Mean values ± SD.

Discussion

Various peroxidase enzymes have been implicated in the bioactivation of xenobiotics. Prostaglandin H synthase has been shown to cooxidize a spectrum of xenobiotic compounds including carcinogens, to potentially harmful reactive intermediates. The majority of these compounds is cooxidized by virtue of their ability to serve as reducing cofactors for the peroxidase moiety of the enzyme. Antioxidants such as BHA, an established forestomach carcinogen in rodents, are effective electron donors and therefore likely substrates for peroxidase enzymes (31). Recently, we have shown that coadministration of prostaglandin H synthase inhibitors (acetylsalicylic acid/indomethacin) significantly inhibited the proliferative effect of BHA in gastro-intestinal tract tissues (24). Moreover: BHA also appeared to be an inhibitor of arachidonic and linoleic acid metabolism by prostaglandin H synthase and lipoxygenase (24). It is therefore probable that BHA or BHA-metabolites such as TBHQ compete with arachidonic acid and/or linoleic acid for prostaglandin H synthase. Subsequent TBHQ conversion into the corresponding quinone yielding active oxygen species, might be responsible for the carcinogenic response elicited by this antioxidant.

ESR measurements, as performed in this study, reveal that autoxidation of TBHQ occurs in phosphate-buffered saline; both the SQ and DMPO-OH signal

appeared after 15 minutes incubation at 37°C. Addition of DTPA inhibited the SQ formation suggesting a role of iron. TBHQ oxidation into SQ is probably caused by a reduction of Fe^{3+} into Fe^{2+}:

$$TBHQ + Fe^{3+} \rightarrow SQ + Fe^{2+} \qquad (1)$$

Subsequently, in presence of oxygen, the SQ radical can generate the superoxide anion radical:

$$SQ + O_2 \rightarrow TBQ + O_2^{-}\cdot \qquad (2)$$

The superoxide anions formed are trapped by DMPO to DMPO-OOH, which is highly unstable under normal experimental conditions and decomposes into a false DMPO-OH adduct. The increase in DMPO-OH signal in the presence of SOD can be explained by dismutation of $O_2^{-}\cdot$ under formation of hydrogen peroxide, subsequent formation of hydroxyl radicals by the Fenton reaction and trapping of hydroxyl radicals by DMPO:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + \cdot OH \qquad (3)$$

Addition of catalase prevented the appearence of the DMPO-OH spectrum. This observation suggests that the formation of the DMPO-OH spectrum was dependent upon the presence of superoxide and hydrogen peroxide, formed via the dismutation of superoxide. Addition of *tert*-butyl alcohol, a hydroxyl radical scavenger, decreased the intensity of the DMPO-OH signal, indicating that hydroxyl radicals are formed under these experimental conditions. $\cdot OH$ may be produced from hydrogen peroxide, formed after dismutation of superoxide, in the presence of a suitable transition metal such as Fe^{2+} (equation 3).
Addition of prostaglandin H synthase in presence and absence of arachidonic acid resulted in a substantial acceleration of SQ formation. This stimulation is even more marked in incubations with prostaglandin H synthase only, suggesting a competition between TBHQ and arachidonic acid for prostaglandin H synthase activity. Addition of catalase and *tert*-butyl alcohol did hardly influence the SQ signal intensity but reduced the DMPO-OH signal significantly. SOD however, increased the intensity of DMPO-OH but decreased the SQ-signal significantly as a result of an equilibrium shift to the right of equation 2. This indicates that SQ formation is not dependent on oxygen radicals, but reactive oxygen species are formed during subsequent oxidation of SQ into TBQ, as described above. Thus, addition of a peroxidase such as prostaglandin H synthase results in a substantial acceleration of TBHQ metabolism into SQ. Subsequently, redoxcycling between the semiquinone radical and quinone occurs, which is accompanied by formation of superoxide anion radicals, hydrogen peroxide and hydroxyl radicals.
Incubations of TBHQ and lipoxygenase had no effect on either SQ formation

and DMPO-OH under the chosen experimental conditions. We have however demonstrated that lipoxygenase is capable of converting TBHQ into TBQ. It remains possible that the period of incubation is too short or that the amount of radicals formed, is too low to be detected by means of ESR.

Quantitation of enzymatic metabolism of TBHQ by prostaglandin H synthase and lipoxygenase showed that the rate of autooxidation of TBHQ in buffer was low (2%) when compared to incubations in which enzymes were available to support redoxcycling. Both prostaglandin H synthase and lipoxygenase were capable of converting approximately 10% of the original amount of TBHQ into its corresponding quinone TBQ. Addition of cofactors resulted in a substantial acceleration of the oxidation of TBHQ into TBQ. These results confirm the finding that prostaglandin H synthase (and lipoxygenase) is (are) capable of oxidizing TBHQ.

Peroxidation of BHA was furthermore studied *in vivo*. The excretion of free-BHA, -TBHQ, -TBQ as well as its conjugated forms in the urine was assessed in male Wistar rats fed 3-BHA, or 3-BHA in combination with prostaglandin H synthase-inhibitors. Most of the dosed 3-BHA was excreted into urine conjugated with glucuronic acid and/or sulphate. Combined excretion of BHA, TBHQ and TBQ into urine did not differ significantly between the BW and BA group (46.9%, resp. 43.5%). Averaged urinary TBHQ excretion was significantly increased in the BA vs the BW group; TBQ excretion was correspondingly lower in the BA and BI vs the BW group. Excretion of free-BHA was however significantly decreased in the BA vs the BW-group, suggesting that acetylsalicylic acid might affect BHA resorption. Regression analysis revealed that the labeling indices of forestomach tissue, as reported previously (24), are negatively correlated with TBQ excretion into urine, e.g. with TBQ formation ($p<0.05$). This indicates that prostaglandin H synthase is involved in the metabolism of TBHQ into TBQ, and that this metabolic pathway contributes to the increase in cell proliferation induced by BHA.

These conclusions are in line with previous results suggesting the role of oxygen species in toxic and carcinogenic effects of BHA. It was demonstrated that both TBHQ and TBQ were capable of stimulating superoxide formation in liver microsomes of phenobarbital-pretreated rats. Moreover, the superoxide production in liver microsomes was accompanied by formation of hydrogen peroxide and hydroxyl radicals (22). Recently, it was shown that the DNA damaging capacity of the quinone, on rat forestomach epithelium was much higher than that of BHA and TBHQ; TBQ maintained its potential for the generation of active oxygen species even after its binding to cellular thiols (23). Furthermore, previous studies on BHA-fed rats revealed small amounts of TBHQ and TBQ to be present in the forestomach epithelium (32).

In summary, we have demonstrated that TBHQ, the hydroquinone metabolite of BHA, is metabolized by the peroxidase moiety of both prostaglandin H synthase and lipoxygenase. Inhibition of prostaglandin H synthase by acetylsalicylic acid or indomethacin resulted in an inhibition of the proliferative

effect induced by BHA. Furthermore, the prostaglandin H synthase-dependent TBHQ-metabolism is accompanied by oxidative stress and may therefore be an important pathway in producing the genetic and cellular damage necessary for BHA-induced toxicity and carcinogenicity.

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Chapter 4

The role of prostaglandin H synthase-mediated metabolism in the induction of oxidative DNA-damage by BHA metabolites

P.A.E.L. Schilderman, J.M.S. van Maanen, F.J. ten Vaarwerk, M.V.M Lafleur[1], E.J. Westmijze[1], F. ten Hoor[2] and J.C.S. Kleinjans.

Departments of Health Risk Analysis and Toxicology and [2]Human Biology, University of Limburg, P.O. Box 616, 6200 MD Maastricht, The Netherlands and [1]Department of Anthropogenetics, Free University, van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands.

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Abstract

The carcinogenicity of the phenolic food antioxidant butylated hydroxyanisole (BHA) may be related to its oxidative biotransformation *in vivo*. In order to determine the ability of BHA, 2-*tert*-butyl(1,4)hydroquinone (TBHQ) and 2-*tert*-butyl(1,4)paraquinone (TBQ) to induce oxidative DNA damage, biological inactivation of ss bacteriophage ϕX-174 DNA, as well as induction of 8-oxodG in dG by these compounds was studied *in vitro*, in presence and absence of peroxidases. Both test systems showed that BHA and TBQ (probably due to lack of reductase activity *in vitro*) were not capable of induction of oxidative DNA damage. TBHQ however appeared to be a strong inactivator of phage DNA as well as a potent inducer of 8-oxodG formation. Addition of radical scavengers showed that this damage was due to formation of superoxide anion, hydrogen peroxide and hydroxyl radicals. Addition of iron chelators and metal ions showed that the one-electron oxidations of TBHQ via the semiquinone radical into TBQ are toxic via the formation of oxygen radicals and are not directly due to the hydroquinone itself or the formation of semiquinone radicals. Although peroxidation of TBHQ by prostaglandin H synthase is indicated to result in a superoxide anion burst, this is not accompanied by an increase in oxidative DNA damage *in vitro*. This might be due to the use of hydrogen peroxide as a substrate by prostaglandin H synthase itself, consequently resulting in less formation of hydroxyl radicals. Oxidation of TBHQ by lipoxygenases showed that no semiquinone radicals or oxygen radicals were formed, probably due to a

two-electron oxidation of TBHQ directly into TBQ.
The present results indicate that metabolic activation of BHA yielding reactive oxygen species may induce a carcinogenic potential, since the BHA-metabolite TBHQ, appeared to be a strong inducer of oxidative DNA damage.

Introduction

The carcinogenic and toxic effects of the synthetic phenolic food antioxidant butylated hydroxyanisole (BHA) have been extensively studied. In animal studies, BHA has been found to induce the formation of papillomas and carcinomas in the forestomach of various rodent species (1-3). These dose-dependent changes in rodent forestomach tissue appeared to be accompanied by an increase in cellular proliferation rate in the early stage of neoplastic development (4, 5). In rodents, however, other tissues are also susceptible for cell-growth-enhancing effects of BHA (4, 6, 7). These effects of BHA have been ascribed to an epigenetic mechanism, because BHA has yielded negative results in most tests for mutagenicity (8 - 12).
Recently, several reports indicate that the antioxidant BHA paradoxically possesses prooxidant capacity, which might be involved in its toxic properties. BHA is oxidatively demethylated by cytochrome p450 to *tert*-butylhydroquinone (TBHQ) *in vivo* in dogs (13), in rats and in man (14), and *in vitro* in rat liver microsomes (15). Induction of cytochrome p450 by phenobarbital increased the oxidative demethylation of BHA into TBHQ but did not affect forestomach labelling index in rat forestomach (16). It is therefore unlikely that the proliferation enhancing effects of BHA are attributable to this p450-mediated biotransformation pathway. A stimulation of NADPH oxidase activity and an increase of hydrogen peroxide formation by BHA was observed in liver microsomes (17, 18). Furthermore, it was demonstrated that addition of BHA to liver microsomes of phenobarbital-pretreated rats leads to excess production of superoxide, hydrogen peroxide as well as hydroxyl radicals (19). Moreover, TBHQ autoxidizes to 2-*tert*-butyl(1,4)paraquinone (TBQ) and TBQ exceeds TBHQ by far in its capacity for superoxide production in microsomes (19). Also, the microsome-mediated clastogenicity of BHA in cultured Chinese hamster ovary cells has been suggested to be due to generation of reactive oxygen species by BHA and more markedly by its quinone-metabolite (20). These reports indicate that the carcinogenic effects of BHA are due to the conversion of the parent compound to more reactive metabolites like TBHQ and TBQ.
Recently, we have demonstrated that coadministration of prostaglandin H synthase inhibitors significantly reduced the proliferative effect of BHA in gastro-intestinal tract tissues (5). We subsequently showed that addition of a peroxidase such as prostaglandin H synthase resulted in a substantial acceleration of TBHQ metabolism into TBQ, which was accompanied by superoxide anion, hydrogen peroxide and hydroxyl radical formation (21). The hydroxyl

radical is the most reactive oxygen metabolite and is suggested to be responsible for some of the toxic effects occurring during redox-cycling processes, causing peroxidation of lipids, protein- and DNA-damage (22). A particular type of oxidative DNA-damage is C8 oxidation of deoxyguanosine (dG). The resulting 8-oxodeoxyguanosine (8-oxodG) may be used as a biological marker of oxidative stress to DNA (23).

We therefore hypothesized that metabolic activation of BHA profoundly contributes to its carcinogenic potential. Prostaglandin H synthase can be involved in the metabolic activation of BHA by converting the cytochrome p450-generated hydroquinone-metabolite to the corresponding quinone thereby generating reactive oxygen species which can induce oxidative DNA damage, ultimately inducing an increase in cell proliferation.

In this study, the oxidation of dG by BHA, TBHQ and TBQ has been quantified *in vitro*, using HPLC with electrochemical detection. By determination of modulating effects of radical scavengers on the oxidation of dG, identification of the reactive oxygen species involved, became possible. Furthermore, in order to determine the effect of enzymatic peroxidative activation of TBHQ on 8-oxodG formation similar incubations have been performed with the peroxidases: prostaglandin H synthase and lipoxygenase.

It has been demonstrated that ss bacteriophage ϕX-DNA can be inactivated by oxidative damage. This assay provides a relatively simple system in which biological consequences of interaction between naked ss phage DNA and BHA (respectively TBHQ and TBQ) as well as generated oxygen radicals can be studied without interference of cellular components. Studying the biological inactivation of ϕX-DNA by BHA or its metabolites TBHQ and TBQ in relation with effects on oxidation of 2'-deoxyguanosine (dG) might contribute to elucidation of the mechanism of BHA toxicity.

Materials and Methods

Materials

BHA (food grade BHA, purity > 99%, 97.5% 3-BHA) was obtained from J. Dekker Co. (Wormerveer; The Netherlands). TBHQ (purity > 97%), and *tert*-butyl alcohol were purchased from Aldrich Chemical Co. (Brussels, Belgium). lipoxygenase, chromium trioxide, diethylenetriaminepentaacetic acid (DTPA), superoxide dismutase (SOD), desferrioxamine mesylate and dG were obtained from Sigma (St Louis, MO), diethylether from BDH (Poole, UK), and prostaglandin H synthase from Cayman (Ann Arbor, MI). Methanol was HPLC grade and was obtained from Rathburn (Walkerburn, UK). Water was purified using a milli-Q water purification system. Analytical reagent-grade chemicals were used in all other instances. TBQ was synthesized by oxidizing 2-*tert*(1,4) butylhydroquinone (TBHQ, 10 mmol) with chromium trioxide (60 mmol) in pyridine as described previously (21).

Induction of 8-oxodG by BHA, TBHQ and TBQ
BHA, TBHQ and TBQ were dissolved in 0.5 ml of 2.7 mM solution of dG in 10 mM Tris/HCl, pH 7.4, and incubated at 37°C for periods for 1 hour up to 17 hours. DMSO was excluded from incubation mixtures because addition of DMSO resulted in elevated background levels of 8-oxodG (data not shown). Furthermore, formation of 8-oxodG was quantified by HPLC after peroxidation of TBHQ by 200 units of prostaglandin H synthase or 350 units of lipoxygenase, with and without the sodium salt of arachidonic acid (0.2 mM) and linoleic acid (0.2 mM) as substrate, respectively. All incubations using prostaglandin H synthase contained 1 µM haematine whereas incubations with lipoxygenase contained 1 nM hydrogen peroxide as additional cofactor. In order to determine the effect of superoxide anion radicals, incubation mixtures were supplemented with SOD (120 units). Incubations with *tert*-butyl alcohol (100 mM) were performed to investigate the role of OH-radicals. To determine the involvement of hydrogen peroxide catalase (1000 units) was added to the incubation mixtures. Furthermore, in order to determine the effect of iron in radical formation, incubation mixtures of TBHQ were supplemented with DTPA (pH adjusted to pH 7.4), desferrioxamine mesylate (1 mM), Fe^{2+} (0.2 mM) and Fe^{3+} (0.2 mM), respectively. All incubations were performed in duplicate.

HPLC-analysis of 8-oxodG
Reversed phase HPLC was performed with a Spectroflow 480 Solvent delivery system coupled with a Kratos spectroflow 783 programmable absorbance detector and an Antec electrochemical detector (850 mV). The working electrode is constructed of a glassy carbon type (the diameter is 3 mm resulting in an effective cell volume of less than 0.35 µl). The reference electrode is of the Ag/AgCl type and consists of a silver wire coated with a melt of AgCl. Electrical contact with the detector cell is accomplished by a salt bridge of saturated KCl. A Supelcosil™ column (Supelco; 250 x 4.6 mm) was used in conjunction with a guard column (ODS pellicular 30 x 2.1 mm). The mobile phase consisted of 10% aqueous methanol containing citric acid (12.5 mM), sodium acetate (25 mM), acetic acid (10 mM) and NaOH (30 mM). Elution was performed at a flow rate of 1.0 ml/min. The limit of detection was 40 fmol absolute for 8-oxodG. Students *t*-test was used for the comparison of averaged oxidation ratios, calculated from duplicate incubations and analyses.

Inactivation of ss ϕX-174 DNA by BHA, TBHQ and TBQ.
Single-stranded ϕX-174 DNA was isolated from wildtype ϕX-174 DNA bacteriophage according to Blok et al. (24). Solutions of ss ϕX-174 DNA (1 µg/ml) in 0.50 ml 0.01 M phosphate buffered saline pH 7.3 were incubated at 37°C for increasing periods of time with BHA, TBHQ and TBQ (0.1mM) in the presence of 1% DMSO. Furthermore, the biological inactivation of ϕX-174 DNA was investigated after peroxidation of TBHQ by prostaglandin H synthase or lipoxygenase, with or without the sodium salt of arachidonic acid and linoleic

acid as substrates, respectively. In order to study the effect of DMSO, which has shown to stabilize the semiquinone radical of TBHQ, on biological inactivation of ss ϕX-174 DNA increasing concentrations of DMSO (1%, 10% and 30%) were added to the incubation mixtures. Concentrations of scavengers, enzymes, cofactors as well as incubation conditions are comparable to those applied in the dG incubations. Data on incubations with DTPA are excluded, since addition of DTPA to incubation mixtures resulted in acidification, which results in depurination of the DNA and subsequently inactivation.
At several time intervals of incubation, samples of 20 µl were taken and diluted 50-fold with ice-cold 0.05 M TRIS-HCl buffer (pH 8.0) to stop the reaction. The biological activity of ϕX-174 DNA was determined by measuring the bacteriophage production after transfection of the DNA to freshly prepared E-coli (AB 1157) spheroplasts as described previously (25). The number of plaques provides a measure for the non-damaged part of the DNA.

Results

Induction of 8-oxodG in dG by BHA, TBHQ and TBQ.
Dose-response relationships between BHA, TBHQ and TBQ concentrations and the ratio of induced 8-oxodG/dG are presented in Figure 4-1. The background level of 8-oxodG appeared to be $2.1 \cdot 10^{-5}$ 8-oxodG/dG during 17 hours of incubation at 37°C. This ratio increases two-fold during 17 hours of incubation with respectively BHA (0.08 mM) and TBQ (0.08 mM). TBHQ incubation (0.1 mM) during the same time-period however resulted in a seventeen-fold increase in 8-oxodG formation. The kinetics of 8-oxodG formation by TBHQ (0.1 mM) show a linear increase in 8-oxodG formation during the first seven hours, followed by a slower phase of 8-oxodG formation from 7-25 hours of incubation. The background level remained low (data not shown).
The effects of TBHQ in presence and absence of radical scavengers, iron chelators and metal ions are given in Table 4-1. Incubation of TBHQ only resulted in a ratio of $34.9 \cdot 10^{-5}$ 8-oxodG/dG. Addition of SOD (200 units), *tert*-butyl alcohol (100 mM) and catalase (1000 units) all significantly ($p<0.05$) decreased the oxidation ratio by respectively 32%, 22%, and 95%. Addition of both SOD (200 units) and *tert*-butyl alcohol (100 mM) showed no additional decrease in 8-oxodG formation (data not shown). No effects of scavengers have been found on background levels of 8-oxodG. DTPA and desferrioxamine mesylate, both iron chelators, almost completely inhibited 8-oxodG formation. Addition of Fe^{2+} however increased the oxidation ratio by 35%, while supplementation of the incubation mixture with Fe^{3+} resulted in a significant decrease of the ratio by 88% ($p<0.05$).

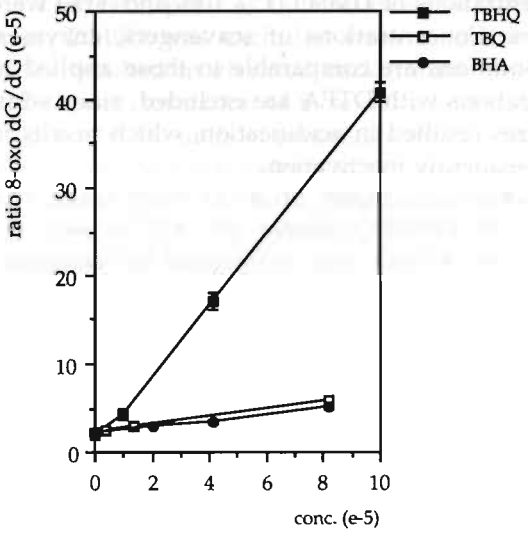


Figure 4-1. Oxidation ratio of dG (2.7 mM) induced by BHA, TBQ and TBHQ during 17 hours of incubation at 37°C, Mean ± SD calculated from duplicate incubations and analyses.

Table 4-1: The effect of autoxidation of TBHQ on 8-oxodG induction in presence and absence of radical scavengers, after 17 hours of incubation at 37 °C.

Incubation	Ratio 8-oxodG/dG (e-5)
TBHQ	34.90 ± 7.21
TBHQ + SOD	23.6 ± 2.83[a]
TBHQ + *tert*-butyl alcohol	27.2 ± 2.55[a]
TBHQ + catalase	1.95 ± 0.21[a]
TBHQ + Fe^{2+}	47.2 ± 0.99[a]
TBHQ + Fe^{3+}	4.20 ± 0.28[a]
TBHQ + DTPA	2.02 ± 1.70[a]
TBHQ + desferrioxamine mesylate	5.48 ± 2.32[a]

Incubation conditions are as indicated in the Method section. Mean ± SD from duplo incubations and analysis. Values marked with superscripts differ significantly (students-*t*-test) as compared to the control incubation. $p<0.05$ is considered significant.

The effect of enzymatic peroxidation of TBHQ by prostaglandin H synthase respectively lipoxygenase in presence and absence of scavengers is shown in Table 4-2. Peroxidation of TBHQ by prostaglandin H synthase resulted in an

equal level of 8-oxodG/dG as compared to 8-oxodG induction by TBHQ spontaneously. Addition of SOD (200 units) to incubation mixtures of TBHQ and prostaglandin H synthase resulted in a two-fold increase in the oxidation ratio ($p<0.05$). Further, addition of *tert*-butyl alcohol (100 mM) decreased the 8-oxodG induction by 50% ($p<0.05$). Addition of catalase (1000 units) decreased the oxidation ratio to background levels ($p<0.01$) Since addition of arachidonic acid as additional substrate resulted in elevated background levels of 8-oxodG indicating side-effects of arachidonic acid on DNA oxidation, incubations have been performed without this substrate. Prostaglandin H synthase/haematine did not affect the background level of 8-oxodG/dG.

Table 4-2: The effect of enzymatic peroxidation of TBHQ by lipoxygenase resp. prostaglandin H synthase on 8-oxodG induction in presence and absence of radical scavengers, after 17 hours of incubation at 37°C.

Incubation	Ratio 8-oxodG/dG (e-5)
TBHQ	34.90 ± 7.21
TBHQ + Lipoxygenase	6.35 ± 0.35[a]
TBHQ + Lipoxygenase + SOD	3.75 ± 0.49
TBHQ + Lipoxygenase + *tert*-butyl alcohol	7.05 ± 1.06
TBHQ + Lipoxygenase + catalase	7.30 ± 1.41
TBHQ + prostaglandin H synthase	39.70 ± 5.23
TBHQ + prostaglandin H synthase + SOD	73.00 ± 9.90[b]
TBHQ + prostaglandin H synthase + *tert*-butyl alcohol	19.80 ± 1.13[b]
TBHQ + prostaglandin H synthase + catalase	3.90 ± 0.99[b]

Incubation conditions are as indicated in the Method section. Mean ± SD from duplo incubations and analysis. Values marked with superscripts differ significantly (students-*t*-test) as compared to the appropriate control incubation. [a]$p<0.05$ as compared to the control incubation without peroxidase; [b]$p<0.05$ by comparison of radical scavenging activity to the appropriate incubation with peroxidase activity.

Peroxidation of TBHQ by lipoxygenase resulted in a significant decrease ($p<0.05$) of the oxidation ratio as compared to induction of 8-oxodG by TBHQ spontaneously. No effects of SOD, *tert*-butyl alcohol and catalase have been found on 8-oxodG levels as compared to incubation mixtures containing TBHQ and lipoxygenase alone. Whereas lipoxygenase/hydrogen peroxide did not affect background levels of 8-oxodG; linoleic acid, an additional substrate of lipoxygenase, increased the background levels of 8-oxodG; therefore, incubations have been performed without linoleate.

Inactivation of single-stranded φX 174 DNA

The surviving fraction of bacteriophage DNA after incubation with BHA, TBQ

and TBHQ is shown in Figure 4-2. The survival curves clearly show that BHA had no effect on ss ϕX DNA-activity. In incubations with TBQ hardly any inactivation was observed, whereas TBHQ caused an inactivation to a degree of 4 decades within 30 hours.

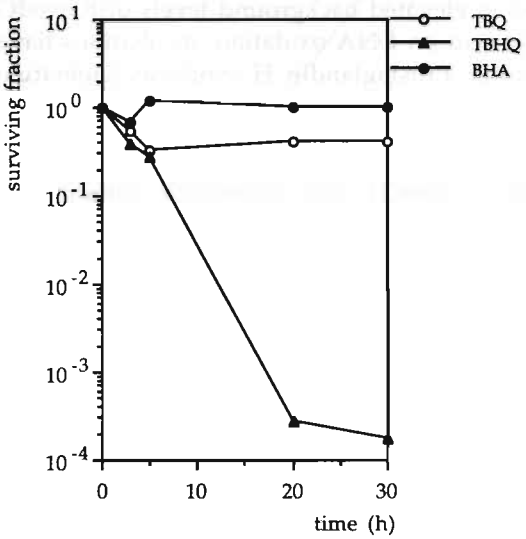


Figure 4-2: Surviving fraction of ss ϕX–174 DNA during 30 hours of incubation at 37°C with respectively 0.1 mM BHA, TBHQ, and TBQ.

Maximal inactivation of ss ϕX DNA was found after 17 hours of incubation of phage DNA with 0.1 mM TBHQ, as is shown in Figure 4-2. Further, addition of *tert*-butyl alcohol, SOD or catalase all resulted in an almost complete reduction of DNA-damage induction by TBHQ (Fig. 4-3).
The survival curves of ss ϕX-174 DNA incubated with TBHQ in presence of 1%, 10% and 30% DMSO showed that an increase in DMSO concentration resulted in less inactivation of ss ϕX DNA (data not shown).
The surviving fraction of DNA after incubation with TBHQ in the presence of lipoxygenase appeared to be about 3 decades higher as compared to the degree of inactivation by TBHQ itself (Fig. 4-4). Addition of *tert*-butyl alcohol, SOD or catalase to incubations of TBHQ with lipoxygenase had no effect on the surviving fraction (data not shown). Addition of linoleic acid as substrate to incubations of TBHQ with lipoxygenase also resulted in less inactivation of ϕX-174 DNA as compared to incubations of TBHQ only (Fig. 4-4). Lipoxygenase did hardly affect the surviving fraction of ss ϕX DNA; however lipoxygenase in combination with linoleic acid was also found to inactivate ϕX DNA by about 3.5 decades in 30 hours (data not shown).

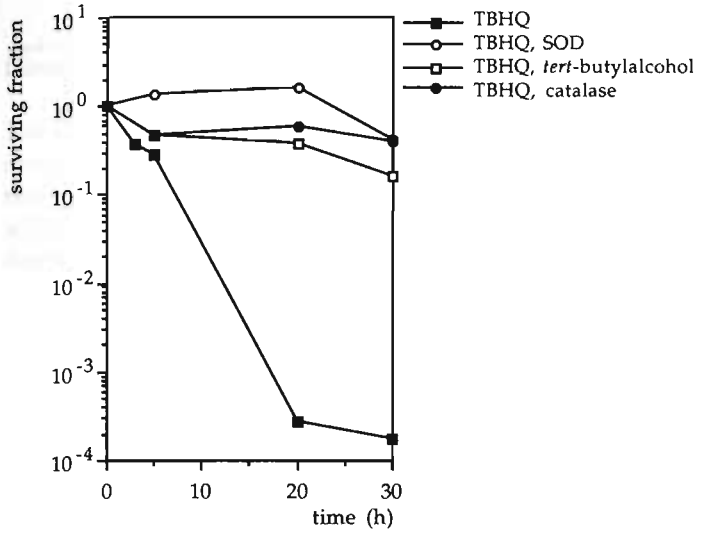


Figure 4-3: Surviving fraction of ss ϕX-174 DNA during 30 hours of incubation at 37°C of 0.1 mM TBHQ; 0.1 mM TBHQ + 120 units SOD; 0.1 mM TBHQ + 1000 units catalase and 0.1 mM TBHQ + 100 mM *tert*-butyl alcohol.

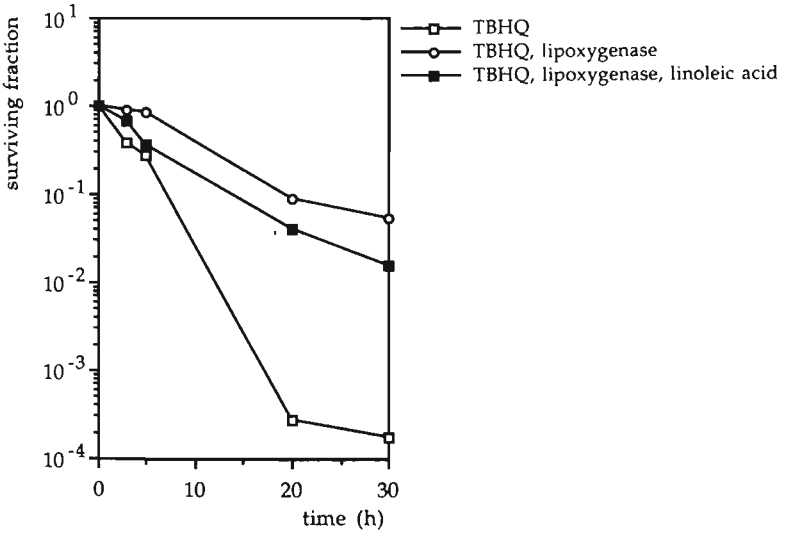


Figure 4-4. Surviving fraction of ss ϕX-174 DNA during 30 hours of incubation at 37°C of 0.1 mM TBHQ; 0.1 mM TBHQ + 350 units lipoxygenase; 0.1 mM TBHQ + 350 units lipoxygenase + 0.2 mM linoleic acid.

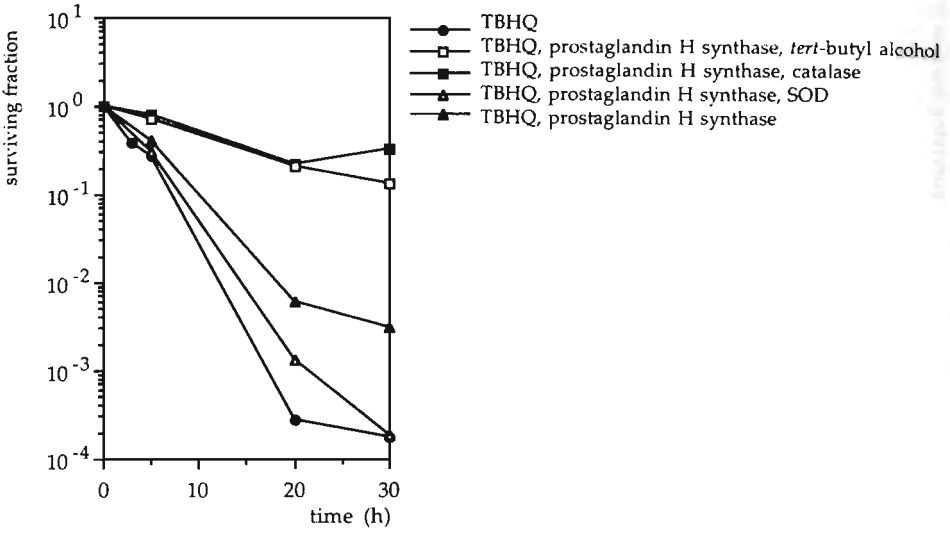


Figure 4-5. Survival curves of ss ϕX DNA during 30 hours of incubation at 37°C of 0.1 mM TBHQ; 0.1 mM TBHQ + 200 units prostaglandin H synthase; 0.1 mM TBHQ + 200 units prostaglandin H synthase + 120 units SOD; 0.1 mM TBHQ + 200 units prostaglandin H synthase + 1000 units catalase and 0.1 mM TBHQ + 200 units prostaglandin H synthase + 100 mM *tert*-butyl alcohol.

Peroxidation of TBHQ by prostaglandin H synthase resulted in almost 2 decades less inactivation of the DNA as compared to spontaneous inactivation by TBHQ (Fig. 4-5). The surviving fraction present after addition of SOD to the incubation mixture of TBHQ and prostaglandin H synthase, appeared to be about one decade higher as compared to inactivation in absence of SOD. Furthermore, addition of *tert*-butyl alcohol and catalase both resulted in an almost complete reduction of DNA-damage induced by prostaglandin H synthase-mediated metabolism of TBHQ. Addition of arachidonic acid as additional substrate to TBHQ incubations with prostaglandin H synthase resulted in less inactivation of ϕX DNA as compared to incubations of prostaglandin H synthase and TBHQ (data not shown).

In summary: both assays showed that BHA and TBQ were not capable of induction of oxidative DNA-damage. The hydroquinone metabolite TBHQ however appeared to be a strong inactivator of phage DNA as well as a potent inducer of 8-oxodG formation *in vitro*. Addition of radical scavengers showed that this damage was due to superoxide anion, hydrogen peroxide as well as hydroxyl radical formation. Although peroxidation of TBHQ by prostaglandin H synthase resulted in a superoxide anion burst as demonstrated by the increase in DNA oxidation and ϕX DNA inactivation after incubation in the presence of SOD, this was not accompanied by an increase in oxidative DNA da-

mage exceeding the effect obtained by TBHQ itself. Oxidation of TBHQ by lipoxygenase resulted in an almost complete reduction of oxidative DNA damage.

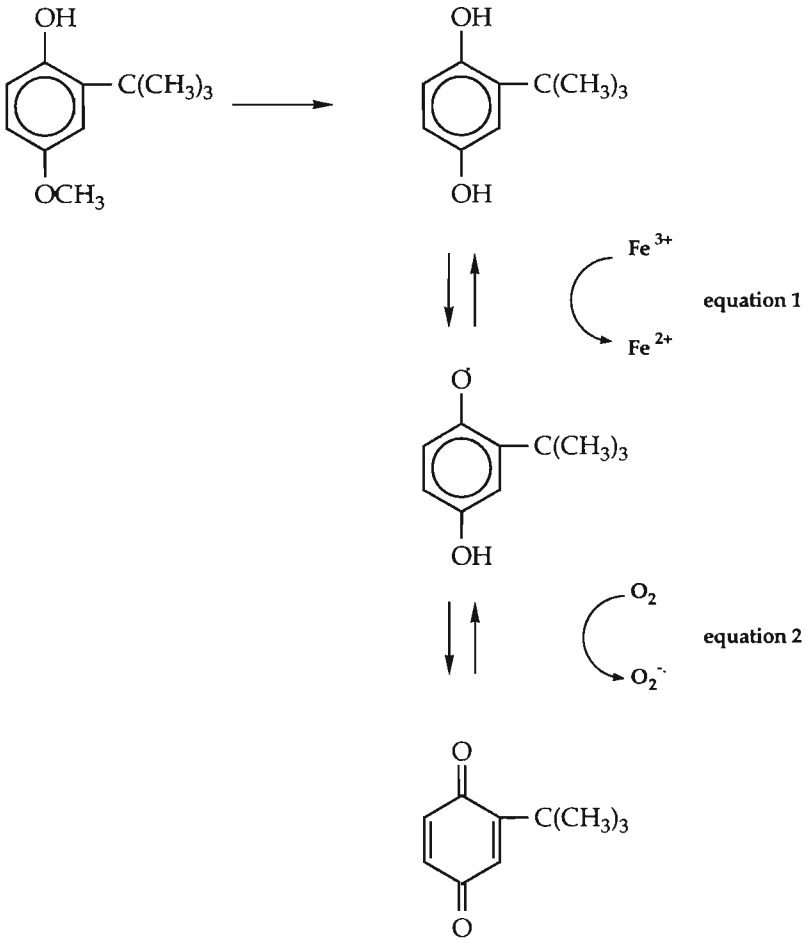
Discussion

The present experiments show that BHA had no effect on the survival curves of ss ϕX-174 DNA. Furthermore, incubations of BHA with dG only resulted in a two-fold increase in 8-oxodG formation. These results confirm earlier results which indicate that the reactivity of BHA itself is probably not high enough to damage cellular macromolecules and that BHA becomes carcinogenic after being metabolized to more reactive compounds (26). In all species studied the main metabolic transformations of BHA are dimerization, conjugations and *O*-demethylation (26).*O*-demethylation of BHA by cytochrome p450 results in formation of TBHQ *in vivo* in rats and man (14) and in dogs (13), as well as *in vitro* in rat liver microsomes (12). Although the oxidative demethylation of BHA into TBHQ is elevated after phenobarbital treatment of rats, it did not affect the proliferation rate in rat forestomach tissue. The proliferation enhancing effects of BHA are therefore probably not attributable to this cytochrome p450 mediated biotransformation pathway (16).

Both test systems showed that TBQ was not capable of induction of oxidative DNA-damage. In general, quinones can be activated by reductive biotransformation *in vivo*. Previous studies have suggested that cytochrome p450 may be involved in the reduction of quinones. For example NADPH-cytochrome p450 reductase is involved in the reductive bioactivation of quinones in mammalian liver (27). Enzymatic one-electron reduction of quinones leads to the formation of a reactive semiquinone radical, which may react with molecular oxygen leading to the generation of the parent quinone and concomitant production of superoxide anion. Enzymes known to catalyse the one-electron reduction of quinones include NADPH cytochrome p450 reductase, NADH cytochrome b5 reductase and xanthine oxidase. BHA has previously shown to increase NADPH-quinone reductase activity in rats *in vivo* (28). This indicates that BHA may indirectly enhance its own carcinogenicity *in vivo* by induction of reductase activity. Moreover, it was shown that the DNA damaging capacity of TBQ on rat forestomach tissue was much higher than that of BHA and TBHQ; TBQ maintained its potential for the generation of reactive oxygen species even after its binding to cellular thiols (29).

Recently, we have demonstrated that coadministration of acetylsalicylic acid and indomethacin, two inhibitors of prostaglandin H synthase, resulted in a significant reduction of the proliferative effect of orally administrated BHA in rat gastro-intestinal tract tissues (5). TBHQ-metabolism into TBQ was significantly decreased in rats treated with BHA and prostaglandin H synthase-inhibitors as compared to rats treated with BHA only. This indicates that prostaglandin H synthase is involved in the metabolism of TBHQ into TBQ

and that this metabolic pathway contributes to the increase in cell proliferation induced by BHA (21).



$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$ **equation 3**

$H_2O_2 + Fe^{2+} \longrightarrow OH^{\cdot} + OH^- + Fe^{3+}$ **equation 4**

Scheme 4-1: proposed mechanism of action.

Short-term feeding studies in rats at doses of 0.25% - 2.0% TBHQ in the diet showed that the 1% TBHQ dose led to hyperplasia of the forestomach epithelium (30). In 2% TBHQ-treated animals brownish discolourations and mild hyperplasia of the forestomach mucosa with focally increased hyperplasia of basal cells was observed (31). TBHQ induced a lesser hyperplastic response than BHA at the maximum dose employed in these studies.

Inactivation studies with ϕX-174 DNA demonstrated the induction of lethal DNA modifications by TBHQ. The mechanism for the generation of reactive oxygen species we previously proposed (scheme 3-1, Chapter 3), is largely confirmed by the present results on TBHQ-induced inactivation of phage DNA in combination with the effects of radical scavengers. Autoxidation of TBHQ is accompanied by formation of superoxide anion radicals, hydrogen peroxide and hydroxyl radicals.

An increase in DMSO-concentration, which as we have previously shown results in an increase in semiquinone radical formation, was found to decrease the inactivation of phage DNA by TBHQ. Oxidation of dG by TBHQ was partly inhibited by SOD and *tert*-butyl alcohol. Catalase however completely inhibited 8-oxodG formation induced by TBHQ. These findings indicate that the one-electron oxidations of TBHQ via SQ into TBQ cause DNA oxidation via the formation of oxygen radicals, and that these effects are not directly due to the hydroquinones themselves or to semiquinone radicals. The effect of DMSO could be explained by the stabilization of the semiquinone radical resulting in decreased redox-cycling and decreased formation of oxygen radicals. This hypothesis is supported by the finding that addition of iron chelators, which prevent semiquinone formation, also inhibited oxidation of dG. Moreover, supplementation of the incubation mixture with Fe^{3+} dramatically decreased 8-oxodG-formation. Addition of Fe^{2+} on the contrary enhances hydroxyl radical formation by acceleration of the Fenton reaction (equation 4, scheme 4-1) which ultimately results in an increase in 8-oxodG-formation.

Since peroxidation of TBHQ by prostaglandin H synthase is known to induce radical formation, it was expected that addition of prostaglandin H synthase would result in higher inactivation ratios of phage DNA. However, during the first hours of incubation the inactivation is found to be equal with or without the presence of prostaglandin H synthase, whereas after 30 hours of incubation total inactivation is observed to be higher without peroxidation. After 17 hours of incubation of TBHQ with dG, the oxidation ratios were also found to be equal with or without peroxidation of TBHQ by prostaglandin H synthase. Apparently, a more rapid metabolism by enzymatic peroxidation of TBHQ resulting in higher superoxide anion and hydrogen peroxide formation does not result in increased oxidative DNA-damage. Addition of catalase completely inhibited the observed effects indicating that formation of hydrogen peroxide is crucial. It is possible that the peroxidase moiety of prostaglandin H synthase utilizes the formed hydrogen peroxide as substrate during its oxidation reaction (32, 33). As a result of this reaction less hydrogen peroxide is available for

hydroxyl radical formation. In both test systems, addition of SOD increased 8-oxodG formation respectively ϕX-174 DNA inactivation by TBHQ in the presence of prostaglandin H synthase. This is in agreement with earlier electron spin resonance results which indicate that peroxidation by prostaglandin H synthase induces a superoxide anion burst. After dismutation (equation 3), hydroxyl radicals are formed by the classical Fenton reaction (equation 4) which are responsible for the induced DNA damage. *In vivo* low levels of hydrogen peroxide can efficiently degraded by enzymes such as catalase. Oxidative DNA-damage can however be induced by oxygen radicals derived from hydrogen peroxide, under conditions in which the rate of production or hydrogen peroxide is too large, for it to be efficiently degraded by the enzymes normally present in the cellular system. These activated oxygen species may both lead to cell death and genetic alterations, thereby explaining the thresholded increase in cell proliferation in rat gastro-intestinal tract tissues.

Although we have shown that lipoxygenase is capable of converting TBHQ into TBQ this reaction appeared not to be accompanied by semiquinone-radical formation (21). This finding is confirmed by the present results on inactivation of phage DNA, respectively oxidation of dG. Oxidation of TBHQ by lipoxygenase resulted in both assays in a statistically significant decrease of oxidative DNA-damage as compared to autoxidation of TBHQ into TBQ. Addition of radical scavengers did not affect the induced damage, which confirms that no oxygen radicals are formed during the oxidation reaction by lipoxygenase. Lipoxygenases are dioxygenases, that produce no semiquinone radicals from TBHQ, which is in agreement with ESR measurements (20).

In conclusion, the results presented here indicate that BHA itself is not reactive enough to induce oxidative DNA damage. The quinone-metabolite also showed very low reactivity, probably due to lack of reductase activity *in vitro* (27). TBHQ however induced inactivation of phage DNA and 8-oxodG formation, due to the generation of reactive oxygen species. Although peroxidation of TBHQ by prostaglandin H synthase resulted in a superoxide anion burst and subsequently in hydrogen peroxide formation this was not accompanied by an increase in oxidative DNA-damage, probably due to hydrogen peroxide metabolism by prostaglandin H synthase itself. These findings do however not exclude the importance of this biotransformation pathway *in vivo*.

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Chapter 5

Induction of oxidative DNA damage and early lesions in rat gastro-intestinal epithelium in relation with prostaglandin H synthase-mediated metabolism of butylated hydroxyanisole.

P.A.E.L. Schilderman, F.J. ten Vaarwerk, J.T. Lutgerink, A. van der Wurff[1], F. ten Hoor[2] and J.C.S. Kleinjans.

Departments of Health Risk Analysis and Toxicology, [1]Pathology and [2]Human Biology, University of Limburg, P.O. Box 616, 6200 MD Maastricht, The Netherlands

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Abstract

In order to determine the effect of metabolic activation of the food additive 3-*tert*-butyl-4-hydroxyanisole by prostaglandin H synthase on the gastro-intestinal cell proliferation we studied the nature and the time-dependency of early lesions in forestomach, glandular stomach and colon/rectum of rats given BHA with and without coadministration of acetylsalicylic acid (an inhibitor of prostaglandin H synthase), in combination with the formation of oxidative DNA damage in the epithelial cells of glandular stomach and colon/rectum as well as in the liver.

BHA appeared to be a strong inducer of oxidative DNA damage in the epithelial cells of the glandular stomach, increasing the level of 7-hydro-8-oxo-2'-deoxyguanosine (8-oxodG) with increasing duration of BHA administration. Similar observations were made in colorectal DNA although levels of oxidative DNA damage tend to be smaller. In liver DNA, BHA appeared to be capable of increasing background 8-oxodG levels only after fourteen days of treatment. This relatively slow response may be related to very low prostaglandin H synthase-activity of liver cells.

The severity of hyperplasia and inflammation in both forestomach and glandular stomach appeared to increase gradually with continued BHA administration. The hyperplasia induced by BHA was paralleled by inflammatory changes. In colorectal tissue however, no tissue abnormalities were observed. This indicates that oxidative DNA damage induced by BHA is not a consequence of early lesions in gastro-intestinal epithelium, but might be the initial step in the stimulation of gastro-intestinal cell proliferation which, as we have shown previously, also occurs in colon epithelium.

Coadministration of the prostaglandin H synthase inhibitor acetylsalicylic acid

resulted in a significant decrease of both epithelial oxidative DNA damage and the incidence of lesions, which indicates that this enzyme system is involved in the enhancement of cellular proliferation induced by BHA. Cooxidation by prostaglandin H synthase of the BHA-metabolite *tert*-butylhydroquinone into *tert*-butylquinone yielding active oxygen species, might therefore be responsible for the carcinogenic effects of this food antioxidant.

Introduction

Butylated hydroxyanisole (BHA) has been used for many years as an additive to prevent oxidative spoilage of foods (1). However, in addition to its action as an antioxidant, BHA also exerts a wide variety of biological effects (2). At low doses, BHA appeared to be effective in inhibiting carcinogen-induced tumor development in rodents, partly as a result of the induction by BHA of specific enzymes in the cytochrome p450 complex (3-8). At high doses, BHA was found to induce carcinomas in rat and hamster forestomach epithelium (9, 10). Time- and dose-dependent changes in the forestomach epithelium include hyperplasia, papillomas and carcinomas (9, 11-14). Although the initial proliferative response is very rapid showing inflammation and hyperplasia with hyperkeratosis of the squamous epithelium (12), it takes a considerably longer period for carcinomas to develop (15). If BHA-induced forestomach lesions are caused by irritation, as expressed by epithelial cell necrosis or inflammation, they might be reversible. Simple hyperplasia and papilloma, in which the epithelium proliferates upwards, with and without basal cell proliferation, appear to be completely reversible (16). Basal cell hyperplasia, on the contrary, in which the epithelium proliferates downwards, persists after cessation of BHA-administration (16-18). Moreover, BHA-induced hyperplasia may not be due merely to regeneration following cell necrosis or reactive change of the epithelium in response to inflammation, because hyperplasia can also be observed in forestomach regions without inflammation or necrosis of epithelial cells (2). This indicates that the irritating potential of BHA is not solely responsible for its carcinogenic action. Furthermore, effects of BHA on cell proliferation are not restricted to the forestomach of rodents (19-23).

The negative results in most tests for mutagenicity (24-28) strongly suggest that BHA by itself does not react with DNA (29, 30). There are however several indications for the generation of reactive oxygen species during the metabolism of BHA or more specifically, of its primary metabolites 2-*tert*-butyl(1,4) hydroquinone (TBHQ) and its corresponding quinone (31-35). Production of excessive amounts of hydrogen peroxide and oxygen radicals may cause induction of enhanced cell proliferation.

Recently, we have demonstrated that coadministration of acetylsalicylic acid and indomethacin, both inhibitors of prostaglandin H synthase, resulted in a significant reduction of the proliferative effect of BHA in rat gastro-intestinal tract tissues (21). Moreover, prostaglandin H synthase appeared to be involved

in the metabolism of TBHQ into 2-*tert*-butyl(1,4)paraquinone (TBQ) causing formation of superoxide anion, hydrogen peroxide and hydroxyl radicals (35). Furthermore, TBHQ but not BHA or TBQ appeared to be a strong inducer of oxidative DNA-damage *in vitro*, due to the generation of reactive oxygen species (36). It is possible that these oxygen radicals directly react with intracellular macromolecules such as DNA, RNA and thiol groups of enzymes.
Reactive oxygen species are very difficult to study *in vivo*. However, detection of specific products of their reaction with DNA is feasible. This is not only indicative for the formation of oxygen radicals; it also shows their ability to penetrate into the nucleus. Reactive oxygen species may cause several types of DNA lesions. The chemical properties of one of them, 7-hydro-8-oxo-2'-deoxyguanosine (8-oxodG), allows for its detection in a DNA hydrolysate by HPLC in combination with electrochemical detection (37). This provides the possibility to investigate the relationship between oxidative damage in target tissue DNA and the carcinogenic potential of oxygen radical-forming agents, such as BHA.
In order to determine the pathogenesis of BHA-induced lesions in gastro-intestinal tract tissues, we studied the nature and the time-dependency of the early epithelial lesions in rat forestomach, glandular stomach and colon/rectum of rats given BHA, in relation with 8-oxodG formation in the epithelial cells of the same tissues as well as in the liver.
We suggest that prostaglandin H synthase-dependent TBHQ-metabolism may be an important pathway in the production of genetic and cellular damage by BHA.

Materials and Methods

Materials

BHA (food grade BHA, purity > 99 %, 97.5% 3-BHA) was obtained from J. Dekker CO. (Wormerveer; The Netherlands). Proteinase K (sp. act. 20 units/mg lyophilisate), RNase A (sp. act. 50 units/mg dry powder), RNase T1 (sp. act. 100.000 units/ml) and α-amylase (sp. act. 1000 units/mg) were purchased from Boehringer Mannheim (West Germany). Alkaline phosphatase (Type VII-N; sp. act. 10.000 units/ml) and nuclease P1 (sp. act. 200 units/mg protein) were obtained from Sigma (St. Louis, MO). Methanol was HPLC grade and was obtained from Rathburn (Walkerburn, UK). Analytical-grade chemicals were used at all other instances. Water was purified by means of a milli-Q water purification system.

Animals and maintenance

Male Wistar rats (Winkelmann, Borchen, Germany), 5 weeks old (weighing 94 ± 6; mean ± SD), were housed individually in metabolic cages in an air-conditioned room at 21-22°C and 50-55% relative humidity with a 12-hr light/dark cycle. Rats were randomly assigned to the different treatment groups. During 4 days of acclimatization the rats had free access to powdered standard labo-

ratory chow (diet No. SRM-A; Hope farms, Woerden, the Netherlands) and drinking water.

Treatment of animals and histopathological examination
Four groups of 18 rats each were studied. Group CB received an experimental diet consisting of powdered laboratory chow supplemented with 1.5% BHA. Group AB received the same diet plus acetylsalicylic acid dissolved in tap water (0.2%). Corresponding control groups consisting of the same number of animals, received the powdered diet without supplementation according to a paired-feeding protocol based on the food consumption of the BHA-groups. These groups were encoded CC respectively AC. The BHA-fed rats had free access to the food. Acetylsalicylic acid was added to the drinking water together with a sucrose carrier (5g/l). The vehicle-treated control rats received drinking water with sucrose carrier only. Drinking water was available to all rats ad libitum. Fresh drinking water was provided at 3 day intervals. Water and food intake were recorded daily in order to calculate the average daily intake of BHA and acetylsalicylic acid. Body weights were determined three times per week.
Groups of six rats within each group were designated for serial sacrifice at 3, 7 and 14 days of treatment. On the last day of the experimental diet, the animals were sacrificed under ether anaesthesia by exsanguination via the aorta. The gastro-intestinal tract tissues (forestomach, glandular stomach and colon/rectum) were dissected, opened lengthwise, cleaned of their contents, washed in ice-cold phosphate buffered saline (pH 7.4), cut into several pieces, partly stored at ice (0°C) for epithelial cell isolation and the remainder fixed in 70% ethanol for histochemistry. After fixation in 70% ethanol, the preparations were cut into strips, embedded in paraffin and sectioned. Slides containing sectioned tissue were stained with haematoxyline and eosine and examined by light microscopy. Histologically, epithelial lesions in forestomach tissue were classified in three categories: hyperplasia, epithelial defects and inflammatory reactions. Depending on the thickness of the mucosa, hyperplasia was classified as mild (0.1-0.25 mm), moderate (0.25-0.5 mm) and severe (> 0.5 mm). In rat glandular stomach, epithelial lesions were classified in two categories : hyperplasia and inflammatory reactions. Hyperplasia was classified in mild (0.3-0.4 mm) and moderate (> 0.4 mm) depending on the thickness of the mucosa.

DNA-extraction procedure
Within 3 hours after sacrificing the animals, epithelial cells were isolated. Organs were incubated at 37°C for 20 minutes in phosphate buffered saline containing 50 mM EDTA, the tubes were vigorously mixed several times in order to release epithelial cells; this procedure was repeated until the supernatant was clear. The fractions of epithelial cells were combined and frozen at -20°C until the DNA-extraction procedure. The livers were immediately frozen in liquid nitrogen and stored at -80°C until isolation of DNA.
DNA of epithelial cells and liver were isolated according to a published procedure (Gupta, 1984). In short: liver samples were homogenized in 1% SDS/

1mM EDTA and subsequently incubated with proteinase K. The homogenate was successively extracted with 1 vol of phenol, 1 vol of phenol/chloroform/isoamyl alcohol (25/24:1; v/v/v) and 1 vol of chloroform/isoamyl alcohol (24/1). After addition of 0.1 vol of 3 M NaAc (pH 6.0), nucleic acid was precipitated with 2 vol of ice-cold ethanol, washed with 70% ethanol to remove salt and dissolved in 5 mM Tris-HCl (pH 7.4). RNA was destroyed by addition of RNase T1 (150 U/mg DNA) and RNase A (300 µg/mg DNA) during 30 min of incubation at 38°C. After extraction of the digest with 1 vol chloroform/isoamyl alcohol (24/1; v/v), DNA was recovered as described above. DNA concentration was assayed spectrophotometrically. The DNA was digested to deoxynucleosides by treatment with nuclease P1 (25 U/mg DNA) and alkaline phosphatase (25 U/mg DNA) according to Lutgerink et al., 1992 (38). The extraction procedure for epithelial cells was the same except for the homogenization step.

HPLC/ECD-analysis of 8-oxodG

8-oxodG was detected by HPLC/ECD, which was performed using a Spectroflow 480 Solvent delivery system coupled with a Kratos spectroflow 783 programmable absorbance detector and an Antec electrochemical detector (850mV). A supelcosil™ column (supelco; 250 x 4.6 mm) was used in conjunction with a guard column (ODS pellicular 30 x 2.1 mm). The mobile phase consisted of 10% aqueous methanol containing sodium acetate (25 mM), citric acid (12.5 mM), NaOH (30 mM) and acetic acid (10 mM). Elution was performed at a flow rate of 1.0 ml/min. The lower limit of detection was 40 fmol absolute for 8-oxodG, or 1.5 residue/10^6 dG requiring a minimum yield of 35 µg DNA per tissue sample. The yield of DNA isolated from epithelial cells of the forestomach varied from 1 to 5 µg. Consequently, by this method no oxidative DNA damage could be assessed in forestomach epithelium. dG was simultaneously monitored at 260 nm. Oxidative DNA damage was expressed as the ratio of 8-oxodG to dG.

Statistics

Results are expressed as mean ± SD of the mean. Student's-*t*-test for unpaired values was applied to evaluate the statistical significance of differences between experimental and respective control groups; $p<0.05$ is considered significant.

Table 5-1: Data for average body weight, relative liverweight, average food-, BHA- and ASA-intake of rats maintained on diets containing 0% or 1.5% BHA and drinking water containing 0% or 0.2% ASA for respectively 3, 7 and 14 days.

Group	treatment	Body weight Initial	Final	Liverweight (%)	food intake (g/kg/day)	BHA-intake (g/kg/day)	ASA-intake (mg/kg/day)
AB-3	ASA + BHA	115 ± 8.6	112 ± 9.2	5.34 ± 0.41	8.0 ± 1.0	1.07 ± 0.13	303 ± 26
CB-3	BHA	116 ± 8.6	120 ± 8.1	5.93 ± 0.52	9.5 ± 1.7	1.18 ± 0.19	
AC-3	ASA	115 ± 4.9	108 ± 3.4[b]	4.06 ± 0.27[a]	8.5 ± 0.7		296 ± 18
CC-3	---	114 ± 1.7	115 ± 4.2	4.25 ± 0.54[a]	10.2 ± 2.1		
AB-7	ASA + BHA	115 ± 7.7	133 ± 6.6	6.14 ± 0.30[a]	11.2 ± 0.8[b]	1.41 ± 0.07	382 ± 83
CB-7	BHA	120 ± 5.8	145 ± 13.0	6.16 ± 0.56[a]	12.9 ± 0.9	1.48 ± 0.07	
AC-7	ASA	117 ± 8.1	129 ± 6.1	4.21 ± 0.31	10.9 ± 0.7[b]		356 ± 111
CC-7	---	114 ± 10.3	133 ± 10.8	4.47 ± 0.48	12.8 ± 0.8		
AB-14	ASA + BHA	112 ± 5.4	166 ± 12.4	6.37 ± 0.59[a]	13.4 ± 1.1	1.51 ± 0.09	369 ± 55
CB-14	BHA	116 ± 8.4	172 ± 9.9	6.17 ± 0.48[a]	13.9 ± 0.4	1.50 ± 0.08	
AC-14	ASA	117 ± 7.3	162 ± 6.1	4.78 ± 0.23	13.2 ± 1.1		307 ± 19[a]
CC-14	---	121 ± 5.2	170 ± 8.5	4.47 ± 0.48	13.9 ± 0.4		

Values marked with superscripts differ significantly (Student's *t*-test) from the corresponding pair-fed control-group ([a]: $p<0.05$), values of the AB resp. AC-group from CB resp. CC-group ([b]: $p<0.05$).
AB: BHA + acetylsalicylic acid; CB: BHA only; AC: acetylsalicylic acid controls; CC: controls.

Results

General responses to treatment

Data for averaged body weight, relative liver weight (% body weight), as well as averaged food-, BHA- and acetylsalicylic acid-intake are presented in Table 5-1. Immediately after the onset of administration of BHA, rats in the AB and CB groups decreased their food intake, probably due to unpalatability of the diet. From day 4 of the experiment onwards, the food consumption remained relatively constant. Acetylsalicylic acid-administration via the drinking water resulted in a slight decrease in food intake by comparison of AB with CB and AC with CC. This only resulted in a significant decrease in food intake of the acetylsalicylic acid-consuming groups as compared to their appropriate control groups (AB<CB; AC<CC) after 7 days of treatment. There were however no significant differences in body weight between the four groups of rats after 3, 7 or 14 days of treatment. BHA-intake did not differ between the two BHA-fed groups. A gradual increase in BHA-intake, expressed as g/kg/day, was seen in the course of the experiment. The only significant decrease in acetylsalicylic acid-intake was seen after 14 days of treatment (AC<AB). Data on the relative liver weight showed that BHA-administration resulted in a significant increase of approximately 35% in the BHA-consuming groups as compared to their PFC-groups.

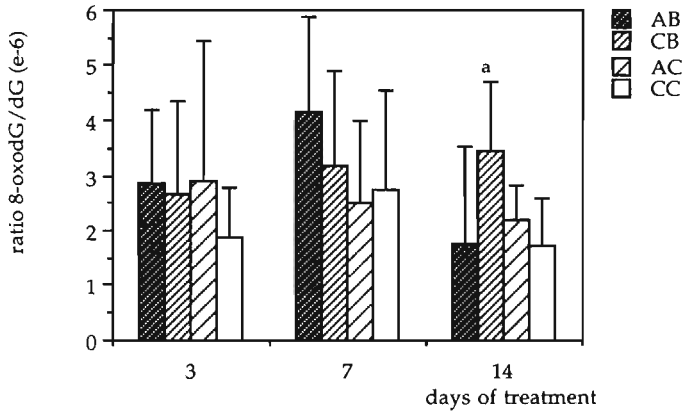

Figure 5-1. Amount of 8-oxodG/dG of liver DNA of rats maintained for respectively 3, 7 and 14 days on diets containing 0% or 1.5% BHA and drinking water containing 0% or 2.0% acetylsalicylic acid. Superscripts indicate the statistical significance of differences (Student's *t*-test). [a]: $p<0.05$ as compared to the appropriate control groups; [b]: $p<0.05$ by comparison of AB with CB or AC with CC.
AB: BHA + acetylsalicylic acid; CB: BHA only; AC: acetylsalicylic acid controls; CC: controls.

Induction of 8-oxodG in vivo

No significant differences in 8-oxodG levels of liver DNA were observed after 3 and 7 days of BHA treatment. BHA-administration increased averaged 8-oxodG levels in the DNA significantly from less than 2 residues/10^6 dG to approximately 4 residues/10^6 dG after 14 days of treatment as compared with the CC group, as shown in Figure 5-1.

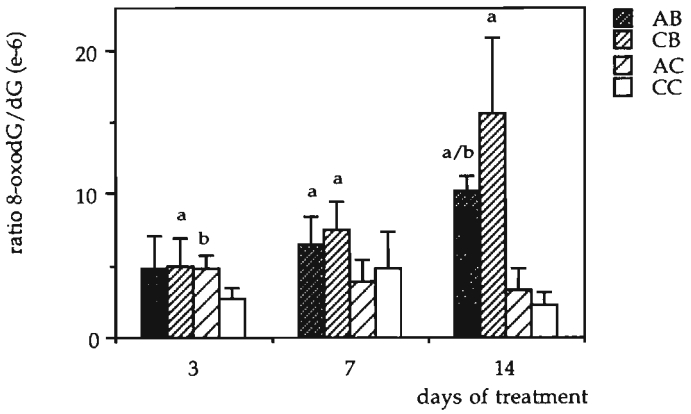

Figure 5-2. Amount of 8-oxodG/dG in the DNA of the epithelial cells of glandular stomach of rats maintained for respectively 3, 7 and 14 days on diets containing 0% or 1.5% BHA and drinking water containing 0% or 2.0% acetylsalicylic acid. Superscripts indicate the statistical significance of differences (Student's *t*-test). [a]: $p<0.05$ as compared to the appropriate control groups; [b]: $p<0.05$ by comparison of AB with CB or AC with CC.
AB: BHA + acetylsalicylic acid; CB: BHA only; AC: acetylsalicylic acid controls; CC: controls.

Figure 5-2 shows the 8-oxodG/dG ratios in the epithelial cell DNA of the glandular stomach. Acetylsalicylic acid-administration hardly affected the amount of 8-oxodG in the DNA (AC vs CC). BHA intake however increased 8-oxodG levels in the DNA significantly from an averaged 5 residues/10^6 dG after 3 days to 16 residues /10^6 dG after 14 days of treatment. Coadministration of acetylsalicylic acid significantly inhibited the oxidative potential of BHA after prolonged administration.

In Figure 5-3, the ratios of 8-oxodG/dG in colon epithelial cells are presented. The most striking feature in these data is the inhibiting effect of 8-oxodG formation in the DNA by acetylsalicylic acid after three and fourteen days of BHA treatment ($p<0.05$; AB<CB). BHA increased, although not significantly, the amount 8-oxodG/dG (e-6) from a mean of 4 residues /10^6 dG up to 6 residues /10^6 dG, whereas in the AB group a mean of 3 residues /10^6 dG was found.

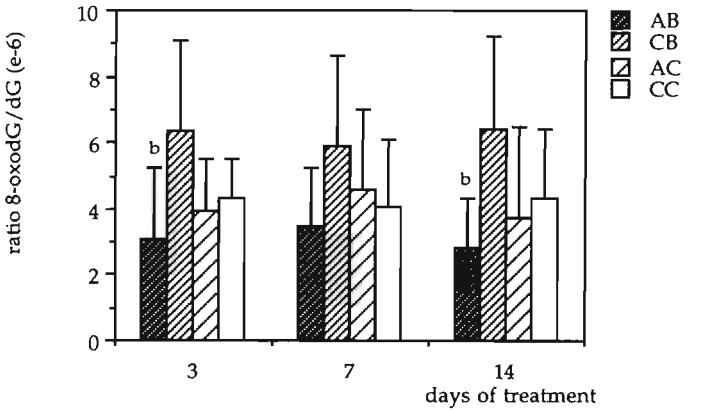

Figure 5-3. Amount of 8-oxodG/dG of the DNA of the epithelial cells of colorectal tissue of rats maintained on diets for respectively 3, 7 and 14 days containing 0% or 1.5% BHA and drinking water containing 0% or 2.0% acetylsalicylic acid. Superscripts indicate the statistical significance of differences (Student's *t*-test). [a]: $p<0.05$ as compared to the appropriate control groups; [b]: $p<0.05$ by comparison of AB with CB or AC with CC.
AB: BHA + acetylsalicylic acid; CB: BHA only; AC: acetylsalicylic acid controls; CC: controls.

Histopathology

The incidences of early lesions of the forestomach in the various groups are summarized in Table 5-2. Figure 5-4a shows the forestomach mucosa of a control rat. After 3 days on the BHA diet, mild epithelial hyperplasia, characterized by thickening of the forestomach mucosa, was observed in most animals. Microscopically, these lesions showed mild hyperkeratosis. In addition, mild inflammatory reactions were observed in both mucosa and submucosa. No marked increase in cellularity or basophilic staining of the epithelium was observed (Fig. 5-4b). Continued administration of BHA for 7 days increased the severity of hyperplastic changes in rat forestomach epithelium. In four of the six animals, moderate hyperplasia was observed (Fig. 5-4c). This consisted of the formation of papillae (cf. dermal papillae of skin) and irregularly spaced, elongated rete ridges (cf. rete pegs of skin). Moreover, slight epithelial defects were found in five out of six animals. This group also showed a marked increase in inflammatory reactions and basophilic staining of the epithelium. Isolated cystic swellings were observed in the submucosa. These findings were most pronounced in the vicinity of the forestomach-fundic stomach junction. In rats fed the BHA diet for fourteen days, the hyperplastic changes in the forestomach mucosa were even more advanced (Fig. 5-4d). Marked hyperplasia and hyperkeratosis had developed, widely spread over the forestomach mucosa, but these changes were more pronounced immediately adjacent to the limiting ridge (Fig. 5-5a). Although the inflammatory reaction was even more pronounced, hardly no epithelial defects were apparent. The muscular layer and serosa of the forestomach were normal.

Table 5-2: Incidence of histopathological lesions in the forestomach of rats maintained on diets containing 0% or 1.5% BHA and drinking water containing 0% or 0.2% ASA for respectively 3, 7 and 14 days.

	No. of rats with lesions after treatment for:											
	3 days				7 days				14 days			
Group:	AB	CB	AC	CC	AB	CB	AC	CC	AB	CB	AC	CC
Lesion:												
Epithelial hyperplasia												
- mild	1	4	0	0	4	1	1	1	4	1	0	0
- moderate	0	0	0	0	0	4	0	0	2	4	0	0
- severe	0	0	0	0	0	0	0	0	0	1	0	0
Epithelial defects	0	0	0	0	0	5	0	0	0	1	0	0
Inflammation												
- mild	2	5	0	0	2	4	0	0	5	2	0	0
- marked	0	1	0	0	0	2	0	0	0	4	0	0

AB: BHA + acetylsalicylic acid; CB: BHA only; AC: acetylsalicylic acid controls; CC: controls.

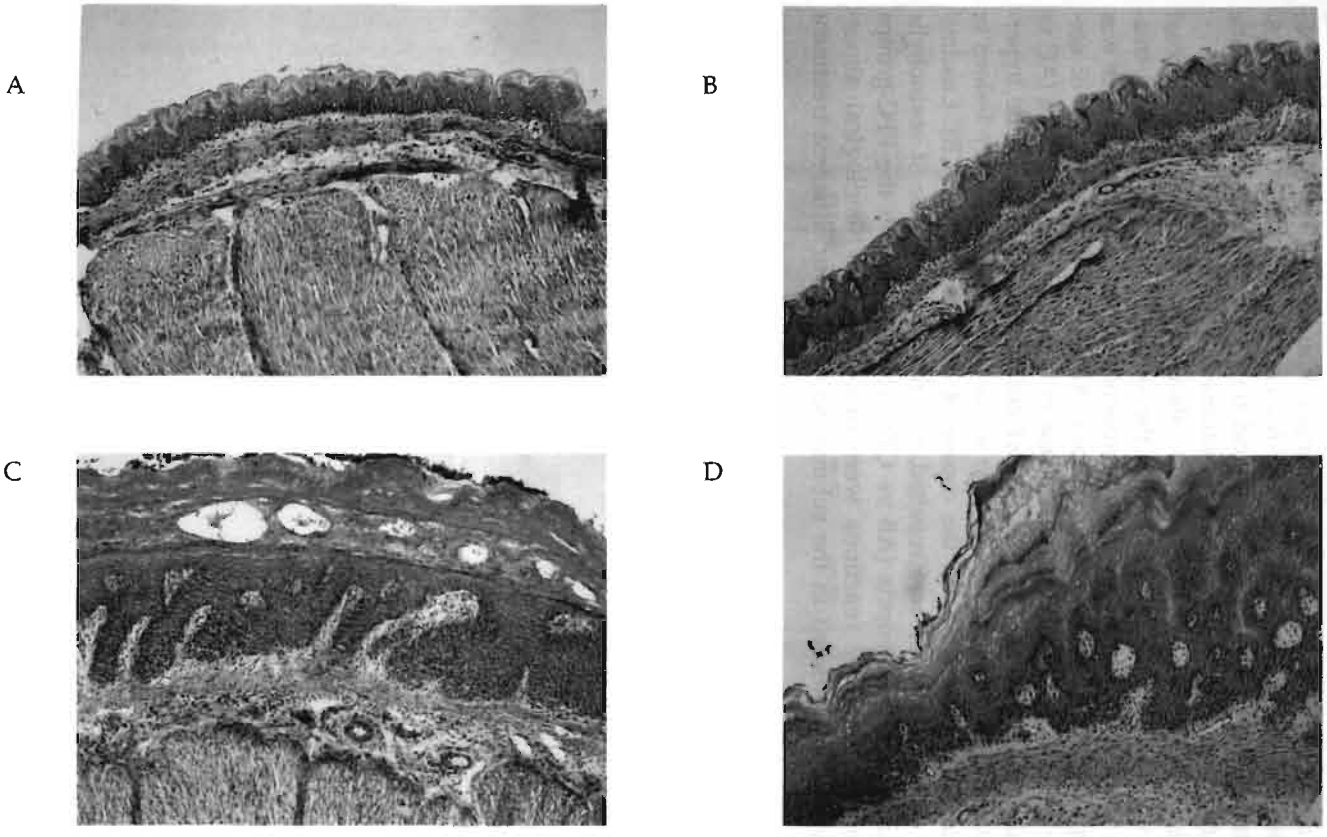

Figure 5-4: Effect of BHA on forestomach epithelium of rats maintained on diets containing (a): 0% BHA, (b): 1.5% BHA for 3 days; (c): 1.5% BHA for seven days; and (d): 1.5% BHA for fourteen days, a gradual increase in hyperplasia and hyperkeratose of the epithelium is observable. H&E x 100.

In summary: the incidences of mild, moderate and severe hyperplasia as well as inflammatory lesions increased roughly with prolonged BHA treatment. Simultaneous administration of acetylsalicylic acid (AB) decreased both the incidence and severity of hyperplastic changes induced by BHA (Fig. 5-5b). No abnormalities were observed in both pair-fed control groups (AC, CC)(Fig. 5-5c/d). Administration of acetylsalicylic acid only did not induce hyperplastic and/or inflammatory reactions in rat forestomach epithelium.
Examination of the glandular stomach showed an increase in mucosal thickness with eosinophylic infiltration in the lamina propria in the BHA-treated group (Table 5-3). The underlying submucosa appeared normal. There was no eosinophylic infiltrate in the submucosa. Although administration of acetylsalicylic acid only induced a mild increase in thickness of the mucosa (AC vs CC), simultaneous administration of acetylsalicylic acid decreased the hyperplasia induced by BHA (AB vs CB). The incidence of inflammatory lesions in the lamina propria increased in the course of BHA treatment (CB). Coadministration of acetylsalicylic acid resulted in a marked decrease of eosinohylic infiltrate in the lamina propria (AB vs CB), whereas in both the PFC-groups hardly no inflammatory reactions were observed. No histological abnormalities were demonstrated in the submucosa of the four different treatment groups.
Microscopic examination of colorectal tissue showed normal epithelium. There were no differences in histological appearances between the four test groups.

Table 5-3: Incidence of histopathological lesions in the glandular stomach of rats maintained on diets containing 0% or 1.5% BHA and drinking water containing 0% or 0.2% ASA for respectively 3, 7 and 14 days.

	No. of rats with lesions after treatment for:											
	3 days				7 days				14 days			
Group:	AB	CB	AC	CC	AB	CB	AC	CC	AB	CB	AC	CC
Lesion:												
Epithelial hyperplasia												
- mild	2	4	2	0	3	1	4	1	2	1	4	0
- moderate	0	0	0	0	1	4	0	0	3	5	0	0
Inflammation												
- mild	2	3	2	0	3	4	1	1	3	2	1	0
- marked	0	2	0	0	0	2	0	0	0	4	0	0

AB: BHA + acetylsalicylic acid; CB: BHA only; AC: acetylsalicylic acid controls; CC: controls.

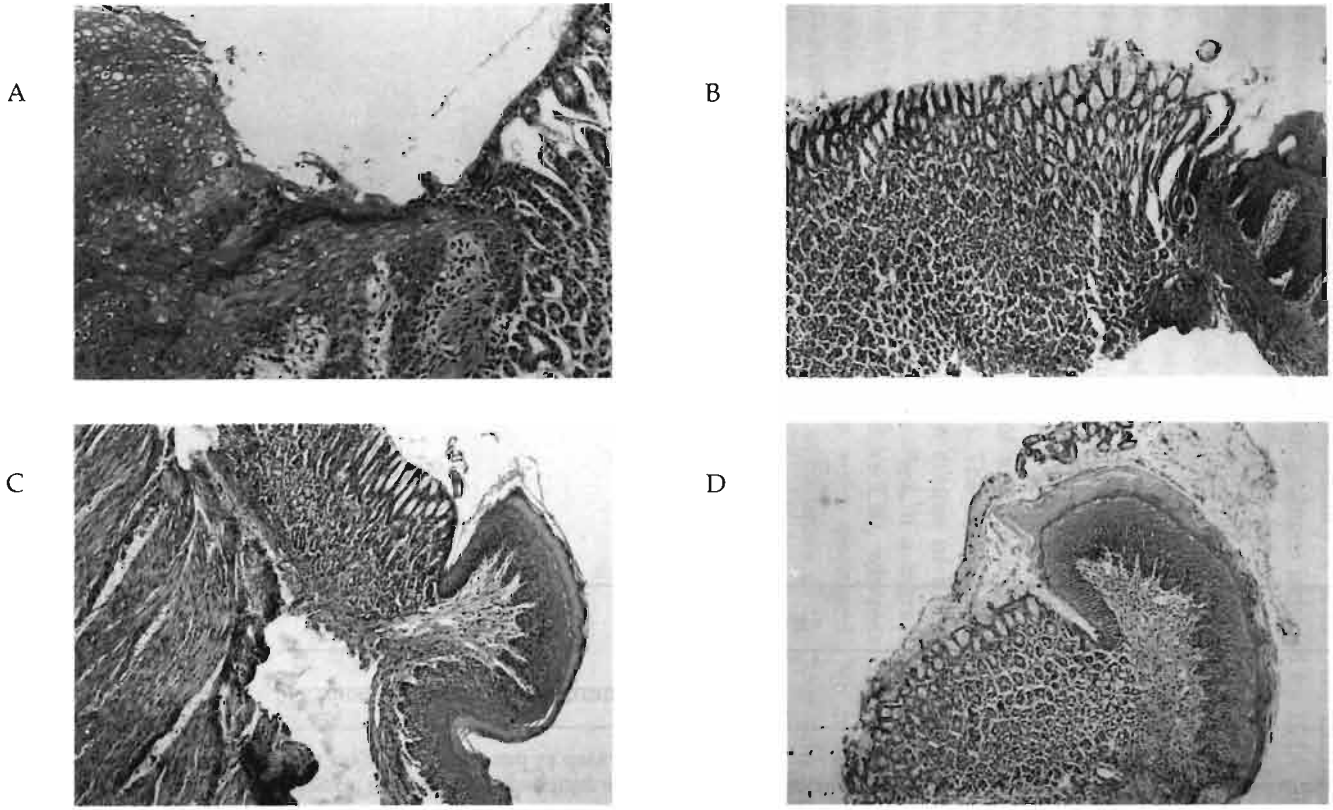

Figure 5-5: Forestomach epithelium of rats maintained on diets containing 0% or 1.5% BHA and drinking water containing 0% or 2.0% acetylsalicylic acid for fourteen days, showing folding of the hyperplastic epithelium near the glandulat/non-glandular junction and massive hyperkeratosis in the BHA-treated animals. (CB)(Fig. 5a); in the AB group a reduction of the irritating effect of BHA was observed (Fig. 5b), whereas in both coth control groups no abnormalities were observed (AC: Fig 5c; CC: Fig. 5d). H%E x 100.
AB: BHA + acetylsalicylic acid; CB: BHA only; AC: acetylsalicylic acid controls; CC: controls.

Discussion

Many mutagens, tumor promotors and carcinogens are known to generate oxygen radicals. Oxygen-derived free radicals are important mediators in several forms of tissue damage, such as injury associated with inflammatory responses, ischaemic injury to organs and tissues, and injuries resulting from the intracellular metabolism of chemicals and drugs. At normal conditions, the deleterious effects of oxygen radicals are balanced by specific antioxidant defenses in tissues. However, as the production of these compounds increases or the host oxidant defenses impair, excessive tissue injury results and physiologic functions of cells and organs are altered (39). The feasibility to detect 8-oxodG, one of the approximately twenty known primary products of oxidative DNA damage, has facilitated research on the relationship between DNA damage by reactive oxygen species and cancer. The C-8 position of deoxyguanosine residues in DNA is oxidized *in vitro* to 8-oxodeoxyguanosine by various oxygen radical producing agents such as reducing agents (40), X-rays (41), asbestos plus hydrogen peroxide (42), polyphenol with hydrogen peroxide and ferric ion (43) as well as the tumor promotor tetradeconyl phorbol acetate (44). *In vivo* studies showed a significant increase of 8-oxodG in target DNA of rats given the ferric complex of nitrilotriacetate (45) and potassium bromate (46). Formation of 8-oxodG in cellular DNA should be considered as an indication for formation of reactive oxygen species.

In the present study, the effect of the food antioxidant BHA on 8-oxodG formation was evaluated in rats in order to elucidate its epigenetic mode of action in the induction of carcinogenic events. Furthermore, in order to determine the role of prostaglandin H synthase in the metabolic activation of BHA we evaluated modulations by an inhibitor of this enzyme system: acetylsalicylic acid. The potential of BHA to induce 8-oxodG in liver DNA appeared to be very low. Only after 14 days of continuous BHA-administration a significant increase in 8-oxodG was found in the CB-group as compared to its pair-fed control-group. This relative lack of response was expected since the prostaglandin H synthase activity of liver cells is known to be very low (47). In the DNA of the epithelial cells of the glandular stomach, chronic feeding of BHA induced a time-dependent increase in 8-oxodG formation. Coadmini-stration of acetylsalicylic acid inhibited the effect of BHA on oxidative DNA damage. Similar observations were made in colorectal DNA although levels of oxidative DNA damage tend to be smaller. It is concluded that BHA is capable of inducing oxidative DNA damage *in vivo* and that prostaglandin H synthase is probably involved in this process.

Reactive oxygen species are also important mediators of both acute and chronic inflammatory reactions. These reactions can both initiate and potentiate inflammatory responses through direct toxic effects on cells and through modification of serum proteins, lipids and structural components of tissues. At sites of inflammation, the reaction products of oxygen radicals are capable of recruiting inflammatory cells to sites of tissue injury and thus amplify the inflammatory

response (39). BHA is known to cause inflammation and hyperplasia with hyperkeratosis of the squamous epithelium after short term administration (12). However, the mechanism of formation of early BHA-induced lesions is not completely understood, nor is it known whether the inflammatory reaction is a cause of the tissue injury, or rather a consequence.

The present results showed that the severity of forestomach hyperplasia as well as the inflammatory reactions increased with prolonged BHA-administration. Simultaneous administration of 1.5% BHA and 0.5% acetylsalicylic acid showed a marked inhibition of forestomach lesions. In contrast to the BHA-treated rats, no proliferation or inflammation was observed in the pair fed-control groups. The hyperplastic lesions induced by BHA were not restricted to forestomach tissue. Similar changes were observed in the glandular stomach. BHA treatment resulted in thickening of the mucosa with eosinophylic infiltration of the lamina propria. In this target organ, coadministration of acetylsalicylic acid resulted in a marked decrease of both hyperplasia and inflammation. In contrast in colorectal tissues, no abnormalities were observed.

The irritating effect of BHA was most pronounced in the upper gastro-intestinal tract tissues, whereas in colorectal tissue no histological effect could be observed. This suggests a dose-response relationship for the effect of BHA on both 8-oxodG formation and irritation of the epithelium. Colorectal tissue is exposed to lower concentrations of BHA after oral intake than gastric tissue as a consequence of resorption as well as dilution by fecal bulk.

In two previous studies we showed that oral intake of BHA at this particular dose increases cell proliferation rates in rat forestomach, glandular stomach and colon/rectum after a fourteen days period of administration (20, 21). This increase in cell proliferation appears to be accompanied, as the present study demonstrates, with the increase of oxidative damage in DNA of both glandular stomach and colon/rectum. Oxidative DNA damage in colon DNA however occurs, as the present study demonstrates, in absence of inflammatory responses in colon epithelium. It seems therefore unlikely that an inflammatory action or other hyperplastic lesion is initially responsible for enhancement of cell proliferation induced by BHA. This is in agreement with the previously demonstrated inhibiting effect of BHA on prostaglandin H synthase-mediated metabolism of arachidonic acid. Metabolites of arachidonic acid have been shown to be involved in inflammatory processes. If BHA would induce an increase in gastro-intestinal cell proliferation via an initial inflammatory response, arachidonic acid metabolism is more likely to be increased, in contrast to the reductions which we have found (21).

The present results are consistent with our previous study in which we demonstrated that the proliferation enhancing effects of BHA in rat gastro-intestinal tract can be inhibited by coadministration of acetylsalicylic acid, the effect being most pronounced in upper gastro-intestinal tract tissues (21). Acetylsalicylic acid inhibited prostaglandin H synthase-mediated metabolism of TBHQ in TBQ *in vivo* (36). Moreover, prostaglandin H synthase-dependent metabolism of TBHQ was accompanied by oxidative stress, which resulted in

oxidative DNA damage *in vitro* (35). Therefore, in combination with the present results, this indicates that the carcinogenic potential of BHA could be ascribed to oxygen radical formation, induced during prostaglandin H synthase-mediated metabolism of the primary BHA-metabolite TBHQ into TBQ, at conditions at which the rate of active oxygen production is too large to be efficiently degraded by antioxidative factors physiologically present in the cellular system. These oxygen radicals may lead to excessive cell proliferation in rat gastro-intestinal tract tissues.

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Chapter 6

Induction of oxidative DNA damage and enhancement of cell proliferation in human lymphocytes by butylated hydroxyanisole

P.A.E.L. Schilderman, E. Rhijnsburger, I. Zwingmann, F. ten Hoor[1] and J.C.S. Kleinjans.

Departments of Health Risk Analysis and Toxicology, [1]Human Biology, University of Limburg, P.O. Box 616, 6200 MD Maastricht, The Netherlands

Abstract

In order to investigate the possible relationship between the induction of oxidative DNA damage and an enhancement of cell proliferation, we analysed the effect of butylated hydroxyanisole (BHA) and its primary metabolites *tert*-butylhydroquinone (TBHQ) and *tert*-butylquinone (TBQ) on 8-oxodeoxyguanosine formation and labeling indices in human lymphocytes, *in vitro*.
Analysis of culture medium and lysated cell fraction after administration of BHA or metabolites of BHA, revealed that BHA and TBHQ can be actively metabolized in whole blood. Moreover, TBQ can be reduced into TBHQ. While in cultures treated with BHA 50-60% of the dose administered was recovered, much lower dose recovery was assessed in cultures treated with either TBHQ or TBQ. This indicates that a considerable binding of these compounds to macromolecules occurred.
All three testcompounds induced a dose-dependent increase in cell proliferation of phytohaemaglutinin-stimulated lymphocytes, 50 μM being the optimal dose. Through metabolism of BHA into TBHQ it is not clear which compound is responsible for the proliferation enhancing effects as observed in culture.
HPLC/ECD-analysis of oxidative DNA damage in lymphocytes exposed to respectively 100 μM BHA, TBHQ or TBQ, showed that BHA was not capable of inducing oxidative DNA damage to a significant degree. TBQ and in particular TBHQ however increased 8-oxodG formation by resp. 66% and 1100% in human lymphocytes cultured *in vitro*.
In this study, no link can be made between induction of oxidative DNA damage and enhancement of cell proliferation in human lymphocytes.

Introduction

3-*Tert*-butyl-4-hydroxyanisole (BHA) is a phenolic antioxidant which is widely used as a food additive. Food additives are not supposed to exert any carcinogenic action. BHA is negative in most assays for genotoxicity (1-9) except for chromosomal aberrations in different cell lines (3, 10). However, BHA has been shown to induce forestomach carcinomas in rats and hamsters when fed daily high doses during lifetime (11-14). Moreover, BHA appears to have initiating potential in two-stage mouse skin carcinogenesis (139) and in two-stage transformation of BALB/3T3 cells (15). The same study shows that the initiating potential of the quinone metabolite of BHA is about 100 times higher than that of BHA (16). Several reports ascribe this suggested carcinogenic potential of BHA to the endogenous formation of *tert*-butylhydroquinone (TBHQ) and *tert*-butylquinone (TBQ) (16, 17).

TBHQ, a primary metabolite of BHA, also produces inconsistent results in different genotoxicity assay systems. TBHQ appears to be non-genotoxic in the Salmonella/microsome assay (2, 3, 18). TBHQ can however produce chromosomal aberrations in bone-marrow cells of mice (19) and in Chinese hamster fibroblast cell line CHL in the presence of rat liver S9 mix (3). Moreover, TBHQ and its corresponding quinone are clastogenic to Chinese hamster ovary cells (10). TBHQ may be mutagenic to V79 Chinese hamster lung cells and seems to induce positive effects in the L5178Y mouse lymphoma test (20). TBHQ furthermore, induces a dose-related increase in SCE frequency in bone marrow cells of mice (21). These results therefore do not exclude a genotoxic action by TBHQ.

Further research revealed that both BHA and TBQ have an initiating action in the two-stage transformation of BALB/3T3 cells, the potency of TBQ being approximately 100 times higher than that of BHA (16). TBQ may therefore also play a role in BHA-induced forestomach carcinogenesis in rodents particularly because it has been shown that TBQ is formed in the forestomach or its vicinity, after oral BHA intake (17). DNA analysis of forestomach epithelium of male F344 rats following oral administration of BHA, shows that the DNA-damaging capacity of TBQ is much stronger than that of TBHQ and BHA (22). Within this respect, the ability of BHA, TBHQ and TBQ to induce the formation of superoxide anion has also been tested in rat forestomach homogenate. All three test agents appear to be capable of stimulating superoxide formation, TBQ by far being the most active (23). These findings indicate that TBHQ and TBQ play a role in the tumor-promoting and carcinogenic action of BHA, probably linked to their potential to generate reactive oxygen species.

Reactive oxygen species are assumed to be involved in tumor promotion and tumor development. Sustained production of low levels of oxidants can contribute to this process. Quinones are potent electrophiles that are capable of attacking nucleophilic cellular constituents and can generate active oxygen species which damage biomolecules as a result of redoxcycling between quinone and hydroquinone forms via semiquinone radicals. SQ is formed from both TBHQ and TBQ in liver microsomes. In the presence of oxygen, these com-

pounds induce excess production of superoxide. Moreover, TBQ damages hepatocyte plasmamembranes. It is concluded that the SQ-dependent superoxide formation may contribute to the toxic actions of BHA (24).
Recently, we demonstrated by means of electron spin resonance spectroscopy that TBHQ is capable of forming superoxide anion, hydrogen peroxide and hydroxyl radicals (25). Subsequently, we tested the oxidative potential of BHA, TBHQ and TBQ in two non-cellular assays. The results again indicate that BHA itself, in contrast to the hydroquinone metabolite, is not capable of inducing oxidative DNA damage. In this study, the oxidative potential of TBQ is also very low, probably due to lack of enzymes capable of reducing TBQ to the semiquinone radical *in vitro* (26).
Several experiments show that superoxide and hydrogen peroxide when added to culture medium can cause growth responses, thereby stimulating the expression of early growth regulating genes such as the proto-oncogene c-fos. Addition of superoxide dismutase or catalase to this culture medium had a progressively inhibitory effect on cell proliferation. Superoxide and/or hydrogen peroxide may therefore function as mitogenic stimuli (27).
In the present study, we investigate the potential of BHA and its primary metabolites TBHQ and TBQ to induce oxidative DNA damage and cell proliferation in human lymphocytes, cultured *in vitro*.

Materials and methods

Materials

BHA (food grade BHA, purity > 99 %, 97.5 % 3-BHA) was obtained from J. Dekker CO. (Wormerveer; The Netherlands). TBHQ (purity>97%), and 3,5-di-*tert*-butyl(4) hydroxyanisole (DBHA) (purity>97%) were purchased from Aldrich Chemical CO. (Brussels, Belgium), diethylether from BDH (Poole, UK), limpet acetone powder (type 1) from Sigma (St Louis, MO), 5-bromodeoxyuridine (BrdU) from Serva (Heidelberg, Germany) and the monoclonal peroxidase-conjugated antibody rabbit anti-mouse IgG (F114) from Dakopatts (Denmark). RPMI 1640, streptomycin, penicillin, L-glutamin, heparine, foetal calf serum (FCS) and phytohaemagglutin (PHA) were all obtained from GIBCO (Europe). Proteinase K (sp. act. 20 units/mg lyophilisate), RNase A (sp. act. 50 units/mg dry powder), RNase T1 (sp. act. 100.000 units/ml) and α-amylase (sp. act. 1000 units/mg) were purchased from Boehringer Mannheim (West Germany). Alkaline phosphatase (Type VII-N; sp. act. 10.000 units/ml) and nuclease P1 (sp. act. 200 units/mg protein) were obtained from Sigma (St. Louis, MO). Methanol was HPLC grade and was obtained from Rathburn (Walkerburn, UK). Analytical-grade chemicals were used at all other instances. Water was purified by means of a milli-Q water purification system.

Lymphocyte culture

Peripheral blood was obtained from a healthy donor. Blood was drawn by venipuncture in heparinized tubes. 0.4 ml of whole blood was added to each culture. Cultures were adjusted to a final volume of 5.0 ml in RPMI 1640 medium, supplemented with 100 mg/ml streptomycin, 100 U/ml penicillin, 5 mM L-glutamin, 50 U/ml heparine and 10% FCS. To all 5.0 ml cultures 0.2 ml PHA was added. Cell proliferation was estimated by incorporation of BrdU. Cultures were incubated at 37°C for 46 h after which 0.05 ml of a filter-sterilized 2.5 mM BrdU solution was added (final concentration: 0.25 mM) for an additional 4 h period. After a total of 50 h of incubation, cells were processed according to standard procedures, including hypotonic treatment with 75 mM KCl solution for 20 min at 37 °C and fixation with a mixture of methanol: glacial acetic acid (3:1; v/v). Following several washes with fresh fixative, the cells were concentrated in a small volume and the resulting suspension spread on clean microscope slides. For detection of BrdU-containing cells on these slides, an indirect enzyme-labeled antibody technique was applied (28, 29) using anti-BrdU-antibody (clone IIB-5) and peroxidase-conjugated rabbit anti-mouse IgG. The peroxidase-containing antibody sites were visualized by means of diaminobenzidine. The sections were counterstained with Giemsa. The labeling index is expressed as the percentage of BrdU-positive cells. Slides were encoded and scored for immunoreactivity by light microscopy. Slides were scored by two independent observers, the interobserver variation appearing low. As all cultures were in duplicate, an average of labeling indices was obtained by counting 2000 nuclei from the slides prepared from each culture. Cell viability was assessed by trypan blue exclusion before and after the experiment and was over 90% before the experiment. Similar cultures were used for determination of BHA, TBHQ and TBQ in culture medium or lysated cell fraction.

Chemical treatment

For chemical treatment, the chemicals to be tested were first dissolved in dimethylsulfoxide (DMSO). Aliquots of these solutions were added to the medium to give the appropriate final concentration. The highest concentration of DMSO in the culture medium was 0.1%; control incubations contained similar concentrations DMSO.

Analysis of the medium

Aliquots of the culture media were adjusted to pH 4.8 with 1M sodium acetate buffer pH 4.8 and 100 µl methanol containing 250 µg/ml DBHA, was added as an internal standard. In order to deconjugate the glucuronic acid and sulphate conjugates of BHA, TBHQ and TBQ, limpet acetone powder was added as described previously (25; Chapter 3). In short: complete 5 ml samples were extracted twice with diethylether, the organic phases were combined and methanol was added. The ether fraction was subsequently evaporated under vacuum until methanol was left, in order to prevent votality of TBQ. The residue was diluted with ammonium formiate buffer pH 3.5 and stored at -20°C until HPLC

analysis. For the determination of free BHA (i.e. unconjugated BHA), free TBHQ and free TBQ, the procedure was the same except for the addition of limpet acetone powder and the overnight incubation step. In order to determine the intracellular levels of test compounds, duplicate cultures were washed with phosphate buffered saline (pH 7.4). After ultrasonic treatment for obtaining lysated cell fractions, the extraction procedure was the same.

High Performance Liquid Chromatography (HPLC) of BHA, TBHQ and TBQ
Reversed phase HPLC was performed as described previously (25; Chapter 3). The mobile phase consisted of methanol and 0.1 M formiate buffer, pH 3.5, using a linear gradient from 0 till 30 min (40 to 85 % methanol, 15 % per min). The retention times of the products were as follows: TBHQ; 8.5 min, TBQ; 14.0 min, BHA; 18.6 min and DBHA at 29.0 min. TBHQ, BHA and the internal standard DBHA were monitored at 290 nm; TBQ at 250 nm. The limit of detection was 1 ng absolute for both BHA and TBHQ and 0.5 ng for TBQ. The recovery of BHA was 88.2 ± 0.5%; TBHQ: 90.4 ± 3.2% and TBQ: 78.1 ± 2.9%.

DNA-extraction procedure
Cultures of whole blood were first treated with lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 10 mM EDTA, pH 7.4) and incubated on ice for 20 min. The white blood cells were harvested and frozen at -20°C until the DNA-extraction procedure.
DNA of white blood cell were isolated according to a published procedure (30). In short: samples were homogenized in SDS/EDTA and subsequently incubated with proteinase K. The homogenate was successively extracted by means of phenol, phenol/chloroform/isoamyl alcohol and chloroform/ isoamyl alcohol. After precipitation of the DNA, RNA was destroyed by addition of RNase T1 and RNase A. After extraction of the digest using chloroform/isoamyl alcohol and subsequently saturated diethylether, DNA concentration was assayed spectrophotometrically. The DNA was digested to deoxynucleosides by treatment with nuclease P1 and alkaline phosphatase according to Lutgerink et al. (31: Chapter 4).

HPLC/ECD-analysis of 8-oxodG
HPLC/ECD detection of 8-oxodG and dG was performed as described previously (Chapter 4). The lower limit of detection for 8-oxodG was 40 fmol absolute. dG was simultaneously monitored at 260 nm. Oxidative DNA damage was expressed as the ratio of 8-oxodG to dG.

Statistics
Results are expressed as mean ± SD of the mean. Student's-*t*-test for unpaired values was applied to evaluate the statistical significance of differences. $p<0.05$ is considered significant.

Table 6-1a: Data on viability (%) of human lymphocytes exposed to BHA, TBHQ and TBQ and recovery of the test compounds in culture medium and lysated cell fractions (%)(Table 6-1b: absolute amounts recovered in μM), after 50 h of incubation at 37°C.

	Dose administered (mM)	Viability (%)	Dose recovered (%) in culture medium			in lysated cell fraction		
			as BHA	as TBHQ	as TBQ	as BHA	as TBHQ	as TBQ
BHA	1.000	54.3 ± 2.2	--	--	--	--	--	--
	0.100	89.9 ±3.4	61.4 ± 3.3	0.20 ± 0.04	0.001 ± 0.00	2.34 ± 0.09	0.99 ± 0.02	nd
	0.050	98.3 ± 2.8	54.8 ± 3.2	0.56 ± 0.08	nd	3.11 ± 0.08	0.89 ± 0.03	nd
	0.025	96.7 ± 1.1	53.9 ± 6.7	0.87 ± 0.04	nd	1.78 ± 0.00	nd	nd
	0.013	94.3 ± 1.8	48.5 ± 4.3	1.87 ± 0.09	nd	0.89 ±0.06	nd	nd
TBHQ	1.000	31.5 ± 4.9	--	--	--	--	--	--
	0.100	72.1 ± 2.2	nd	1.32 ± 0.03	0.67 ± 0.01	nd	0.40 ± 0.09	nd
	0.050	94.4 ± 3.8	nd	1.31 ± 0.09	0.89 ± 0.01	nd	nd	nd
	0.025	96.8 ± 1.1	nd	3.21 ± 0.06	0.76 ± 0.02	nd	nd	nd
	0.013	95.7 ± 0.9	nd	5.82 ± 1.10	2.20 ± 0.01	nd	nd	nd
TBQ	1.000	43.1 ± 6.8	--	--	--	--	--	--
	0.100	92.3 ± 2.2	nd	0.09 ± 0.001	1.11 ± 0.03	nd	nd	0.09 ± 0.01
	0.050	96.6 ± 3.0	nd	nd	0.99 ± 0.01	nd	nd	nd
	0.025	96.5 ± 4.4	nd	nd	0.11 ± 0.00	nd	nd	nd
	0.013	94.6 ± 0.1	nd	nd	nd	nd	nd	nd

Table 6-1b:

	Dose administered (mM)	Dose recovered (abs: µM) in culture medium			in lysated cell fraction		
		as BHA	as TBHQ	as TBQ	as BHA	as TBHQ	as TBQ
BHA	1.000	--	--	--	--	--	--
	0.100	61.4 ± 3.3	0.20 ± 0.04	0.001 ± 0.00	2.34 ± 0.09	0.99 ± 0.02	nd
	0.050	27.4 ± 1.6	0.28 ± 0.04	nd	1.56 ± 0.04	0.45 ± 0.02	nd
	0.025	13.5 ± 1.70	0.22 ± 0.01	nd	0.45 ± 0.00	nd	nd
	0.013	6.06 ± 0.54	0.23 ± 0.01	nd	0.10 ±0.01	nd	nd
TBHQ	1.000	--	--	--	--	--	--
	0.100	nd	1.32 ± 0.03	0.67 ± 0.01	nd	0.40 ± 0.09	nd
	0.050	nd	0.66 ± 0.05	0.45 ± 0.01	nd	nd	nd
	0.025	nd	0.80 ± 0.02	0.19 ± 0.01	nd	nd	nd
	0.013	nd	0.73 ± 0.01	0.28 ± 0.00	nd	nd	nd
TBQ	1.000	--	--	--	--	--	--
	0.100	nd	0.09 ± 0.00	1.11 ± 0.03	nd	nd	0.09 ± 0.01
	0.050	nd	nd	0.50 ± 0.01	nd	nd	nd
	0.025	nd	nd	0.03 ± 0.00	nd	nd	nd
	0.013	nd	nd	nd	nd	nd	nd

Results

In Table 6-1a data are presented on the viability of the exposed lymphocytes at the end of the experiment and on the dose recovered in culture medium and lysated cell fraction (additionally, the amount the dose recovered is also expressed in absolute amounts (μM) in Table 6-1b). Addition of BHA, TBHQ and TBQ to isolated lymphocytes resulted in a dose-dependent increase in cytotoxicity. The onset of cell death (assessed by % trypan blue uptake) resulted in dark colouring of the medium, indicating that also erythrocytes were lysated. At concentrations of 1 mM and higher, all three test compounds exhibited toxic effects. Cultures treated with 100 mM TBHQ or TBQ, also showed cytotoxicity. At concentrations of 0.100 μM and higher, TBHQ appeared to be more cytotoxic than TBQ; TBQ more than BHA ($p<0.05$).

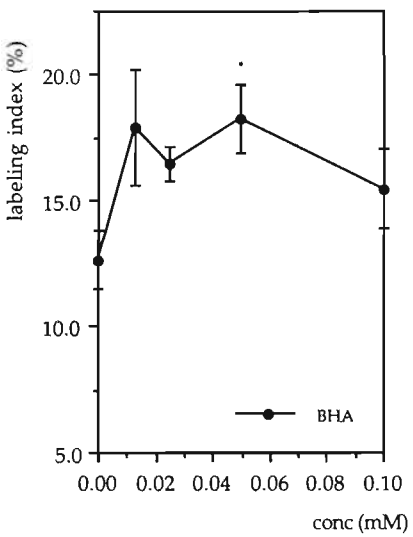

Figure 6-1: Labeling indices of lymphocytes exposed to respectively 0, 0.0125, 0.025, 0,050 and 0.100 mM BHA. Mean ± SD from duplicate incubations and analysis. Values marked with superscripts differ significantly (Student's *t*-test) as compared to the control incubation. *: $p<0.05$. Incubation conditions are as indicated in Materials and methods.

In incubations containing different concentrations of BHA, 55% on the average was recovered from the culture medium as the parent compound while only 1% on the average was converted into TBHQ and even smaller amounts were recovered as TBQ. The lower the BHA dose administered, the higher the amount of TBHQ recovered. In the lysated cell fractions about 2% was recovered as BHA and approximately 1% as TBHQ in the highest dose cultures. TBQ could not be detected. In cultures treated with different doses of TBHQ, only 2% could be recovered as TBHQ while on the average 1% was detected as

TBQ. In the lysated cell fractions only 1% of TBHQ at the highest dose administered, was recovered. In cultures to which different amounts of TBQ were administered, about 1% was detected as TBQ and only traces of TBHQ were observed. Deconjugation by means of limpet acetone powder revealed no extra amounts of BHA, TBHQ or TBQ which indicates that no conjugation with glucuronic acid or sulphate has occurred.
Figure 6-1 shows the dose-response curve of the mean labeling indices (LI) in cultures treated with different amounts of BHA. BHA was capable of inducing a significant increase in LI, the optimal concentration being 50 µM BHA. The effect of TBHQ on cell proliferation of human lymphocytes is shown in Figure 6-2. The dose-dependent increase in LI induced by TBHQ showed a maximum response at 50 µM TBHQ, increasing the LI from 12.63% to 18.55%; 100 µM TBHQ being slightly cytotoxic. The proliferation enhancing effects of TBQ, were also statistically significant, as is shown in Figure 6-3. At 100 µM TBQ in culture cytotoxicity was observed.

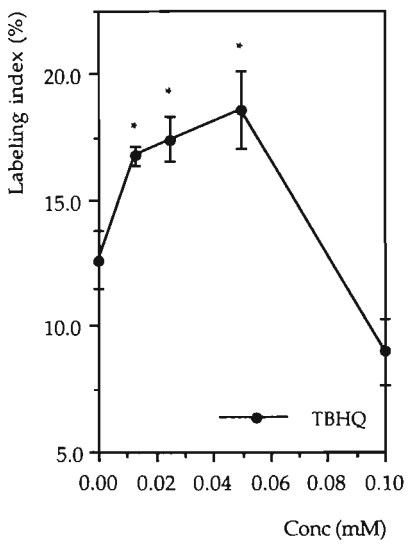

Figure 6-2: Labeling indices of lymphocytes exposed to respectively 0, 0.0125, 0.025, 0,050 and 0.100 mM TBHQ. Mean ± SD from duplicate incubations and analysis. Values marked superscripts show significant differences (Student's *t*-test) as compared to the control incubation. *: $p<0.05$. Incubation conditions are as indicated in Materials and methods.

Data on 8-oxodG formation in human lymphocytes cultured for 50 h at 37°C in presence of respectively 100 µM BHA, TBHQ and TBQ, are presented in Table 6-2. The background level of 8-oxodG appeared to be 0.32 ± 0.07 8-oxodG/dG (10^{-3}). BHA administration enhanced, although not significantly, this ratio to 0.42 ± 0.02 8-oxodG/dG (10^{-3}). The ratio of 8-oxodG/dG of human lymphocytes

exposed to 100 µM TBQ was significantly increased to 0.53 ± 0.01 8-oxodG/dG (10^{-3}). 100 µM TBHQ induced an 11-fold increase in 8-oxodG/dG formation (3.48 8-oxodG/dG (10^{-3})). However, TBHQ appeared to be cytotoxic at this particular dose, which resulted in fragmentation of the DNA (as observed during DNA isolation) and consequently increased susceptibility of the DNA to oxidative damage.

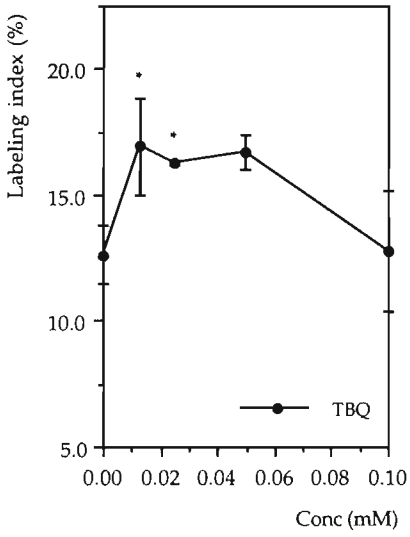

Figure 6-3: Labeling indices of lymphocytes exposed to respectively 0, 0.0125, 0.025, 0,050 and 0.100 mM TBQ. Mean ± SD from duplicate incubations and analysis. Values marked with superscripts differ significantly (Student's *t*-test) as compared to the control incubation. *: $p<0.05$. Incubation conditions are as indicated in Materials and methods.

Table 6-2: the effect of 100 µM BHA, TBHQ, resp. TBQ on 8-oxodG formation in human lymphocytes after 50 h of incubation at 37°C.

	Ratio 8-oxodG/dG (e-3)
control	0.32 ± 0.074
BHA	0.42 ± 0.023
TBHQ	3.48[a]
TBQ	0.53 ± 0.008[a]

Mean ± SD from duplicate incubation and analysis. Values marked with superscript differ significantly (Student's *t*-test) as compared to the control incubation; [a]: $p<0.05$.

Discussion

Analysis of culture medium and lysated cell fractions shows that both BHA and TBHQ can be actively metabolized in whole blood. Moreover, reduction of TBQ into TBHQ occurs. No conjugation of BHA or its metabolites to glucuronic acid or sulphate was observed. However, in view of the low recovery after administration of these compounds, in both culture medium and lysated cell fraction, a considerable binding of in particular TBHQ and TBQ to macromolecules must have taken place,

Reports on the metabolism of BHA *in vivo* and *in vitro* have revealed that the main metabolic pathways are *O*-demethylation by cytochrome p450, conjugation to glucuronic acid and/or sulphate, and dimerization (17, 32, 33). *O*-demethylation by cytochrome p450, yielding TBHQ, seems to be rate-limiting. *In vitro* high concentrations of BHA appear to result in uncoupling of cytochrome p450 from its reductase thereby blocking its own oxidative metabolism (34). Similar results are found *in vivo*; a tenfold increase in the dose of BHA administered to rats is not reflected by a comparable enhancement in absolute amounts of TBHQ formed. Apparently, relatively more TBHQ is formed at lower dose-levels of BHA (35). This is in agreement with the present results on BHA metabolism in whole blood. The lower the dose of BHA administered, the higher the amount (%) of TBHQ detected.

Furthermore, we have presented evidence that BHA, TBHQ and its corresponding quinone are cytotoxic to lymphocytes cultured in whole blood. This toxic effect may be related to the formation of reactive metabolites or active oxygen species, but may also be due to the effects of these compounds on lipid membranes. Cytotoxicity of BHA has been shown in several *in vitro* systems. BHA is cytotoxic to freshly isolated hepatocytes and its cytotoxicity appears to be related to its effect on the mitochondrial membrane. Effects on biomembranes include inactivation of lipid containing viruses, increasing fluidity of cellular membranes in hydrophobic but not in polar regions and perturbing phospholipid packing. These membrane effects even occur in cells which metabolize these compounds (36). Antioxidants prevent damage to lipid membranes by terminating free radical chain reactions, while on the other hand they can cause damage by intercalating into the hydrophobic bilayer thereby possibly interfering with membrane bound enzyme function and membrane integrity (36, 37).

Cytotoxicity to whole blood also indicates that erythrocytes are lysated, which results in loss of enzyme activity. Erythrocytes possess prostaglandin H synthase activity, an enzyme system involved in the metabolism of TBHQ into TBQ, which is accompanied by formation of reactive oxygen species (Chapter 3). Loss of prostaglandin H synthase, as we suggest, can therefore result in less metabolism of TBHQ and less production of oxygen radicals.

All three test compounds are capable of inducing cell proliferation of lymphocytes in culture to a similar degree. For each compound, 50 μM of the test substance appears to be the optimum concentration for induction of cell proli-

feration. Subsequent analysis of 8-oxodG formation in lymphocytes exposed to 100 μM of BHA, TBHQ and TBQ reveals that both TBQ and TBHQ are capable of inducing 8-oxodG formation, the effect of TBHQ being dramatically. However, the dose of TBHQ and probably also of TBQ as applied in this study for determination of 8-oxodG formation in lymphocyte cultures, appears to be cytotoxic. Therefore, determination of 8-oxodG formation must be performed at lower doses. 8-OxodG analysis of incubations with the different test compounds using this established optimal dose for enhancement of cell proliferation, is currently under investigation.

Although the rates of metabolism of TBHQ into TBQ, after administration of either BHA or TBHQ, correlates with the production of oxidative DNA damage, no relationship can be made between intracellular concentrations of TBHQ and the induction of lymphocyte proliferation. Therefore, these preliminary results do not provide indication for a relation between the increase of oxidative DNA damage by BHA and induction of lymphocyte proliferation. *O*-demethylation of BHA by cytochrome p450 yields TBHQ. Inhibition of this enzyme system by par example piperonyl butoxide prevents hydroquinone formation. Similarly, inhibition of prostaglandin H synthase by acetylsalicylic acid prevents oxidation of TBHQ into SQ. Therefore, lymphocyte cultures have to be treated with these known inhibitors of BHA resp. TBHQ metabolism. In this way, the effects of BHA on cell proliferation and induction of oxidative DNA damage can be distinguished from the effect of its metabolite TBHQ on these parameters.

Additionally, it may be hypothesized that intracellular conditions in peripheral human lymphocytes stimulated by PHA to divide *in vitro*, are not similar to those in gastro-intestinal epithelium *in vivo*, which may imply different responses to oxidative attacks between these cell systems. Therefore at present, it is not clear whether human lymphocytes can be used as a test system for assessing genetic damage of BHA and its metabolites *in vitro*.

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Chapter 7

Modulation by dietary factors of BHA-induced alterations in cell kinetics of gastro-intestinal tract tissues in rats

P.A.E.L. Schilderman, H. Verhagen*, B. Schutte[1], F. ten Hoor and J.C.S. Kleinjans.

Departments of Health Risk Analysis and Toxicology and [1]Molecular Cell Biology, University of Limburg, PO Box 616, 6200 MD, Maastricht, The Netherlands.*: Present address: TNO-CIVO Toxicology and Nutrition Institute, PO Box 360, 3700 AJ, Zeist, The Netherlands.

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Abstract

To determine the effects of dietary ethanol or fibre on 2(3)-*tert*-butyl-4-hydroxyanisole (BHA)-induced alterations in cell kinetics in gastro-intestinal tract tissues, groups of six male Wistar rats were fed diets containing 0% (control) or 1.5% BHA for two weeks. One group fed 1.5% BHA-fed and one pair-fed control (PFC) group received 10% ethanol in the drinking water; two similarly fed groups received drinking water only. Another group fed 1.5% BHA and a pair-fed control group received a diet supplemented with 20% cellulose; two similar groups received no fibre supplementation. Cell kinetics in forestomach-, glandular stomach-, and oesophagus-tissue were determined, after 14 days of experimental feeding, by means of bivariate 5-bromodeoxyuridine (BrdU)/DNA-analysis using immunocytochemistry and flowcytometry. In the fibre experiment, colo/rectal-tissue was examined as well.

In both experiments the labeling indices (LI) in all the gastro-intestinal tract tissues were significantly altered in the BHA-fed groups compared with the corresponding control-groups. In the ethanol experiment no statistically significant difference in the labeling indices was observed in the forestomach or glandular stomach between the two control-groups or between the two BHA-fed groups. However, intake of ethanol-supplemented drinking water induced oesophageal LI increases in rats fed a BHA-free diet. Thus 14 days of simultaneous ethanol administration has no effect on BHA-induced alterations in cell kinetics in the oesophagus, glandular stomach or forestomach of rats.

In the forestomach- and colo/rectal-tissue, a high cellulose diet resulted in a significant decrease of the BHA-induced elevation of LI. Thus dietary cellulose provides a partial protection against the proliferation enhancing effects of BHA in the rat gastro-intestinal tract.

Introduction

The synthetic food-antioxidant 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) is a carcinogen in the forestomach of rodents. Furthermore, BHA is a modulator of chemically induced mutagenesis and carcinogenesis (1-6). Time- and dose dependent changes in the forestomach epithelium include hyperplasia, papillomas and carcinomas (7-9). The greatest increase in cellular proliferation was preferentially found along the lesser curvature (10). These changes are accompanied by an increase in labeling index (LI)(8, 9, 11-17). It is therefore suggested that LI-increases can be used as early markers of the carcinogenic potential of BHA. Recently we found that in rats not only forestomach-, but also glandular stomach-, small intestine- and colo/rectal- and possibly oesophagus-tissues are susceptible to the proliferation enhancing effects of BHA (18). In primates, the mitotic index was increased by 40% at the lower end of the oesophagus, after BHA had been administered by gavage (19). The oesophagus of pigs showed proliferative and parakeratotic changes after sub-chronic dietary BHA-feeding (20). In rodents other tissues appear to be target-organs for growth enhancing effects of BHA (18, 21, 22).
Although doses of BHA used in these experimental designs, are extremely high compared to approved concentrations of 0.001-0.02% in food for BHA alone or in combination with other antioxidants the possible interactions of BHA with other food constituents are relevant to the assessment of the risks of BHA intake. Simultaneous intake of ethanol and BHA is likely within a Western-type consumption pattern. Chronic alcohol consumption is considered a major risk factor for cancer in man. Main targets for ethanol-related carcinogenesis are the liver and the upper gastro-intestinal tract. The association is particularly strong for cancer of the oesophagus (23-26). Since ethanol is not carcinogenic itself, its effect may be explained by its modifying effects on other carcinogens (23). Following from our previous findings on the effects of BHA on cell proliferation index in rat upper gastro-intestinal tissues, the present study was intended to investigate the effects of ethanol on BHA-induced alterations in cell kinetic parameters in oesophagus, forestomach and glandular stomach.
Since high fibre diets have been shown to possess anticarcinogenic capacity in experimental carcinogenesis studies (27-29), we furthermore analyzed modulations of BHA-induced changes in gastro-intestinal cell kinetics in rats consuming a high cellulose diet.

Methods

Animals and maintenance
Male Wistar rats (Winkelmann, Borchen, FRG), five weeks old (89 $\pm$ 3g; mean $\pm$ SD), were housed individually in metabolic cages in an air-conditioned room at 21-22°C and 50-55% humidity with a 12-h dark/12-h light cycle. Rats were divided into groups of six animals each. During a seven days acclimatization

period, the rats had free access to powdered standard laboratory chow (diet nr. SRM-A; Hope Farms, Woerden, The Netherlands). The diet consisted of 27.5% protein, 7.3% fat, 4.4% fibre, 54.0% nitrogen free extract (carbohydrats), 6.6% minerals, vitamins and essential trace elements. Rats were given an experimental diet consisting of powdered laboratory chow supplemented with 0 (controls) or 1.5% BHA (food-grade BHA, purity >99%, 93% 3-BHA, J.Dekker Company, Wormerveer, The Netherlands). A homogeneous mixture of BHA and laboratory chow was obtained using a food processor to crush the pellets, and a blender for thorough mixing. Rats consumed the experimental diet for a 14 days.

The BHA-fed rats had free access to the food; drinking water was available to all rats *ad libitum*. Food consumption and drinking water were measured daily and body weights were determined three times per week. It has been demonstrated that dietary restriction reduces the incidence of both naturally occurring and chemically induced tumours in several organs, and inhibits cellular proliferation as well (30). Rats fed a diet supplemented with BHA reduce their food consumption immediately, probably due to non-palatability of the diet. Since a reduced dietary intake may interfere with the effects of BHA on cell kinetic parameters, we used pair-fed control groups (PFC). PFC-groups were given control diet restricted to the mean daily food intake (g/day) of rats in the corresponding BHA group. The animals were given access to food every day at 10.00 a.m.; regularly six hours later, food consumption was checked. By then the restricted animals had consumed their food ration by approximately 50%. Since our previous experiments indicated a sub-maximal response of forestomach LI at levels of 1.5% of BHA in the diet (18), we applied this BHA food concentration in the present studies rather than 2%.

Test procedure

In the first experiment, four groups of 6 male Wistar rats (88 $\pm$ 5 g; mean $\pm$ SD) were fed a diet containing 0% (PFC-control) or 1.5% BHA for two weeks. One 0% and one 1.5% BHA-fed group received 10% ethanol in the drinking water (*ad libitum*). These two groups of rats were encoded CE and BE, respectively. Two other groups of rats also fed 0% or 1.5% BHA, received normal drinking water (CW and BW, respectively). To evaluate the contribution of ethanol to the mean daily caloric intake of rats, the caloric value of the food consumption was determined by means of standard bomb calorimetry (IKA Germany). The caloric intake of rats (kJ/rat/day) was calculated as the sum of the caloric value of the food consumption and the amount of 10% ethanol consumed in the drinking water.

In the second experiment, another 4 groups of 6 rats (90 $\pm$ 4 g; mean $\pm$ SD) were fed 0 (PFC-control) or 1.5% BHA in the diet for two weeks. In one 0% and one 1.5% BHA-group the diet was supplemented with 20% dietary fibre (cellulose B; a generous gift of Mr Wijnen, Unilever, Rotterdam, The Netherlands). These groups were encoded CF and BF, respectively. Another PFC and another BHA group received no further dietary supplementation (BC and CC, respectively).

On the 14th day of the experimental diet, the animals were injected ip with 1 ml of 7.5 mg/ml 5-Bromodeoxyuridine (BrdU) (Serva, Heidelberg, FRG) in phosphate-buffered saline (pH 7.4). After four hours, rats were sacrificed by exsanguination through the aorta, under ether anaesthesia. Food was not withheld before killing in order to avoid inter-group differences in the period of last food consumption. The gastro-intestinal tract tissues (oesophagus, glandular stomach and forestomach) were dissected, opened lengthwise, cleaned from their contents, washed, fixed in 70% ethanol and stored at 4 °C in the dark. In experiment 2, in addition the same procedure was followed for colo/rectal tissues.

Assessment of cell kinetic parameters in gastro-intestinal tract tissues
Preparation of the oesophagus, glandular stomach and forestomach for determination of cell kinetics (LI) by flow cytometry was performed according to the procedure described by Schutte et al., (1987a)(31). Four samples per tissue per rat were randomly taken from the ethanol-fixed tissues. These samples were cut into tiny pieces and treated with 0.4 mg/ml pepsine in 0.1N HCl at room temperature. The released nuclei were isolated and subsequently treated with 2N HCl at 37°C for DNA-denaturation. Isolated nuclei were incubated with a monoclonal anti-BrdU-antibody (clone II B5) for 60 min. at room temperature and subsequently incubated with fluorescein-conjugated rabbit anti-mouse IgG-FITC (F313, Dakopatts, Denmark) for 60 min. at 4°C. Total DNA was stained with propidium iodide (Calbiochem., Behring Diagnostics, San Diego, CA). Double stained cells were analyzed on a FACS IV cell sorter (Becton and Dickinson, CA). Log green fluorescence was measured and recorded as the amount of bound anti-BrdU-antibody; red fluorescence was measured and recorded as the amount of bound propidium iodide. The LI is expressed as the percentage of BrdU-positive cells. No distinction can be made between epithelial and non-epithelial cell types. Otherwise, this method provides a reliable alternative for the ^{3}H-thymidine incorporation/ autoradiography technique (16). The LI detection limit is approximately 0.5%. Besides the LI, the potential doubling time (Tpot: the time needed to double the number of cells in a particular tissue without taking loss into account) and the mean transit time (Ts) were determined. Cell kinetic parameters were calculated as described by Begg et al. (1985)(32).
Because the LI in colon/rectum cannot be analyzed by flow cytometry adequately (16), randomized samples from colo/rectal-tissue were embedded in paraffin. For detection of BrdU-containing cells in these samples, an indirect enzyme-labeled antibody technique with peroxidase-conjugated second step reagents was applied (33). In short, sections were mounted on glass slides, deparaffinated and subsequently treated with H_2O_2 in methanol. After pepsine digestion and DNA denaturation, tissue sections were incubated with peroxidase-conjugated rabbit anti-mouse IgG (F114, Dakopatts, Denmark) for 60 min. at room temperature. Peroxidase-containing antibody sites were visualized by means of diaminobenzidine incubation for 5 min. The sections were counter-

stained with haematoxylin. Slides were encoded and epithelial cells were scored by light microscopy for immunoreactivity. Per animal 20 crypts were counted by two independent observers. By making cross-sectional slices of the crypts a discrimination was made between the proliferating and non-proliferating compartment. To exclude false changes in the overall labeling of the total crypt cell population, possibly as a consequence of effects on relative compartimental size, the number of cells per crypt cross-section were also counted. The LI is expressed as the percentage of BrdU-positively stained cells. Since there appeared to be a good correspondence between the values scored by the two observers (inter-observer variation was 7 $\pm$ 6% (mean $\pm$ SD)) values were averaged.

Statistics

Student's *t*-test for unpaired values was applied to evaluate statistical significance of differences between experimental and respective control groups.

Results

Experiment 1:

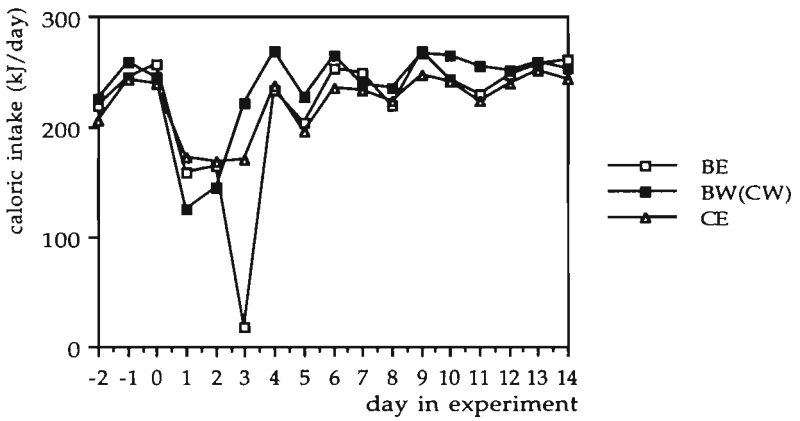

Figure 7-1: Mean caloric intake (kJ/day) of male Wistar rats fed an experimental diet containing 0 or 1.5% BHA and 0 or 10% ethanol in the drinking water for 14 days. There were no significant differences between the BHA-fed rats and their appropriate control groups or between the two BHA-groups or the two control-groups, during the 14-day period.
BE: BHA + ethanol; BW: BHA only; CE: ethanol controls; CW: controls.

Mean caloric intake and body weights are shown in Figures 7-1 and 7-2, respectively. Rats fed a diet supplemented with 1.5% BHA, reduced their food consumption immediately. After four days, their food consumption was more or less constant. Rats with 10% ethanol in the drinking water consumed

significantly lower amounts of food ($p<0.01$) per day, compared with the rats with normal drinking water. During the whole experiment the BHA intake of the BE-group was lower, although not significantly, than the BHA consumption of the BW-group (BE: 1.18 ± 0.27 mg/kg/day; BW; 1.30 ± 0.23 mg/kg/day). There were however no significant differences in mean caloric intake or BHA intake between the four groups of rats. The caloric intake in all groups was reflected by the body weight throughout the experiment.

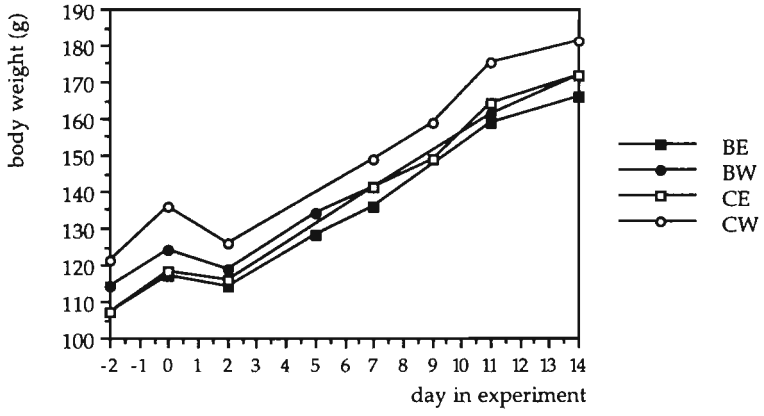

Figure 7-2: Mean body weights of rats fed 0 or 1.5% BHA in the diet and 0 or 10% ethanol in the drinking water. There were no significant differences between the four groups of rats.
BE: BHA + ethanol; BW: BHA only; CE: ethanol controls; CW: controls.

LIs in randomized samples of upper gastro-intestinal tract tissues as measured by flow cytometry are presented in Table 7-1.

Table 7-1: Labeling indices in rat oesophagus, forestomach and glandular stomach after simultaneous consumption of BHA and ethanol.

Tissue	BE-group	BW-group	CE-group	CW-group
oesophagus	4.7 ± 1.4[a]	5.1 ± 1.9[a]	3.4 ± 0.8[b]	2.1 ± 1.0
forestomach	21.6 ± 7.2[a]	18.3 ± 5.7[a]	5.6 ± 2.0	4.8 ± 2.2
glandular stomach	3.5 ± 1.3[a]	4.0 ± 1.9[a]	2.1 ± 0.6	2.1 ± 1.0

Cell kinetic parameters were determined by flow cytometry. LI is expressed as % BrdU-positive cells. Superscripts indicate the statistical significance of differences. a: $p<0.001$ vs appropriate control group; b: $p<0.001$ vs CW.
BE: BHA + ethanol; BW: BHA only; CE: ethanol controls; CW: controls.

The forestomach LIs of BHA-fed rats were significantly higher than those of the corresponding control-groups. There were no significant differences between the two BHA-fed groups (BE vs BW) or between the two control-groups (CE vs CW).
In rat glandular stomach, proliferation enhancing effects as indicated by an increase in LI, were found in the BHA-fed groups as compared to their appropriate control groups. Ethanol had no significant effect on the LI in this organ, since there were no differences in LI between the two control groups (CE vs CW).
In the oesophagus not only an increase in LI was found in the 1.5% BHA-fed groups compared with the corresponding PFC-groups, but there was also a significant difference in cell kinetics between the control groups (CE>CW; $p<0.001$). There was no significant difference between the BHA-fed groups (BE vs BW). In all three organs the increase in LI, as a result of short-term BHA administration, was accompanied by a decrease in Tpot. Ts was not altered.

Experiment 2:

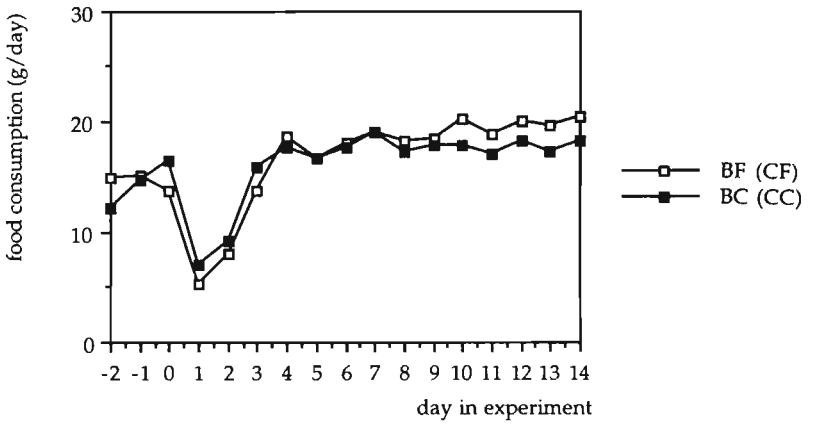

Figure 7-3: Mean daily food consumption by male Wistar rats fed an experimental diet containing 0 or 1.5% BHA and 0 or 20% extra cellulose for a period of 14 days. There were no significant differences between the BF-, BC-, CF- or CC-rats.
BC: BHA only; BF: BHA + fibre; CC: controls; CF: fibre controls.

Mean daily food consumption and body weights are shown in Figures 7-3 and 7-4, respectively. Immediately after the onset of administration of BHA, rats in the BF- and BC-group decreased their food intake. From day 4 of the experiment onwards, the food consumption remained relatively constant. There appeared to be no significant difference in BHA intake between the two BHA-fed groups (BF: 1.38 $\pm$ 0.27 g/kg/day; BC: 1.49 $\pm$ 0.22 g/kg/day) or in caloric intake between all groups of rats (BC and CC: 282 $\pm$ 61 kJ/day; BF and CF: 290 $\pm$ 81 kJ/day). The food intake in all groups was reflected by the body weight

throughout the experiment. There were no significant differences in mean body weight between the four groups of rats.

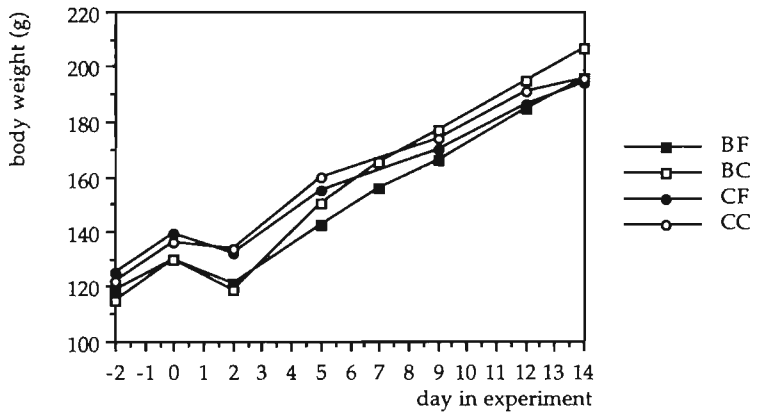

Figure 7-4: Mean body weights of rats fed 0 or 1.5% BHA and 0 or 20% extra cellulose in the diet for 14 days. There were no significant differences between the four groups of rats.
BC: BHA only; BF: BHA + fibre; CC: controls; CF: fibre controls.

Table 7-2:.Labeling indices in rat oesophagus, forestomach, glandular stomach and colon/rectum after simultaneous intake of BHA and dietary cellulose.

Tissue	BF-group	BC-group	CF-group	CC-group
oesophagus	5.5 ± 2.5[a]	6.2 ± 2.6[a]	3.5 ± 0.4	3.6 ± 1.0
forestomach	10.9 ± 2.2[a/b]	14.3 ± 2.1[a]	3.6 ± 0.2[b]	5.0 ± 0.4
glandular stomach	3.6 ± 0.4[a]	3.4 ± 0.3[a]	2.5 ± 0.3	2.5 ± 0.2
colo/rectal	14.2 ± 1.3[a/c]	16.3 ± 1.6[a]	9.3 ± 2.1[c]	11.9 ± 1.9

The labeling indices (% BrdU positive cells) in oesophagus, glandular stomach and forestomach were determined by flow cytometry, the LI in colon/rectum by means of immunocytochemistry. Superscripts indicate the statistical significance of differences. [a]: $p<0.001$ vs appropriate control group; [b]: $p<0.001$: BF<BC; CF<CC; c: $p<0.05$: BF<BC; CF<CC.
BC: BHA only; BF: BHA + fibre; CC: controls; CF: fibre controls.

Data on LI in randomized samples of forestomach-, glandular stomach-, oesophagus- and colon-tissue are presented in Table 7-2. In all of the tissues

examined, BHA induced a significant increase in LI as compared to the appropriate PFC-group. In rat forestomach tissue, simultaneous administration of 20% cellulose and 1.5% BHA resulted in a 3.4% lower LI as compared to the BHA group not given added fibre (BF<BC). Colorectal LI was decreased by 2.1% in groups of rats fed 1.5% BHA vs 1.5% BHA plus 20% fibre. Fibre supplementation of the diet *per se* resulted in a significant decrease (CF vs CC) of 1.4% in forestomach tissue LI and of 2.6% in colo/rectal LI. Furthermore, the increase in colo/rectal epithelial LI was clearly observable as increased immunoreactive staining in the proliferative compartment of the colonic crypts. There were no significant differences in colon crypt height between the four groups of rats. Tissue samples from the glandular stomach and oesophagus showed no significant alteration in LI as a result of simultaneous fibre administration. The proliferative effects of BHA, which resulted in an increase in LI were paralleled by a decrease in Tpot, while Ts was not altered.

Discussion

BHA is a rodent forestomach carcinogen (1, 6, 7, 34) and enhances cell proliferation in other gastro-intestinal tract tissues (19, 20) and in non-intestinal organs (21, 22). The present studies were intended to study possible interactions of BHA with other food constituents, namely ethanol and dietary fibre, in rats.
Results from the present studies confirm our previous results (18) that in rats several tissues from the gastro-intestinal tract (forestomach, glandular stomach, colon/rectum and possibly the oesophagus) are susceptible to the proliferation-enhancing potential of BHA. In both of the present experiments, the proliferation-enhancing effects of BHA after 14 days of administration at 1.5% BHA in the diet of rats were statistically significant in all of the tissues examined compared with the corresponding PFC groups. The LI values in rat forestomach are consistent with previously reported dose-response relations (11-14, 18, 35). The increase in LI was accompanied by a decrease in Tpot, while Ts was not altered. This indicates that BHA increases the growth fraction of cells but not the individual cell duplication rate.
Although ethanol is not carcinogenic itself (26), its effect may result from its modification of the effects of other carcinogens. A number of studies have indicated that ethanol increases the incidence of chemically induced tumors in laboratory animals (23). Chronic alcohol consumption has an enhancing effect on oesophageal carcinogenesis induced by methylbenzyl-nitrosamine (36) and diethylnitrosamine (37). Ethanol consumption can increase the tumor incidence in animals exposed to benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene (38), vinyl-chloride (liver) and polycyclic hydrocarbons (39), N-nitroso-pyrrolidine (nasal cavity and tracheal tumors)(40), nitropyrrolidine (liver, oesophagus and lung)(41) and dimethylhydrazine (rectum)(37). The mechanisms by which ethanol enhances chemical carcinogenesis, are unknown (42). Epidemiological studies indicate an association between the consumption of large amounts of

alcohol and cancer in those areas of the gastro-intestinal tract that are exposed either directly to concentrated ethanol solutions (oesophagus) or where most of the ethanol is metabolized (liver) (43).
Since ethanol consumption is associated with an increased tumor incidence in upper gastro-intestinal tract, synergistic effects of simultaneous administration of ethanol and BHA on cell kinetic parameters in rats may be hypothesized. The only significant result in this study, attributable to ethanol administration, is an increase in oesophageal LI in the CE-group as compared to its PFC-group (CW)($p<0.001$) which is consistent with previous reports (23, 43-46). This phenomenon may be due to direct exposure of the oesophagus to concentrated ethanol-solutions. It is therefore concluded that simultaneous administration of 10% ethanol in the drinking water during a 14-day period, has no effect on BHA-induced alterations in cell kinetics in oesophagus-, forestomach- and glandular stomach-tissue.
Dietary fibre is another factor that might influence cell kinetics in the gastro-intestinal tract. Studies with liquid diets indicate that cell proliferation in colon is reduced when fibre is completely excluded from the diet; this implies that fibre is physiologically important in maintaining colonic cell proliferation at normal levels (28). High fibre diets have been reported to reduce colon cancer risk (27, 28, 47, 48). Current understanding of the role of dietary factors in the process of carcinogenesis implicates the metabolic activity of the bacterial population of the gastro-intestinal tract. The flora composition can be influenced by changes in the diet, possibly by alterations in its fibre-content (27, 47, 48). Secondly, fibre might influence cell proliferation by some direct physicochemical action (shortening of colonic transit time) or by increasing food bulk, thereby diluting and possibly minimising any contact between the mucosa and possible carcinogens (27, 47). The third possible mechanism is the binding of carcinogens to fibres or uptake in fibres which also results in a lower carcinogen concentration (27).
In the present study, the effect of a high fibre intake on BHA-induced alterations in cell kinetic parameters was evaluated in rats. The present study also demonstrates that the organs examined (oesophagus-, forestomach-, glandular stomach- and colon/ rectum-tissue) are a target for proliferation enhancing effects of BHA. Concomitant administration of 20% extra cellulose reduced the LI in forestomach- and colon-tissue in the BHA-fed groups as well as in the control groups. In colon tissue, the LI-reduction in the BF- vs. BC-group was comparable with the decrease in LI in the control groups (CF vs. CC). It is concluded that fibre induces an overall reduction in LI in this organ and does not specifically antagonize the proliferation enhancing effects of BHA. In rat forestomach however, the reduction in LI in the BHA-fed groups was 2.4 times higher as the LI-decrease in the control-groups. This indicates that adding extra fibre to the diet provides a partial protection against the proliferation enhancing effects of BHA in forestomach. This may be explained by BHA-dilution, BHA-uptake in or binding to dietary fibre, or decrease of food transit time in the forestomach. High fibre diets furthermore appear to have no effect on LI in glandular stomach and oesophageal tissue.

It is therefore concluded that in rat forestomach a high fibre-intake decreases the possible tumorigenic risk associated with BHA-intake. However, the BHA-cellulose interaction as reported in this study, has been observed at antioxidant and fibre doses which are not representative for human diet composition. These results therefore have only limited relevance for health risk assessment of BHA consumption.

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Chapter 8

General discussion

Overview of the action of BHA

The synthetic antioxidant BHA is widely used as a stabilizer in preserving edible fats, oils and lipid-containing foods (1). Phenolic antioxidants, such as BHA, have beneficial effects by protecting against toxic compounds that are derived from the destruction of nutrients and the oxidation of fatty acids in food. BHA, at least in small amounts, is present in many commercial food products and thus constitutes a fraction of human and animal diet. For this reason, numerous studies on the biological effects of antioxidative food ingredients have been performed. Initially, BHA has been screened for possible toxicological properties. These toxicity tests have not revealed any severe pathological effects; only high oral doses produced liver hypertrophy and growth retardation in dogs and rodents (2-4) while in primates the mitotic index of the oesophagus increased (5).

In the second half of the seventies, a number of reports drew attention to some unexpected physiologic properties of BHA. At low doses, BHA appeared to be effective in inhibiting carcinogen-induced tumor development in rodents (6-8). The mechanism of chemoprevention could involve: scavenging of radicals, or enhancement of activities of enzymes involved in detoxication of xenobiotics, which decreases the production of ultimate carcinogenic metabolites of xenobiotics probably by inhibiting the enzyme systems involved in their metabolism; furthermore, inhibition of binding of carcinogenic metabolites to cellular DNA and finally modification of the immune response (3, 6). This may imply that BHA could give rise to a reduction of cancer in man. Further studies however revealed that, despite its inhibitory influence on carcinogenesis, BHA may not be as safe as previously assumed.

In 1983, BHA was first demonstrated to be carcinogenic in rat forestomach epithelium (9). This finding stimulated extensive carcinogenicity studies in other animal species. Subsequently, BHA was shown also to be carcinogenic to male Syrian golden hamsters (10-12). The carcinogenic dose appeared to be 2% in rats and 1% in hamsters and the resulting incidence of squamous cell carcinomas was in the range of 10-35% in both species (11, 12). Based on data in experimental animals the International Agency for Research on Cancer (IARC) concluded that there is *"sufficient evidence for carcinogenicity"* (13).

Several reports revealed that the sequence of effects in rat forestomach epithelium includes lesions, inflammation, hyperplasia, papillomas and finally carcinomas, depending on dose-level and duration of BHA-administration (14-16). Both carcinomas and papillomas were induced in a range of experiments by 2%

BHA, only papillomas were found with 1% BHA and only hyperplastic lesions at 0.5% BHA (17, 18). However, several studies showed that simple hyperplasia and papillomas induced by BHA appeared to be completely reversible after withdrawal of BHA, which indicates that continues feeding of BHA is necessary for the increase in size and the progression to carcinoma (12, 19, 20). The proliferation enhancing effects of BHA are not limited to forestomach epithelium; other tissues of the gastro-intestinal tract (21, 22) as well as non-intestinal organs (21, 23) appear to be susceptible for BHA.
Several possible mechanisms for the carcinogenic potential of BHA in the forestomach of rodents have been postulated, such as continuous background exposure to small quantities of initiating agents either exogenously derived or endogenously formed; aberrant methylation from S-adenosylmethionine to the N^7 and O^6 positions of guanine as a result of the excessive proliferative changes in the tissue; formation of reactive oxygen species as a result of redox cycling of metabolites of BHA; uncoupling of membrane function; selective binding of BHA metabolites to microsomal protein resulting in a depletion of tissue thiols; instability of forestomach tissue resulting in oncogene activation; increase in spontaneous mutations as a result of an increased number of mitosis (24, 26). No evidence has however been provided to support any of these hypotheses.

Most genotoxic carcinogens are known to interact with cellular macromolecules and metabolites of many genotoxic carcinogens interact with DNA bases. If BHA or metabolites of BHA interacts with cellular macromolecules in the forestomach epithelium to exert its carcinogenic or toxic action, metabolites of BHA should be detectable in forestomach epithelium; this is recently confirmed (17). However, using either radio-isotopically labelled DNA, or ^{32}P post-labelling-analysis, BHA, TBQ or TBHQ form no adducts with DNA (27, 28). This indicates that BHA-induced carcinogenesis may not be initiated by the formation of aromatic DNA-adducts in rat forestomach (28). BHA did bind, although aspecifically, to forestomach protein (28). The negative results in most tests for mutagenicity (29-33) also strongly suggest that BHA by itself does not react with DNA (34, 35). There are however several indications for the generation of reactive oxygen species during the metabolism of BHA, and more specifically, of its primary metabolites TBHQ and its corresponding quinone (36-39). This indicates, that at a sufficiently high concentration BHA or its metabolites might overcome the intracellular defence mechanisms that control risk for excess levels of potentially injurious natural intermediates, such as hydrogen peroxide. Production of excessive amounts of hydrogen peroxide and oxygen radicals, and the consequent induction of excessive cellular proliferation in the forestomach might be a possible mechanism of indicated carcinogenic action of BHA. Established non-mutagenicity, lesion reversibility and the dependence of cell proliferation on the continuous presence of BHA in the diet, are factors that strongly indicate that BHA is a non-genotoxic carcinogen.

However, recently it has been reported that BHA has a weak initiating potential in two-stage mouse skin carcinogenesis (40) and in a two-stage BALB/3T3 cell transformation assay (41).

Hypothesis

Coadministration of acetylsalicylic acid, an inhibitor of prostaglandin H synthase, has been shown to induce a significant reduction of the proliferation enhancing effects of BHA in rat forestomach (42). Prostaglandin H synthase, an enzyme composed of both cyclooxygenase and peroxidase activity, is involved in the biosynthesis of prostaglandins, thromboxane and prostacycline (20, 43). Virtually all mammalian tissues display some prostaglandin H synthase activity (20, 44, 45). Prostaglandin H synthase is a membrane protein predominantly located in the endoplasmic reticulum, with lesser amounts in nuclear membrane and occasionally in the plasma membrane (43, 46, 47). The first reaction catalysed by prostaglandin H synthase is the conversion of arachidonic acid to prostaglandin G_2. Prostaglandin G_2 is subsequently reduced to the corresponding alcohol prostaglandin H_2 by the peroxidase activity (46). The cyclooxygenase reaction of prostaglandin H synthase is characterized by a high substrate specificity allowing only a small group of polyunsaturated fatty acids to be oxygenated, with arachidonic acid acting as the preferential substrate *in vivo* (45). The lack of specificity of prostaglandin H synthase peroxidase enables the peroxidase to cycle equally well with lipid hydroperoxides, organic hydroperoxides and even with hydrogen peroxide, although prostaglandin H_2 is the primary substrate (45). Free arachidonic acid, or the products of its metabolism are normally not found intra- or extracellularly at considerable levels in mammalian tissues (46). Increased synthesis of prostaglandins is induced by chemical, physical or hormonal stimulation and may represent a physiological defense mechanism (48). This defense mechanism is for instance probably necessary to maintain cellular integrity of the gastro-intestinal mucosa (49). Prostaglandins, thromboxane and prostacycline appeared to be involved in inflammatory processes, wound repair and proliferative skin diseases (50). Prostaglandins of the E-type have often been associated with the regulation of cell proliferation (50-52). Moreover, several studies have demonstrated that simultaneous administration of inhibitors of prostaglandin H synthase activity (acetylsalicylic acid, indomethacin) with certain tumor promotors or chemical carcinogens results in an inhibition of tumor promotion and tumor development, respectively (53-55).
BHA has strong irritating properties and may therefore cause an enhanced phospholipid turnover, especially a release of arachidonic acid. An increase in arachidonic acid metabolism could ultimately result in a disturbance of gastro-intestinal tissue homoeostasis i.e. the steady state between cell gain and cell loss, possibly resulting in hyperplasia. This hyperplastic response may be

characterized by inflammatory reactions, stimulation of the arachidonic acid-metabolism, desensitization of antiproliferative signals and interruption of intercellular communication (51, 56). Furthermore, arachidonic acid itself appeared to be capable of stimulating superoxide anion radical production *in vitro* in inflammatory cells (57). The inflammatory reaction may be accompanied by enhanced amounts of reactive oxygen intermediates produced by polymorphonuclear cells (58). This suggests that arachidonic acid itself or metabolites of arachidonic acid formed as a result of BHA treatment might also be involved in the process of BHA-induced carcinogenesis through enhanced production of reactive oxygen species. In addition, BHA itself has been shown to exert prooxidant activity (37, 38, 41, 59-62). In summary, the working hypothesis for this thesis was that products of prostaglandin H synthase-mediated metabolism, in particular the production of prostaglandin E_2, might be involved in the mechanism of BHA-induced enhancement of cell proliferation.

Cell kinetics in gastro-intestinal tract tissues.

Results from the studies which are described in Chapter 2 and 7, confirm our previous results (22) that in rats several tissues from the gastro-intestinal tract (forestomach, glandular stomach and colon/rectum) are susceptible to the proliferation-enhancing potential of BHA. In both experiments, the proliferation-enhancing effects of BHA after 14 days of administration at 1.5% BHA in the diet of rats were statistically significant in all tissues examined as compared with the corresponding pair-fed control groups. The LI values in rat fore-stomach comply with previously reported dose-response relations (14, 17, 22, 63-65). The increase in LI was accompanied by a decrease in Tpot, while Ts was not altered. This indicates that BHA increases the growth fraction of cells but not the individual cell duplication rate. Moreover, BHA affects not only squa-mous epithelium, but other cell types lining the lumen of the digestive tract as well.

Arachidonic acid and linoleic acid release in relation to gastro-intestinal cell kinetics.

In Chapter 2, the effect of acetylsalicylic acid and indomethacin on BHA-induced alterations in cell kinetic parameters in relation to PGE_2-release was evaluated in rats. In forestomach and glandular stomach, both inhibitors decreased the BHA-induced enhancement of cell proliferation significantly. Acetylsalicylic acid completely counteracted the effect of BHA on LI in colorectal tissue whereas indomethacin exhibited no effect in this organ. Administration of acetylsalicylic acid or indomethacin only did not affect cell proliferation in the tissues examined. Both inhibitors reduced PGE_2 release in

all tissues examined in both the BHA-fed groups and the PFC groups. BHA itself however, appeared to be a potent inhibitor of PGE_2-production in these organs. Similar results were found for the two major hydroxy fatty acids formed by prostaglandin H synthase-mediated metabolism of arachidonic acid. It is therefore concluded that enhancement of cell proliferation induced by BHA in forestomach, glandular stomach and colon/rectum is not attributable to an increase of arachidonic acid metabolism, in particular PGE_2 formation, in these organs.

Arachidonic acid is a substrate for both the prostaglandin H synthase and lipoxygenase pathways. Inhibition of the prostaglandin H synthase-mediated pathway could therefore result in an enhanced metabolism of arachidonic acid by the lipoxygenase pathway. Acetylsalicylic acid exerted an additional inhibitory action on lipoxygenase-mediated metabolism of arachidonic acid in forestomach tissue. Indomethacin had no effect on 5-/12-/15-HETE release in all tissues examined. BHA appeared to be an inhibitor of arachidonic acid metabolism by lipoxygenase in forestomach tissue. In summary: acetylsalicylic acid and indomethacin did not affect linoleic acid metabolism by either the prostaglandin H synthase and the lipoxygenase pathway. BHA treatment however, resulted in a significant inhibition of HODEs release by both pathways.

The effects observed were most pronounced in the upper gastro-intestinal tract tissues, whereas in colorectal tissue the effect was negligible. Forestomach tissue is exposed to higher concentrations of BHA after oral intake than colorectal tissue where BHA which remains after resorption (66), is diluted by faecal bulk. This suggests a dose-response relationship for the inhibitory action of BHA on both linoleic acid and arachidonic acid metabolism.

It is concluded that the increase in cell proliferation induced by BHA in all the organs examined, is not attributable to an increase in linoleic acid- or arachidonic acid-derived hydroxy fatty acids.

However, BHA is a strong inducer of cell proliferation in rat gastro-intestinal tract tissues Coadministration of inhibitors of prostaglandin H synthase significantly inhibited the effects of BHA on labeling indices. This indicates that this enzyme system is involved in the mechanism underlying the carcinogenicity of BHA. It has been demonstrated that prostaglandin H synthase can bioactivate many chemical carcinogens to their ultimate reactive forms (45, 46, 67). Generally, prostaglandin H synthase-dependent metabolic activation is most important in extrahepatic tissues with low monooxygenase activity (46). Several reports have indicated that the reactivity of BHA itself is not high enough to damage cellular macromolecules (68). The negative results in most tests for mutagenicity also strongly suggest that BHA is not DNA-reactive (29-33). It is however possible that BHA becomes carcinogenic after being metabolized to more reactive compounds. Earlier reports indicate that the hydroquinone-metabolite of BHA: TBHQ and its corresponding quinone: TBQ are most likely to represent the active metabolites (37, 41, 68). Quinone metabolites can enter into a redoxcycling in which one-electron reductions to semiquinones

and hydroquinones occur and their subsequent oxidation back to quinones. At these conditions, hydrogen peroxide and oxygen radicals are formed (36). BHA carcinogenesis can therefore be caused by the attack of metabolites of BHA or oxygen radicals produced by these metabolites on cellular constituents. We therefore suggest that prostaglandin H synthase is involved in the metabolic activation of BHA into more reactive metabolites. The peroxidase component of prostaglandin H synthase could be involved by the conversion of TBHQ into TBQ.
Reports on the metabolism of BHA *in vivo* and *in vitro* revealed that the major metabolic pathways for BHA are conjugation reactions of the phenolic hydroxyl group with glucuronic acid and sulphate and *O*-demethylation of BHA into TBHQ followed by conjugation with glucuronic acid and sulphate (66, 69-73). In addition to these major biotransformation pathways several minor metabolites have been identified (35, 59, 68, 70, 74-76).

Metabolism of BHA *in vivo*.

Therefore, peroxidation of BHA was studied *in vivo*, as is described in Chapter 3. The excretion of free-BHA, -TBHQ, -TBQ as well as its conjugated forms in the urine was assessed in male Wistar rats fed BHA, or BHA in combination with prostaglandin H synthase-inhibitors. Most of the administered BHA was excreted into urine conjugated with glucuronic acid and/or sulphate. Averaged urinary TBHQ excretion was significantly increased in rats treated with BHA and acetylsalicylic acid as compared to rats treated with BHA only; TBQ excretion was correspondingly lower in rats treated with BHA and an inhibitor of prostaglandin H synthase (acetylsalicylic acid or indomethacin) as compared to rats treated with BHA only. Regression analysis revealed that the labeling indices of forestomach tissue, as reported in Chapter 2, are negatively correlated with TBQ excretion into urine, e.g. with TBQ formation ($p<0.05$).
We therefore suggest that prostaglandin H synthase is involved in the metabolism of TBHQ into TBQ, and that this metabolic pathway contributes to the increase in cell proliferation induced by BHA. This would also explain why oral intake of BHA decreased the metabolism of both arachidonic acid and linoleic acid by prostaglandin H synthase. This reduction in arachidonic acid- and linoleic acid-derived metabolites could be due to a competition between these hydroxy fatty acids and BHA or metabolites of BHA for prostaglandin H synthase-activity.

Peroxidation of TBHQ *in vitro*.

Quantitation of enzymatic metabolism of TBHQ by prostaglandin H synthase and lipoxygenase *in vitro*, as described in Chapter 3, showed that the rate of

autooxidation of TBHQ in buffer was low (2%) when compared to incubations in which peroxidases were available to support redoxcycling. Both prostaglandin H synthase and lipoxygenase were capable of metabolizing approximately 10% of the original amount of TBHQ into TBQ, under the chosen experimental conditions. Addition of cofactors resulted in an acceleration of the oxidation of TBHQ into TBQ. These results confirm the finding that prostaglandin H synthase (and lipoxygenase) is (are) capable of oxidizing TBHQ.

Induction of oxygen radical formation.

In Chapter 3, the ability of TBHQ to induce the formation of active oxygen species in presence of both prostaglandin H synthase and lipoxygenase was tested by means of electron spin resonance (ESR) spectroscopy measurements. ESR measurements revealed that autooxidation of TBHQ into the SQ radical appeared to be iron-dependent causing a reduction of Fe^{3+} into Fe^{2+}. Subsequently, in presence of oxygen, oxidation of the SQ radical into TBQ occurs generating the superoxide anion radical. These superoxide anions dismute in the presence of SOD under formation of hydrogen peroxide. Hydrogen peroxide can subsequently result in formation of hydroxyl radicals by the classical Fenton reaction. SQ formation appeared not to be dependent of oxygen radicals; reactive oxygen species are formed during subsequent oxidation of SQ into TBQ. Subsequently, redoxcycling between the semiquinone radical and quinone occurs, which is accompanied by formation of superoxide anion radicals, hydrogen peroxide and hydroxyl radicals. Addition of prostaglandin H synthase in presence and absence of arachidonic acid results in a substantial acceleration of SQ formation. This stimulation is even more marked in incubations with prostaglandin H synthase only, confirming competition between TBHQ and arachidonic acid for prostaglandin H synthase activity.
Incubations of TBHQ and lipoxygenase had no effect on either SQ formation and no oxygen radicals were formed.

It is concluded that after prostaglandin H synthase-mediated metabolism of TBHQ into SQ, subsequent redoxcycling between SQ and TBQ yielding reactive oxygen species formation might induce oxidative DNA damage. Several mechanisms are possible which relate endogenous DNA damage to cancer:

- Mutagenic oxidants could produce somatic mutations in nuclear DNA, such as point mutations and/or clastogenic effects such as deletions. A somatic mutation could ultimately result in a disruption of the cell by altering structural genes and their regulation.
- Oxidative damage resulting in mutations of mitochondrial DNA could cause energy deficiencies in cells. Energy deficiencies in cells can lead to deficiencies in energy-dependent maintenance enzymes and also to cell death. Cell death from DNA damage (mitochondrial or nuclear) could cause

neighbouring cells to proliferate. Proliferating cells contain more ss DNA, a much more sensitive target for oxidants. Moreover: proliferating cells can much more effectively convert adducts to mutations than nonproliferating cells.
- Oxidation could result in loss of 5-methylcytosine. 5-Methylcytosine appears to be important in turning off genes in differentiation. Loss of 5-methylcytosine could cause dedifferentiation and subsequent cell proliferation and thus contribute to cancer.
- Oxidative cell damage could activate oncogenes and activate cell proliferation (77).

We therefore hypothesized that enzymatic peroxidation of TBHQ by prostaglandin H synthase into TBQ, thereby generating superoxide anion, hydrogen peroxide and hydroxyl radicals might induce DNA damage. 8-Oxodeoxyguanosine (8-oxodG) is one of the about 20 different oxidized DNA damage products which have been identified and may therefore be used as a biological marker of oxidative stress to DNA (78).

Induction of oxidative DNA damage *in vitro*.

The capacity of BHA, TBHQ and TBQ to induce oxidative DNA damage was therefore studied *in vitro*, as described in Chapter 4. It has been demonstrated that single stranded (ss) bacteriophage ϕX DNA can be inactivated by oxygen radicals. In this test system, the biological consequences of interaction between naked ss phage DNA and BHA (respectively TBHQ and TBQ) as well as generated oxygen radicals can be studied without interference by cellular components. Studying the biological inactivation of ϕX-174 DNA by BHA or its metabolites in relation to effects on oxidation on deoxyguanosine (dG) might contribute to elucidation of the mechanism of BHA toxicity.
The present experiments show that BHA did not affect the survival curves of ss ϕX-174 DNA, while incubations of BHA with dG only resulted in a two-fold increase in 7-hydro-8-oxo-2'-deoxyguanosine (8-oxo-dG) formation. This indicates that the reactivity of BHA itself is probably not high enough to damage cellular macromolecules.
Both test systems showed that TBQ was not capable of induction of oxidative DNA-damage, which is probably due to lack of reductase activity *in vitro*. Quinones accept a single electron to yield semiquinone radicals which can participate in a redoxcycling of superoxide generation by transferring the electron to molecular oxygen. In general, quinones can be activated via one-electron reductions to reactive semiquinone radicals by enzymes such as NADPH-cytochrome p450-reductase and other flavoproteins (79).
However, TBHQ appeared to be a strong inducer of oxidative DNA damage resulting in inactivation of ϕX-174 DNA and oxidation of dG. The mechanism for the generation of reactive oxygen species we previously proposed (Chapter

3, scheme 1), is largely confirmed by the results on TBHQ-induced inactivation of phage DNA in combination with the effects of radical scavengers. Autooxidation of TBHQ is accompanied by formation of superoxide anion radicals, hydrogen peroxide and hydroxyl radicals.
Furthermore, we observed that the one-electron oxidations of TBHQ via SQ into TBQ cause oxidative DNA damage via the formation of oxygen radicals, and that these effects are not due to the hydroquinones themselves nor to semiquinone radicals.
Although peroxidation of TBHQ by prostaglandin H synthase is accompanied by active oxygen formation, addition of prostaglandin H synthase did not result in higher inactivation ratios of phage DNA. Apparently, enzymatic peroxidation of TBHQ causing a superoxide anion burst and consequently higher amounts of hydrogen peroxide, results in hydrogen peroxide utilization by prostaglandin H synthase itself. Consequently, less hydroxyl radical will be formed resulting in less DNA-oxidation. *In vivo*, low levels of hydrogen peroxide can efficiently be degraded by enzymes such as catalase. Oxidative DNA-damage can however be induced by oxygen radicals derived from hydrogen peroxide, at conditions at which the rate of production of hydrogen peroxide is too large for effective degradation by antioxidative enzymes normally present in the cellular system. These activated oxygen species may lead to cell death as well as genetic alterations; this may explain the observed threshold in the dose-response relationship for the induction of cell proliferation in rat gastro-intestinal tract tissues.

Although spectrophotometric measurements revealed that lipoxygenase is capable of converting TBHQ into TBQ, this reaction appeared not to be accompanied by semiquinone-radical or oxygen-radical formation (Chapter 3). This finding is in agreement with the results on oxidation of dG, respectively inactivation of phage DNA. Oxidation of TBHQ by lipoxygenase resulted in both test systems in a statistically significant decrease of oxidative DNA-damage as compared to autooxidation of TBHQ into TBQ. Addition of radical scavengers had no effect on the induced damage, which confirms that no oxygen radicals are formed during the oxidation reaction by lipoxygenase. Lipoxygenases are dioxygenases; consequently a two-electron oxidation of TBHQ into TBQ occurs without semiquinone radical formation, which is in agreement with the described ESR measurements (Chapter 3).

In conclusion, the results presented here indicate that BHA itself does not induce oxidative DNA damage. The quinone-metabolite also showed very low reactivity. TBHQ however induced inactivation of phage DNA and formation of 8-oxodG, due to the generation of reactive oxygen species. *In vivo* and *in vitro* studies on the metabolism of BHA have revealed that the main metabolic routes are *O*-demethylation, conjugations and dimerization (68). Several reports showed that demethylation of BHA by cytochrome p450 yields TBHQ

(35, 80, 81). The oxidative demethylation of BHA into TBHQ can be elevated after cytochrome p450 induction by phenobarbital in rats (82). The increase in TBHQ formation did however not affect the labeling indices in these animals, which makes it unlikely that this biotransformation pathway is responsible for the proliferation enhancing effects of BHA (82). Hydroquinones like TBHQ are subject to various detoxifying conjugation reactions *in vivo*. Only a very small portion will escape conjugation to form the quinone via the semiquinone radical and by this route may produce large amounts of superoxide anion radicals. *In vivo*, the enzymatic reduction of quinone yielding semiquinone radicals depends on the activity of reductases. Although quinones are also electrophylic species which easily bind to cellular macromolecules, they maintain their potential for the generation of active oxygen species even after binding to cellular thiols (79). Moreover, several reports indicate that the prooxidant activity of BHA can be attributed to its metabolites TBHQ and in particular TBQ (35, 68, 79). We have shown that prostaglandin H synthase is capable of converting TBHQ into its semiquinone radical, and that subsequent redox-cycling occurs, yielding reactive oxygen species. As a next step, it is of relevance to determine the significance of the prostaglandin H synthase pathway in the mechanism of carcinogenicity of BHA *in vivo*.

Induction of oxidative DNA damage *in vivo*/histology.

In Chapter 5, we evaluated the effect of metabolic activation of BHA by prostaglandin H synthase on the induction of oxidative DNA damage in the epithelial cells of the glandular stomach and colon/rectum as well as in the liver. Simultaneously, we determined the nature and the time-dependency of the early lesions in rat forestomach, glandular stomach and colon/rectum in the same rats.

BHA appeared to be capable to induce oxidative damage in liver DNA only after 14 days of continuous BHA-administration. This relative lack of response may be related to low prostaglandin H synthase activity of liver cells (20). In the DNA of the epithelial cells of the glandular stomach, BHA induced an increase in 8-oxodG formation gradually with the duration of BHA treatment. Coadministration of acetylsalicylic acid inhibited the effect of BHA on oxidative DNA damage. In colorectal DNA, similar observations were made; the levels of oxidative DNA damage however tend to be smaller.

Histological examination of gastro-intestinal tract tissues showed that the severity of forestomach and glandular stomach hyperplasia as well as the inflammatory reactions increased while BHA-administration continued. BHA treatment resulted in thickening of the mucosa with eosinophylic infiltration of the lamina propria. Coadministration of acetylsalicylic acid showed a marked inhibition of forestomach lesions. In the pair fed-control groups, no proliferation or inflammation was observed. Coadministration of acetylsalicylic

acid resulted in a marked decrease of both hyperplasia and inflammation in these target organs. In colorectal tissues no histological abnormalities were observed.
The irritating effect of BHA was most pronounced in the upper gastro-intestinal tract tissues, whereas in colorectal tissue no histological effect was observable. This suggest a dose-response relationship for the effect of BHA on both 8-oxodG formation and irritation of the epithelium which paralleled the effect on arachidonic and linoleic acid metabolism, as described in Chapter 2.

In Chapter 2 and 7, we showed that oral intake of BHA at this particular dose increases labeling indices in rat forestomach, glandular stomach and colon/rectum after a fourteen days period of administration. This increase in cell proliferation appears to be accompanied with an enhancement of oxidative DNA damage in both glandular stomach and colon/rectum. Oxidative DNA damage however also occurs in regions which demonstrated no indication of inflammation, e.g. colon epithelium. An inflammatory action or other hyperplastic lesion can therefore not be directly responsible for enhancement of cell proliferation by BHA. This is in agreement with the effect of BHA on arachidonic acid and linoleic acid metabolism. Compounds derived from prostaglandin H synthase-mediated metabolites of these fatty acids have been shown to be involved in inflammatory processes. If an initial inflammatory response would be responsible for the induction of gastro-intestinal cell proliferation, arachidonic acid and linoleic acid metabolism is more likely to be increased, in contrast to the reductions as we have found (Chapter 2). Moreover, earlier reports indicate that hyperplasia can also be observed in forestomach epithelium without signs of inflammation or cell necrosis (4). Therefore, we suggest that the production of excessive amounts of hydrogen peroxide and oxygen radicals and the consequent induction of excessive cell proliferation may be the underlying mechanism of carcinogenicity of BHA. No oxygen activating properties can be ascribed to BHA itself. The BHA-metabolite TBHQ can enter redoxcycling after autoxidation or more likely, peroxidation by prostaglandin H synthase into the semiquinone radical. TBQ undergoes redoxcycling leading to an oxidative burst in the presence of enzymes capable of one-electron reduction forming the semiquinone radical.

Induction of oxidative DNA damage and cell proliferation *in vitro*.

In Chapter 6, we have tested the potential of BHA, TBHQ and TBQ to induce cell proliferation and oxidative DNA damage in human lymphocytes. Cultures of human lymphocytes are commonly used as a test system to assess induced DNA damage and for studies on cell kinetics. The results showed that both BHA and TBHQ can be metabolized in whole blood cultures. The low recovery of in particular TBHQ and TBQ indicates that a considerable binding of these

metabolites to macromolecules must have taken place. All three testcompounds were capable of increasing labeling indices of human lymphocytes in culture. Analysis of 8-oxodG formation in the DNA of lymphocytes showed that TBHQ at the applied concentration induced an 11-fold increase in oxidative DNA damage. The toxicity of the dose employed must however be taken into account. TBQ also increased the ratio 8-oxodG/dG significantly. The oxidative effect of BHA on DNA of lymphocytes was less pronounced. Metabolism of BHA however results in TBHQ formation. It is therefore not clear whether the increase in labeling indices and the observed cytotoxicity is attributable to BHA itself or to its hydroquinone metabolite. Therefore, the effects of inhibitors of cytochrome p450 (e.g. piperonyl butoxide) to prevent TBHQ formation and of prostaglandin H synthase (acetylsalicylic acid) which prevents oxidation of TBHQ into SQ have to be assessed, in order to determine the effect of BHA resp. TBHQ on cell proliferation and oxidative DNA damage. For this study, no conclusions can be made about the particular relation between oxidative DNA damage and induction of cell proliferation.

Finally, in Chapter 7, we evaluated the possible interactions of BHA with other food constituents, which might be relevant for the assessment of health risks of BHA intake. Simultaneous intake of ethanol and BHA is likely to occur in a Western consumption pattern. However, 14 days of simultaneous administration of BHA and ethanol had no effect on BHA-induced alterations in cell kinetics. Since high-fiber diets have been shown to possess anticarcinogenic capacity in experimental carcinogenesis, we also analysed modulations of BHA-induced changes in gastro-intestinal cell kinetics in rats consuming a high-cellulose diet. Dietary fibre appeared to provide a partial protection against the proliferation-enhancing effects of BHA in the gastro-intestinal tract of the rat (Chapter 7).

Table 8-1: Overview of the outcome of the thesis

In vivo

BHA:

- Induction of cell proliferation in several gastro-intestinal tract tissues (forestomach, glandular stomach and colon/rectum) of the rat (Chapter 2 and 7).
- Inhibition of both arachidonic acid and linoleic acid metabolism in the same gastro-intestinal tract tissues (Chapter 2).
- Coadministration of acetylsalicylic acid or indomethacin inhibited the BHA-induced enhancement of cell proliferation in the tissues examined (Chapter 2).
- Coadministration of acetylsalicylic acid or indomethacin resulted in a significant inhibition of TBHQ metabolism into TBQ by prostaglandin H synthase (Chapter 3).
- Induction of oxidative DNA damage in liver cells and in the epithelial cells of glandular stomach and colon/rectum of the rat (Chapter 5).
- Induction of hyperplasia and inflammation in both forestomach and glandular stomach of the rat. Absence of tissue abnormalities in colon/rectum of the rat (Chapter 5).
- Coadministration of acetylsalicylic acid resulted in a significant decrease of both epithelial oxidative DNA damage and the incidence of early lesions induced by BHA in the rat (Chapter 5).
- Coadministration of ethanol had no effect on BHA-induced alterations in cell kinetics in oesophagus, forestomach and glandular stomach of the rat (Chapter 7).
- Coadministration of dietary fibre provides a partial protection against the proliferation enhancing effects of BHA in the rat (Chapter 7).

In vitro

BHA

- No induction of oxidative DNA damage in two test systems using ϕX-174 bacteriophage DNA or 2'-deoxyguanosine (Chapter 4).
- Induction of cell proliferation of human cell lymphocytes, concentrations of 1 mM or higher being cytotoxic (Chapter 6).
- BHA was not capable of inducing oxidative DNA damage in human lymphocytes (Chapter 6)

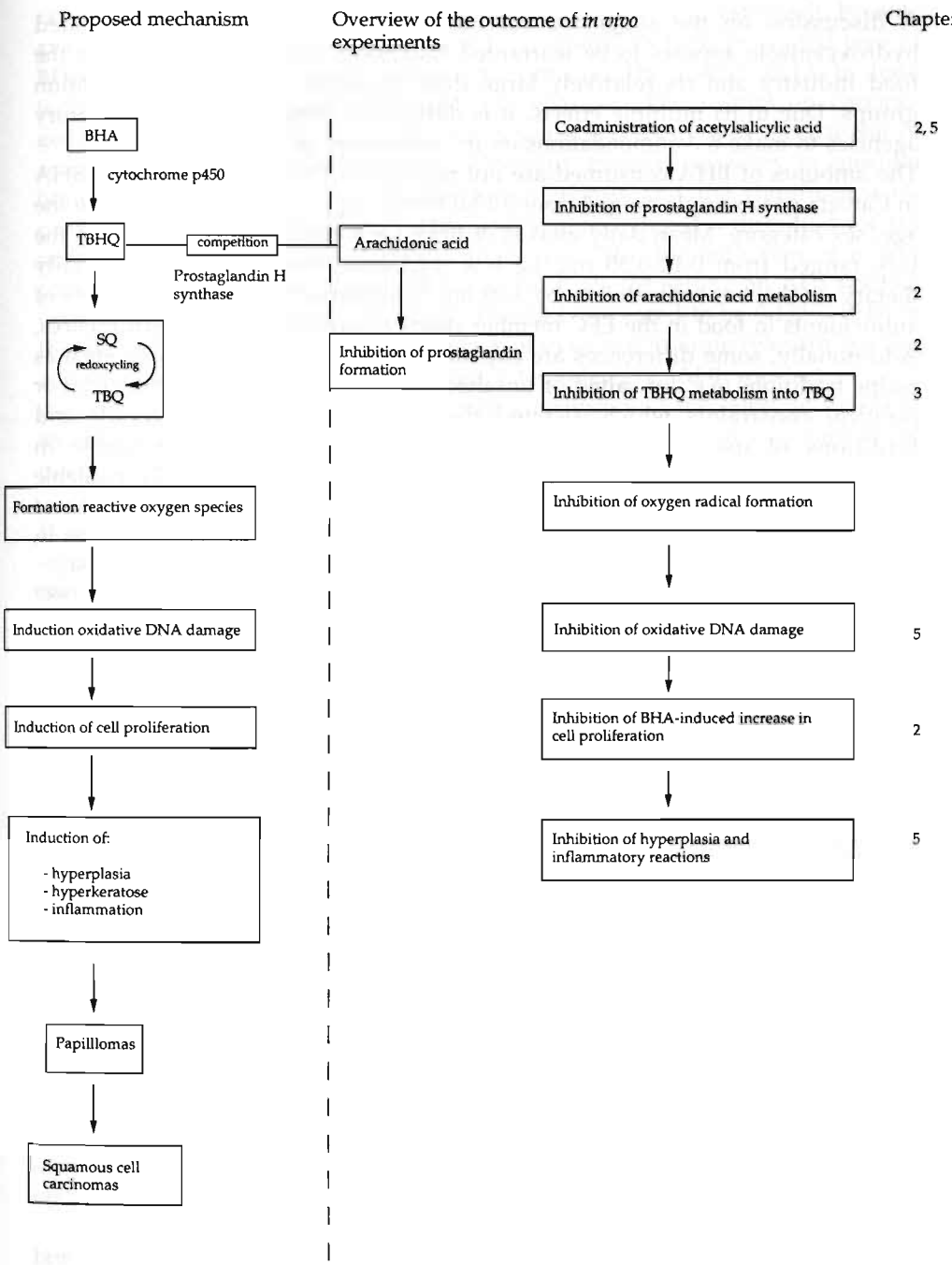
TBHQ

- Autooxidation of TBHQ in phosphate buffered saline resulted in semiquinone radical formation, this reaction being iron-dependent (Chapter 3).
- Redoxcycling between SQ and TBQ is accompanied by formation of superoxide anion radicals, hydrogen peroxide and hydroxyl radicals (Chapter 3).
- Coadministration of prostaglandin H synthase resulted in a substantial acceleration of SQ formation and subsequent active oxygen formation (Chapter 3).
- Incubations of TBHQ and linoleic acid did not result in an enhancement of SQ formation or oxygen radical formation (Chapter 3).
- Spectrophotometric measurements revealed that both prostaglandin H synthase and lipoxygenase can convert TBHQ into TBQ (Chapter 3).

- Strong induction of 8-oxodG formation resp. inactivation of phage DNA due to formation of active oxygen, TBHQ and SQ being inactive (Chapter 4).
- Induction of cell proliferation of human cell lymphocytes, concentrations of 100 μM or higher being cytotoxic (Chapter 6).
- TBHQ was a strong inducer of oxidative DNA damage in human lymphocytes (Chapter 6).

TBQ

- No induction of oxidative DNA damage in the two used assays (Chapter 4).
- Induction of cell proliferation of human cell lymphocytes, concentrations of 1 mM or higher being cytotoxic (Chapter 6).
- TBQ was capable of inducing oxidative DNA damage in human lymphocytes (Chapter 6).



Scheme 8-1: Hypothesis for the possible mechanism underlying the tumorigenicity and the induction of proliferative changes of BHA in gastro-intestinal tract tissues of rodents

A discussion on the suggested carcinogenicity of the synthetic butylated hydroxyanisole appears to be warranted because of its widespread use in the food industry and its relatively large daily ingestion by human population groups. Due to its multiple effects, it is difficult at present time for regulatory agencies to make recommendations on its application as a food additive.
The amounts of BHA consumed are not negligible. The dietary intake of BHA in Canada par example ranged from 0.13-0.19 mg/kg b.w./day depending on the age/sex category. Mean daily intakes of BHA for comparable age groups in the U.S. ranged from 0.12-0.35 mg/kg b.w. In Japan, the maximum total daily dietary intake was estimated to be 3.09 mg. Information of application levels of antioxidants in food in the EEC member states showed considerable differences. Additionally, some differences are explained by consumer preferences such as recipe traditions (e.g. for salted or unsalted magarines), others by commercial or political constraints which dictated the availability of raw materials and traditions of use of antioxidants (tocopherols are readily obtainable in Germany; BHA and gallates in the UK). Only limited information is available on the intake of antioxidants in the EEC. In 1982, the estimated daily intake of BHA in The Netherlands appeared to be about 4 mg per person, whereas in Belgium the daily intake of BHA and/or BHT was 1.45 mg on average (83, 84).
Results from the first carcinogenicity study on BHA became available in 1982 which showed that this additive produces cancer of the forestomach in rats when fed at high dose levels. This matter was reviewed by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) and questions were raised concerning the relevance of this outcome for human health, particularly in view of the fact that man does not have a forestomach, the lack of explanation of the mechanism of action of BHA and finally the fact that the animal response was seen at doses estimated to be 10.000 times or higher than that to which the consumer is exposed. In view of the nature of the lesions reported, the specific sites of the lesions, the absence of systemic carcinogenicity and the negative results in *in vitro* tests for genotoxicity, further information was requested in order to determine the mechanisms involved in the effects of BHA on the forestomach. Moreover, the effects of BHA should be examined in species without a forestomach (e.g. dog or monkey)(85). JECFA set a temporary ADI of 0-0.5 mg/kg b.w. pending results of the additional studies. In 1983, the Scientific Committee for Food (SCF) of the EEC concluded that there was no short-term risk to health in relation with the continued use of BHA. Additional studies on BHA were requested to resolve questions related to possible degradation products, reversibility of the early lesions, possible genotoxicity, kinetics and mechanism of action, the determination of a clear no-effect-level for the induction of forestomach hyperplasia and studies on animals without a forestomach (86).
In the mid eighties, further results on genotoxicity, involving mammalian and bacterial cells, supported the conclusion that BHA was not mutagenic. Additional information on proliferative changes noted in the forestomach of

rats and on the stomach of species that do not possess a forestomach, became available. On basis of these data, the SCF concluded that the permitted uses of BHA did not pose a health hazard to humans. The use of BHA in food technology was maintained without any changes. The results on carcinogenicity studies of BHA together with data on specificity and mechanism of action and the lack of evidence for genotoxicity made it necessary to consider both the concept of a threshold for tumorigenesis and the significance for prediction of effects on human health. The SCF recognized a threshold for the non-genotoxic tumorigenic effects of BHA. The NEL for induction of hyperplasia in the rat forestomach was used as the basis for setting an ADI without using a larger safety factor than usual. The NEL appeared to be 0.125% BHA in the diet (62.6 mg/kg b.w.). Given the lack of evidence in more relevant species, a safety factor of 100 was considered adequate. The ADI was set at 0-0.5 mg/kg/day (87). JECFA reached a similar conclusion on the safety of BHA (87, 88). In evaluating the genotoxic potential of BHA, no attention was paid to the possible genotoxicity of metabolites of BHA.

With respect to TBHQ, *in vitro* and *in vivo* mutagenicity data have become available which do not exclude the possible genotoxicity of TBHQ. Therefore, the SCF of the EEC did not establish an ADI for this compound. The substance is not acceptable for use as an antioxidant in food in the EEC and in Canada.
This means that in several countries the application of BHA as a dietary antioxidant is generally accepted although the use of its metabolite TBHQ is prohibited because of its supposed genotoxicity.
In 1975, the Joint FAO/WHO Committee however established a temporary ADI of 0-0.75 mg/kg b.w. for TBHQ based on results of a long term feeding study in dogs in which a NEL of 1.5 g/kg (75 mg/kg b.w.) of the diet was observed. However, the structural similarity of this compound to BHA and butylated hydroxytoluene was noted and therefore the Committee requested reproduction studies (89). In 1978, the Committee decided, after reevaluating the existing data, that the previously required study of the effect on reproduction with mixtures of antioxidants was no longer needed. The temporary ADI was changed to a regular ADI. The ADI was set at 0-0.5 mg/kg b.w. based on the same feeding study in dogs (90). In 1986, mutagenicity data for TBHQ were reviewed. There appeared to exist some, although conflicting, evidence that TBHQ exerted genotoxic activity. Additional studies to resolve questions related to the mutagenicity of TBHQ were requested as well as life-time feeding studies in two rodent species. Feeding studies should take into account the normal degradation products of TBHQ in food. The existing specifications were revised. Again a temporary ADI was established at 0-0.2 mg/kg b.w., which implies the use of a larger safety factor than usual (91). In 1991, the Committee reviewed the data on tumor promoting activities, lung toxicity, genotoxicity and the induction of hyperplasia by TBHQ. There was however still no adequate carcinogenicity study available. The temporary ADI was therefore exten-

ded until 1994 (92).

Rats fed a diet supplemented with BHA reduce their food intake immediately, probably as a consequence of non-palatability of the diet. A reduced dietary intake, or more specifically a diminished caloric intake, has been shown to inhibit cellular proliferation in several tissues of the mouse (93, 94). Moreover, a reduced caloric intake inhibited the incidence of both naturally occurring and chemically induced tumours in several organs of experimental animals (93, 94). In experimental animals treated with dietary dose levels of BHA above the maximum tolerated dose (0.5 %), both food consumption and body weight were significantly reduced as compared to the control groups. Therefore, in performing (long-term) carcinogenicity studies with dietary BHA, effects of a reduced caloric intake cannot be ruled out. A reduced dietary intake may interfere with the effects of BHA on cell kinetic parameters. Therefore, we used pair-fed control groups. Pair fed control groups were given a control diet restricted to the mean daily food intake (g/day) of rats in the corresponding BHA group. By this method, we discovered cell proliferation enhancing effects not only in rat forestomach but also in rat glandular stomach and colon/rectum. These findings underline the significance of using pair-fed control groups in BHA carcinogenicity studies.

Survey of the results available at present, indicates that the hyperplasiogenic potential of BHA is not limited to rat forestomach epithelium but that other cell types lining the gastro-intestinal tract as well as non-intestinal organs are susceptible for the proliferation enhancing effects of BHA. Although the chemical reactivity of BHA is not high enough to damage cellular macromolecules, reactive metabolites such as TBHQ and TBQ, do exert genotoxicity. After oral administration of low doses of BHA to both rat and man, about 9% will be metabolized into TBHQ which can subsequently be converted into its corresponding quinone, causing oxygen radical formation. Therefore, risk assessment of BHA should also be based on the reactivity of those two metabolites. In view of the observed genotoxic potential of these metabolites, the use of BHA as a food additive is questionable. A reassessment of BHA cancer risk should be considered.

However, there is still no definite proof for carcinogenicity of BHA. Additional studies have to be carried out to resolve questions related to the carcinogenicity and genotoxicity of BHA in relation with the metabolism of BHA into reactive compounds. Therefore the following studies are required for reevaluation of the cancer risk of BHA (and TBHQ):

- An adequate carcinogenicity study, focusing on total gastro-intestinal tract, in at least two different species with and without forestomach, using pair-fed control groups.
- Reevaluation of the possible genotoxic potential BHA in short-term tests of

mutagenicity and genotoxicity, in presence of metabolic activation systems (e.g. cytochrome p450; prostaglandin H synthase).

However, in view of the suspected genotoxicity of TBHQ and the proliferation enhancing effects of BHA in several organs of the rat other than the forestomach, we suggest the use of a larger safety factor for assessment of an ADI for BHA pending the results of the above mentioned studies.

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Summary

Our environment contains many synthetic and naturally occurring antioxidants. Antioxidants are for example present in many food stuffs, tobacco, plants, cosmetics, oils, soap, medicines, rubbers and plastics and are widely used as industrial chemicals. They play a very important role as food additives by preventing rancidity of fats and oils by inhibition of radical formation, decomposition of peroxides or inactivation of metals. Synthetic food antioxidants which are used in food include BHA, BHT, TBHQ, propyl gallate, and ethoxyquin.

BHA has been included at low concentrations in the human diet for many years, without evidence of adverse effects. Toxicity testing of BHA has not revealed any severe pathological abnormalities. However, BHA has carcinogenic potential. Life-time exposure to relatively high doses resulted in dose-dependent changes (hyperplasia, papillomas and finally carcinomas) in the forestomach of rats, hamsters and probably mice. In non-rodents, proliferation-enhancing effects are found in the oesophagus of primates and pigs. In view of the established non-mutagenicity of BHA, this compound is however considered to be a "non-genotoxic" ("epigenetic") carcinogen. Although several mechanisms for the carcinogenic potential of BHA have been postulated, no evidence has been provided to support any of them. In this thesis, several studies on the mechanism underlying the carcinogenicity of BHA in laboratory animals as well as in several *in vitro* assays, are described.

In Chapter 1, a review is given on the current state of toxicological knowledge on BHA.

In Chapter 2 and 7, the effect of BHA on cell kinetic parameters in several tissues of the gastro-intestinal tract (forestomach, glandular stomach and colon/rectum) of the rat is evaluated. Results from both experiments confirm previous results that BHA affects not only the squamous epithelium of rat forestomach tissue but other cell types lining the lumen of the gastro-intestinal tract as well.

In Chapter 2, the effect of coadministration of acetylsalicylic acid and indomethacin, both inhibitors of prostaglandin H synthase, on BHA-induced alterations in cell kinetic parameters in relation to the release of certain hydroxy fatty acids (in particular: PGE_2) is evaluated in rat gastro-intestinal tract tissues. Generally, both inhibitors decreased the BHA-induced enhancement in cell proliferation in rat gastro-intestinal tract tissues. Products of prostaglandin H synthase-mediated metabolism may therefore be involved in BHA-induced enhancement of cell proliferation. Analysis of the release of these hydroxy fatty acids in rat forestomach, glandular stomach and colon/rectum however shows that BHA also inhibited prostaglandin H synthase-mediated metabolism of arachidonic acid and linoleic acid. It is therefore concluded that products of arachidonic acid metabolism are not responsible for the enhancement of cell proliferation induced by BHA.

In view of the negative results in most tests for mutagenicity it is possible that

BHA becomes carcinogenic after being metabolized into more reactive compounds. Prostaglandin H synthase has been shown to be involved in the bioactivation of many chemical carcinogens. Therefore the effect of inhibition of this enzyme system in relation to BHA metabolism *in vivo* is evaluated in rats (Chapter 3). Analysis of the urines of these animals on free-BHA, -TBHQ, -TBQ as well as its conjugated forms showed that the averaged urinary TBHQ excretion in rats treated with BHA and prostaglandin H synthase inhibitors is significantly increased as compared to rats treated with BHA only; TBQ excretion is correspondingly lower. This indicates that prostaglandin H synthase is involved in the metabolism of TBHQ into TBQ and that this metabolic pathway contributes to the proliferation enhancing effects of BHA. Subsequent analysis of TBHQ-metabolism in presence and absence of prostaglandin H synthase *in vitro*, confirms the finding that this enzyme is capable of oxidizing TBHQ into its corresponding quinone (Chapter 3).
Several reports have indicated that the carcinogenic potential of BHA is probably due to these two metabolites, by inducing reactive oxygen species. By using electron spin resonance measurements, as described in Chapter 3, it has been demonstrated that TBHQ is capable of inducing active oxygen species. Spontaneous semiquinone radical formation appears to be dependent on the presence of metal ions. In presence of prostaglandin H synthase TBSQ formation is substantially accelerated. Subsequent oxidation of TBSQ into TBQ is accompanied by superoxide anion formation. A reaction scheme is suggested for the generation of semiquinone radicals as well as reactive oxygen species, involving the formation of superoxide anion radicals which ultimately results in the formation of hydroxyl radicals.
In Chapter 4, the capacity of BHA, TBHQ and TBQ to induce oxidative DNA damage is studied. The hypothesis concerning the prooxidant activity of these compounds has been tested using two *in vitro* assays. TBHQ is found to induce 2'-deoxy-7,8-dihydro-8-oxoguanosine (8-oxodG), a marker for oxidative DNA damage. The mutagenic potential of TBHQ is found to be reduced by addition of oxygen radical scavengers, confirming the significance of oxidative DNA-damage in the underlying mechanism of carcinogenicity of BHA. The oxidative potential of both BHA and TBQ appears to be low. Inactivation of bacteriophage ϕX-174 DNA shows comparable results.
The relevance of the prostaglandin H synthase pathway in BHA carcinogenesis is subsequently studied *in vivo* (Chapter 5). Analysis of the DNA of liver cells and epithelial cells of glandular stomach and colorectal tissue of the rat shows that BHA is capable of inducing oxidative DNA damage. Moreover, subchronic BHA-administration results in an increase in 8-oxodG levels in these organs. Histological examination of gastro-intestinal tract tissues on the induction of hyperplasia and inflammation by BHA, shows a time-dependent increase in the severity of these early lesions in rat forestomach and glandular stomach. In colorectal tissue no signs of inflammation or hyperplasia is observed. An increase in cell proliferation appears to be accompanied by an enhancement of oxidative DNA damage. Moreover, an increase in these parameters is also

observed in absence of inflammatory reactions. It is therefore unlikely that an inflammatory response is initially responsible for the increase in cell proliferation induced by BHA. Coadministration of acetylsalicylic acid inhibited both epithelial oxidative DNA damage and the incidence of early lesions in gastro-intestinal tract tissues. This indicates that this enzyme system is involved in the induction of cell proliferation by BHA. Cooxidation of TBHQ into TBQ by prostaglandin H synthase yielding active oxygen species may therefore be responsible for the carcinogenic effects of BHA.

In Chapter 6, we studied the relationship between the induction of cell proliferation and oxidative DNA damage in human lymphocytes in an *in vitro* assay. BHA, TBHQ and TBQ are all capable of increasing cell proliferation rate of human lymphocytes. However, BHA metabolism in whole blood results in TBHQ formation. It is therefore not clear whether the cell proliferation enhancing effects are attributable to BHA itself or to TBHQ. TBQ and in particular TBHQ increases 8-oxodG formation in human lymphocytes cultured *in vitro*; the applied dose being cytotoxic. Based on these preliminary results no relation can be established between the induction of oxidative DNA damage and an increase in cell proliferation in human lymphocytes.

In Chapter 7, the interaction of BHA with other food constituents, namely dietary fibre and ethanol, on cell kinetics in gastro-intestinal tract tissues is studied. While ethanol has no effect on BHA-induced alterations in cell kinetic parameters, dietary fibre does provide a partial protection against the proliferation-enhancing effects of BHA in the gastro-intestinal tract of the rat.

Finally, it is concluded that in view of the genotoxic potential of TBHQ *in vitro* as well as the ability of BHA to affect various gastro-intestinal tract tissues *in vivo*, the use of BHA as a food additive should be reconsidered. Suggestions for further research are given. Pending the results of these studies, application of a larger safety factor for assessment of an ADI for BHA as a food additive is suggested.

Samenvatting

Onze omgeving bevat vele synthetische en van nature voorkomende anti-oxydanten. Anti-oxydanten worden aangetroffen in veel voedingsmiddelen, tabak, planten, cosmetica, oliën, zeep, medicijnen, rubber en plastics. Verder worden ze veelvuldig gebruikt in de industrie. Zij spelen een zeer belangrijke rol als voedingsmiddelenadditief vanwege hun vermogen om het ranzig worden van vetten en oliën tegen te gaan door middel van remming van radicaalvorming, ontleding van peroxiden en inactivering van metalen. Synthetische anti-oxydanten die worden gebruikt in de voeding zijn BHA, BHT, TBHQ, propylgallaat en ethoxyquin.
Sinds vele jaren wordt BHA in lage concentraties toegevoegd aan voedingsmiddelen zonder dat nadelige effecten aantoonbaar waren. Onderzoek naar de toxiciteit van BHA toonde geen ernstige pathologische effecten aan. BHA is echter kankerverwekkend. Levenslange blootstelling aan relatief hoge doses BHA resulteert in dosis-afhankelijke veranderingen (hyperplasiën, papillomen en carcinomen) in de voormaag van ratten, hamsters en waarschijnlijk ook bij muizen. Bij niet-knaagdieren zoals apen en varkens zijn in de slokdarm proliferatie-verhogende effecten aangetoond. Gezien de negatieve resultaten van BHA in kortdurende testen voor genetische activiteit wordt BHA beschouwd als een niet-genotoxisch ("epigenetisch") carcinogeen. Alhoewel er verschillende hypotheses zijn beschreven met betrekking tot het werkingsmechanisme dat ten grondslag ligt aan de carcinogeniteit van BHA is tot op heden onvoldoende bewijs geleverd om een van deze hypotheses te onderbouwen.
In dit proefschrift worden verschillende experimenten beschreven die er op gericht zijn meer inzicht te verkrijgen in het werkingsmechanisme van de carcinogeniteit van BHA.
In hoofdstuk 1 wordt in het kort weergegeven wat de huidige stand van zaken is met betrekking tot met name de toxicologie van BHA.
In de hoofstukken 2 en 7 wordt het effect van BHA op de celkinetiek in verschillende organen van het spijsverteringsstelsel (voormaag, kliermaag en dikke darm) van de rat beschreven. De resultaten van deze beide experimenten zijn in overeenstemming met eerdere bevindingen dat BHA niet alleen effect heeft op de plaveisel-epitheelcellen van de voormaag, maar ook op andere celtypen die de wand van het maagdarmkanaal bekleden.
In hoofdstuk 2 is gekeken naar het effect van gelijktijdige toediening van remmers van prostaglandine H synthase (acetylsalicylzuur en indomethacine) en BHA op veranderingen in celkinetiek en de vrijmaking van bepaalde vetzuren (met name PGE_2) in het spijsverteringsstelsel van de rat. In het algemeen verlaagden beide remmers de door BHA geïnduceerde verhoging in celproliferatie in de diverse weefsels van het spijsverteringskanaal van de rat. Het is daarom mogelijk dat door prostaglandine H synthase produkten worden gevormd die betrokken zijn bij de door BHA geïnduceerde verhoging van de celproliferatie. Resultaten met betrekking tot de vrijmaking van deze vetzuren toonden ech-

ter aan dat ook BHA het metabolisme van arachidonzuur en linolzuur via prostaglandine H synthase remt. Dit betekent dat produkten van arachidonzuur- (en linolzuur-) metabolisme niet verantwoordelijk zijn voor de door BHA geïnduceerde verhoging van de celproliferatie.
Gezien de negatieve resultaten van BHA in verschillende kortdurende mutageniteitstesten is het mogelijk dat BHA carcinogeen wordt na metabole omzetting in reactievere stoffen. Diverse onderzoeken hebben aangetoond dat prostaglandine H synthase betrokken is bij de metabole activering van veel chemische carcinogenen. Het effect van remming van dit enzym systeem in relatie tot het metabolisme van BHA is vervolgens bestudeerd *in vivo* in ratten (hoofdstuk 3). Analyse van de urines van deze dieren naar zowel de vrije als geconjugeerde vorm van BHA, TBHQ en TBQ toonde aan dat de gemiddelde urinaire uitscheiding van TBHQ van ratten, behandeld met BHA en remmers van prostaglandine H synthase, significant verhoogd is bij vergelijking met ratten die alleen met BHA behandeld zijn; de TBQ uitscheiding was overeenstemmend lager. Dit betekent dat prostaglandine H synthase betrokken is bij de metabole omzetting van TBHQ in TBQ en dat deze metabole omzettingsroute een bijdrage levert aan de celproliferatie verhogende effecten van BHA. Spectrofotometrische bepalingen van TBHQ in aan- en afwezigheid van peroxidases bevestigen de bevinding dat dit enzym in staat is TBHQ, om te zetten in zijn chinon (hoofdstuk 3).
Verschillende onderzoeken toonden aan dat het carcinogene vermogen van BHA waarschijnlijk is toe te schrijven aan deze twee metabolieten, door vorming van reactieve zuurstofspecies. Met behulp van electron spin resonantie spectroscopie is aangetoond dat TBHQ in staat is reactieve vormen van zuurstof te induceren (hoofdstuk 3). De spontane vorming van het semichinon radicaal blijkt afhankelijk te zijn van de aanwezigheid van metaalionen. In aanwezigheid van prostaglandin H synthase is de vorming semichinon radicalen echter enorm versneld. De daarop volgende oxidatie van het semichinon radicaal in the chinon gaat gepaard met de vorming van superoxide anion-radicalen. Een reactieschema is opgesteld voor de vorming van semichinonradicalen en reactieve zuurstofspecies, uitgaande van de vorming van superoxide anionradicalen, hetgeen uiteindelijk resulteert in de vorming van hydroxylradicalen.
In hoofdstuk 4 is het vermogen van BHA, TBHQ en TBQ om oxydatieve DNA schade te induceren bestudeerd. Om de hypothese te testen ten aanzien van de pro-oxidant activiteit van deze stoffen is gebruik gemaakt van twee *in vitro* test-systemen. TBHQ blijkt in staat te zijn om 2'-deoxy-7,8-dihydro-8-oxoguanosine (8-oxodG) te induceren, een specifieke vorm van oxydatieve DNA-schade. De reactiviteit van TBHQ wordt gereduceerd door toediening van radicaal scavengers, hetgeen het belang van oxydatieve DNA-schade in het werkingsmechanisme dat ten grondslag ligt aan de carcinogeniteit van BHA verder bevestigt. Het oxyderend vermogen van zowel BHA als TBQ blijkt erg laag te

zijn. Wanneer gebruik gemaakt wordt van bacteriofaag ϕX174 DNA worden vergelijkbare resultaten gevonden.
Het belang van prostaglandine H synthase in de carcinogenese van BHA is vervolgens *in vivo* onderzocht (hoofdstuk 5). Analyse van het DNA van levercellen en epitheelcellen van de kliermaag en de dikke darm van de rat toonde aan dat BHA in staat is oxydatieve DNA schade te induceren. Subchronische toediening van BHA resulteerde in een verhoging van de 8-oxodG concentraties in deze organen. Voorts zijn diverse organen van het spijsverteringskanaal onderzocht op histologische afwijkingen zoals hyperplasie en ontstekingsreacties. BHA-toediening resulteerde in een tijds-afhankelijke toename in de ernst van deze vroege laesies in de voormaag en de kliermaag van de rat. In de dikke darm zijn geen tekenen van ontsteking of hyperplasie waargenomen. Een verhoging in celproliferatie blijkt gepaard te gaan met een verhoging van oxidatieve DNA schade. Maar een verhoging in deze parameters is ook waargenomen in gebieden waarin geen ontstekingsreacties waarneembaar zijn. Het lijkt daarom zeer onwaarschijnlijk dat een ontstekingsreactie primair verantwoordelijk is voor de verhoging in celproliferatie. Gelijktijdige toediening van acetylsalicylzuur resulteerde in een remming van zowel de oxydatieve DNA schade in epitheelcellen als in de incidentie van histologische afwijkingen in dezelfde weefsels van het spijsverteringsstelsel van de rat. Dit betekent dat dit enzym systeem betrokken is bij de door BHA geïnduceerde verhoging van de celproliferatie. Coöxidatie van TBHQ door prostaglandin H synthase, gepaard gaande met de vorming van reactieve zuurstof species, kan daarom verantwoordelijk worden geacht voor de carcinogene effecten van BHA.
In hoofdstuk 6 is de relatie bestudeerd tussen de inductie van cel proliferatie en oxidatieve DNA schade in humane lymphocyten in een *in vitro* testsysteem. BHA, TBHQ en TBQ zijn alle drie in staat de cel proliferatie te verhogen. Aangezien, metabolisme van BHA in vol bloed resulteert in de vorming van TBHQ is het onduidelijk of de verhoging in celproliferatie is toe te schrijven aan BHA zelf of aan TBHQ. TBQ en met name TBHQ verhoogden de 8-oxodG vorming in humane lymphocyten, de gebruikte dosis was echter cytotoxisch. Het is dan ook niet mogelijk om op basis van deze resultaten een relatie te leggen tussen de inductie van oxydatieve DNA schade en een verhoging in celproliferatie in humane lymphocyten.
In hoofdstuk 7 is gekeken naar het effect van de interactie tussen BHA en andere voedselbestanddelen (voedingsvezel en ethanol) op de celkinetiek van weefsels van het spijsverteringskanaal van de rat. Ethanol heeft geen effect op de door BHA geïnduceerde veranderingen in celkinetiek. Voedingsvezel daarentegen vormt een gedeeltelijke bescherming tegen de proliferatie-verhogende effecten van BHA in het spijsverteringskanaal van de rat.
Tenslotte is geconcludeerd dat, gezien de genotoxiciteit van TBHQ *in vitro* en het vermogen van BHA om verschillende organen van het spijsverteringskanaal *in vivo* te beïnvloeden, het gebruik van BHA als een voedingsadditief opnieuw beoordeeld moet worden. Voorts zijn enkele suggesties voor vervolg-

onderzoek gedaan. In afwachting van de resultaten van deze onderzoeken is het raadzaam een grotere veiligheidsfactor voor vaststelling van de ADI voor BHA als voedingsadditief te hanteren.

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Full papers

- Schilderman,P.A.E.L, Verhagen,H., Schutte,B., ten Hoor,F. and Kleinjans,J.C.S., (1991) Modulation by dietary factors of DNA-induced alterations in cell kinetics of gastro-intestinal tract tissues in rats. *Fd Chem Toxicol.*, **29** , 79-85.
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Curriculum Vitae

Pauline Anne Elisabeth Louise Schilderman werd geboren op 26 oktober 1961 te Heerlen. In 1981 behaalde zij het Atheneum-B diploma aan het Coriovallum college te Heerlen. Daarna startte zij met de studie Fysiotherapie aan de Academie voor Fysiotherapie te Heerlen (niet voltooid). Vervolgens studeerde zij tussen 1985 en 1989 Gezondheidswetenschappen met als afstudeerrichting Biologische Gezondheidkunde aan de Rijksuniversiteit Limburg te Maastricht. Vanaf september 1989 is zij als assistent in opleiding werkzaam geweest aan de Rijksuniversiteit Limburg bij de vakgroep Humane Biologie/Biologische gezondheidkunde (i.o) later Gezondheidsrisicoanalyse en Toxicologie (prof. dr. F. ten Hoor, prof. dr. J.C.S. Kleinjans) alwaar het onderzoek zoals beschreven is in dit proefschrift werd uitgevoerd. Vanaf januari 1994 is zij als universitair docent verbonden aan dezelfde vakgroep.

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