The toxicity of antioxidants and their metabolites

Aalt Bast *, Guido R.M.M. Haenen

Department of Pharmacology and Toxicology, Faculty of Medicine, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands

Received 27 July 2001; received in revised form 2 November 2001; accepted 9 November 2001

Abstract

Antioxidants are used on a very large scale to try to obtain and preserve optimal health. Nutraceuticals and food supplements frequently contain huge dosages of antioxidants. It is not generally recognised that high intake of antioxidants may also have adverse effects. Three antioxidants i.e. vitamin E, β-carotene and lipoic acid are used to illustrate general considerations on the toxicity of antioxidants. Based on the examples the following recommendations for the evaluation of the toxicity of antioxidants are made: (i) classical safety factors should not be used. (ii) Knowledge on the mechanism of the efficacy and toxicity of antioxidants should be increased. (iii) Bio-kinetic/bio-efficacy modelling might be of help to optimise dosage. (iv) When antioxidant supplementation changes into therapy, a more accurate risk/benefit analyses is warranted. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Vitamin E; β-Carotene; Lipoic acid; Antioxidant; Toxicity; Safety

1. Introduction

Antioxidants, in particular antioxidant vitamins have the annotation of being healthy and safe. This probably goes back to the discovery of vitamins in the beginning of the 20th century where the intake of vitamins was found important in preventing deficiencies. These deficiencies led to diseases like scurvy (shortage of vitamin C) or neurological disorders (shortage of vitamin E).

During the last two decades, the use of specific food components changed from treating deficiencies to preventing myriad diseases. Many diseases are associated with oxidative stress and the use of antioxidant rich food or antioxidant food supplements became immensely popular. In this hype, the term nutraceuticals was introduced in order to indicate the use of purified or concentrated food components in a pharmaceutical formulation with a presumed health or performance increasing effect. The general idea that nutraceuticals can still be regarded as food remained. Subsequent careless application resulted. Without appropriate knowledge on side effects, biotransformation or biokinetics of the nutraceuticals, relatively high doses were administered to large populations. Toxicologists, by nature not guided by whim or fancy, regularly warned against the delusive safety of nutraceuticals. Also in retrospect it can be concluded that for food-based compounds lack of toxicological risk should not be taken for granted. However, large populations are subjected to huge doses of for example antioxidants. Where lie the toxicological risks for these compounds? In this paper three antioxidants i.e. vitamin E, β-carotene and lipoic acid will be reviewed. Vitamin E and β-carotene are dietary antioxidants that are also present in numerous supplements. Vitamin E is generally regarded as safe, while the safety of β-carotene is heavily debated. Lipoic acid is an antioxidant that is used as a drug for the treatment of diabetic polyneuropathy.

2. Vitamin E

Vitamin E is the overall collective term for all biological active tocopherols and tocotrienols and their derivatives which exhibit the biological activity of \( R,R,R\)-α-tocopherol, the natural occurring vitamer with the highest activity (Witting, 1980). The chemical...
The aromatic ring of the chroman head is fully methylated (R1, R2 are H in \( \alpha \)-tocopherol and a phytyl chain. The phytyl chain intercalates with the phospholipids, while the chroman head—responsible for the actual antioxidant effect—faces the cytosol, although the chroman ring is still located in the hydrophobic zone of the lipid bilayer. An important characteristic of vitamin E is the presence of three asymmetrical carbon atoms in the phytyl tail. The biological half-life of the eight stereoisomers of a vitamer greatly differs. The half-life also affects the number of the different vitamers such as \( \alpha \)-tocopherol or to the other stereoisomers such as \( \gamma \)-tocopherol and \( \delta \)-tocopherol resides in its long retention in the body compared with the other vitamers such as \( R,R,R-\alpha \)-tocopherol or to the other stereoisomers such as \( S,S,S-\alpha \)-tocopherol (Witting, 1980). This is due to the high affinity of \( R,R,R-\alpha \)-tocopherol for transfer and binding proteins (Burton and Traber, 1990; Zimmer et al., 2000).

After ingestion of vitamin E, it has to be incorporated in micelles for efficient absorption. In the gastrointestinal tract vitamin E esters are hydrolysed to free \( \alpha \)-tocopherol and is transported via the lymph to blood. The absorption greatly depends on the composition of diet, i.e. the amount of fat consumed. All body tissues, in particular all cell membranes and subcellular organelles (mitochondria, cell nuclei, endoplasmatic reticulum) are supplied with vitamin E (Burton and Traber, 1990).

The best-studied activity of vitamin E is the protection of membranes against free radical damage. In the antioxidant activity of tocopherol, a radical abstracts a hydrogen atom from the aromatic hydroxyl group and a chromanoxyl radical is formed (Fig. 2). This chromanoxyl radical is fairly stable due to delocalization of the unpaired electron. The oxygen in the heterocyclic ring of the chroman ring is fixed in such a position that there is a considerable overlap between the 2p-type orbital of a lone pair of the oxygen and the aromatic \( \pi \)-system. This permits stabilisation of the chromanoxyl radical by interaction of the unpaired electron with a lone pair of that oxygen (Burton et al., 1985) In this way the degree of delocalization is enhanced.

The chromanoxyl radical can be converted back into tocopherol in several ways, e.g. by the interaction with vitamin C (Packer et al., 1979) or reduced glutathione (GSH) (Haenen and Bast, 1983). The reaction of GSH and the chromanoxyl radical is catalysed by a free radical reductase. Despite this interplay with other antioxidants, tocopherol can be converted into a quinone. By this oxidation, one of the rings of the chroman head is opened (Fig. 2). This quinone can be reduced into a hydroquinone (Bindoli et al., 1985) It has been reported that the antioxidant activity of this hydroquinone is superior to that of tocopherol. Provocatively, it has been stated that \( \alpha \)-tocopherol may serve as a reservoir of \( \alpha \)-tocopherol hydroquinone (Kohar et al., 1995).

Experiments using deuterated \( \alpha \)-tocopherol quinone demonstrate that \( \alpha \)-tocopherol quinone can be converted into \( \alpha \)-tocopherol in man (Moore and Ingold, 1997). During oxidation, numerous other products, including dimers, are formed (Kamal-Eldin and Appelqvist, 1996).

Beside oxidation of the chroman head, a conversion directly linked to the antioxidant function of tocopherol, tocopherols undergo \( \omega \)-oxidation and subsequent \( \beta \)-oxidation of the phytyl chain (Brigelius-Flohé and Traber, 1999; Pope et al., 2001; Van Houte et al., 2001). Several metabolites generated via the latter route have been described.

Insertion of vitamin E into lipid bilayers decreases membrane fluidity (Patel and Edwards, 1988). This decrease is similar to that observed after cholesterol enrichment. More recently, other non-antioxidant effects of tocopherol have been reported. \( R,R,R-\alpha \)-tocopherol stimulates protein phosphatase 2A in a concentration-dependent way. This activation may lead to dephosphorylation and inactivation of protein kinase C, a key enzyme in the proliferation of a number of cells. This pathway is suggested to be involved in the inhibition of the proliferation of vascular smooth mus-
cle cells by $R,R,R$-$\alpha$-tocopherol (Azzi and Stocker, 2000). Interestingly, $R,R,R$-$\beta$-tocopherol has no effect on proliferation of these cells. This difference in effect of both vitamers cannot be explained by a difference in uptake in the cells nor by a difference in free radical scavenging activity since these properties are very similar for both compounds (Azzi and Stocker, 2000).

The recommended daily allowance of vitamin E for an adult is 10 mg $R,R,R$-$\alpha$-tocopherol equivalents per day. It is generally believed that $\alpha$-tocopherol is relatively safe in a ‘therapeutic’ daily dose up to 300 mg (Kappus and Diplock, 1992). The major adverse effect reported was that oral intake of high levels of vitamin E can exacerbate an impaired blood coagulation due to vitamin K deficiency caused by malabsorption or anti-coagulant therapy (Kappus and Diplock, 1992). Recently, we have found that tocopherols and several tocopherol esters inhibit glutathione S-transferase P 1-1 (GST P 1-1) (Van Haaften et al., 2001a). It is known that GST P1-1 is present in the skin (Singhal et al., 1993). Mice lacking GST P1-1 have an increased risk for skin tumorgenesis (Henderson et al., 1998). Interestingly, vitamin E has been reported to be a complete tumour promoter in mouse skin (Mitchel and McCann, 1993). Combining these data, it is tempting to suggest that the promoter effect of vitamin E might be caused by GSTP1-1 inhibition. It can be calculated that the concentration of tocopherol esters in numerous cosmetic products is sufficient for blocking GST P1-1 (Van Haaften et al., 2001b). This urges to evaluate the potential risk of the application of these products.

Paradoxically, a pro-oxidant effect of tocopherol has also been described. Similar to the pro-oxidant effect of vitamin C, this activity is caused by the reducing power of tocopherol that, beside scavenging free radicals, is also responsible for the reduction of transition state metals. This reduction facilitates the formation of free radicals (Minoti and Aust, 1992). It has been stated that this pro-oxidant effect of tocopherol is involved in the increase in fatal myocardial infarctions that was observed in a clinical study with vitamin E supplements (Halliwell, 2000).

As mentioned above, quinones are metabolites from vitamin E generated when it exerts its antioxidant activity. In general, quinones have to be regarded as cytotoxic due to their ability to generate oxygen radicals, their ability to oxidise cellular components, and their ability to form Michael adducts with cellular thiol. The Michael addition of thiol to quinones depends on the nature, the number and the position of substituents on the quinone. $\alpha$-Tocopheryl quinone, the fully substituted tocopherol quinone, is incapable of forming these adducts with thiol, while $\gamma$-tocopheryl and $\delta$-tocopheryl quinone, that are only partially substituted, do. It has been found that $\gamma$-tocopherol quinone and $\delta$-tocopherol quinone are far more cytotoxic in cell culture than $\alpha$-tocopheryl quinone (Thornton et al., 1995). This difference in toxicity can be explained by the difference in ability of these different quinones to form Michael adducts. This effect of substituents on the toxicity of $\alpha$-tocopherol quinone parallels that of the effect of substituents on the toxicity of N-acetaminophen quinonimide. The toxicity of the fully substituted quinonimide is far less compared with the partially or non-substituted derivatives. Additionally, it has been shown that $\alpha$-tocopherol quinone can antagonise the reduction of the chromanoxyl radical of tocopherol by the GSH-dependent free radical reductase (Van Haaften et al., 2001c).

The difference in toxicity between $\alpha$-tocopheryl quinone and the other tocopheryl quinones is in line with the results of a study performed in 1930 with natural oils that were treated with FeCl₃ to destroy vitamin E (Taylor and Nelson, 1930). Although at that time it was not realised that tocopherol quinones were formed, they undoubtedly were. Processed cod liver oil supported growth in experimental animals, whereas processed germ oil was highly toxic. Cod liver oil mainly contains $\alpha$-tocopherol, whereas germ oil also contains $\gamma$ and $\delta$-tocopherol. The contrasting biological effect between both processed oils may now be explained by the difference in toxicity of the different quinones formed.

Recently, another metabolite of a vitamin E, has gained much attention, 2,7,8-trimethyl-2-($\beta$-carboxyethyl)-6-hydroxychroman, a metabolite from $\gamma$-tocopherol that is formed by $\beta$-oxidation of the phytol chain (Fig. 3), was reported to possess a strong natriuretic effect (Wechter et al., 1996). It was claimed that this metabolite is identical to a putative hormone proposed four decades ago, that controls the body’s pool of extracellular fluid. This is an important determinant in hypertension, congestive heart failure and cirrhosis. Surprisingly, the comparable metabolite formed from $\alpha$-tocopherol does not display a natriuretic effect, suggesting a great specificity of the metabolite of $\gamma$-tocopherol (Wechter et al., 1996).

A similar difference in efficacy between vitamins and their metabolites is also found in the inhibition of cyclooxygenase. $\gamma$-Tocopherol and one of its major metabolites $\gamma$-CEHC were effective inhibitors of this enzyme, while $\alpha$-tocopherol only had a minor effect (Jiang et al., 2000). It was hypothesised that this anti-inflammatory effect may contribute to disease prevention by $\gamma$-tocopherol.

![Chemical structure of 2,7,8-trimethyl-2-($\beta$-carboxyethyl)-6-hydroxychroman](image-url)

Fig. 3. Chemical structure of 2,7,8-trimethyl-2-($\beta$-carboxyethyl)-6-hydroxychroman. This metabolite of $\gamma$-tocopherol has a natriuretic effect.
The work of Azzi et al. (Azzi and Stocker, 2000; Stocker et al., 1999) suggests that the antioxidant effect of vitamin E is not the primary action of vitamin E. It is a challenging thought that vitamin E is in the first place a hormone-like compound that controls several cellular functions. Due to its oxidisability, it would act as a sensor of oxidative stress rather than a direct antioxidant. So the primary effect of scavenging of free radicals would not be to detoxify these radicals but rather to trigger the cellular response toward oxidative stress.

The new and diverse biological effects of vitamin E, advocate a re-evaluation of the effect of vitamin E administration. In this re-evaluation the metabolism and the biological effects of the metabolites should be included. Also the difference between the vitamers and the stereoisomers of the vitamers have to be considered. As discussed above the kinetics, efficacy, metabolism and toxicity of these vitamers may greatly differ.

3. β-carotene

β-Carotene, of which the chemical structure is depicted in Fig. 4, is a very lipophylic carotenoid that has provitamin activity. It can be converted into retinol (vitamin A) by a dioxygenase. Observational epidemiological studies indicate that the consumption of fruit and vegetables prevents cancer. Not only carotenoid rich fruit and vegetables but also a relatively high β-carotene serum level has been associated with a reduced incidence of for example lung cancer. The proposed beneficial properties of β-carotene were emphasised in a few small supplementation studies (Pryor et al., 2000). Cigarette smokers generally have subnormal serum concentrations of various carotenoids (including β-carotene). This is commonly attributed to oxidative depletion of carotenoids by oxidative stress and an increased inflammatory response, both associated with smoking (Van der Vliet, 2000).

Despite this suggested beneficial role of β-carotene, several large, randomised supplementation trial could not support this suggestion. Two major trials even found increased cancer incidence after β-carotene supplementation in both smokers and asbestos workers (Pryor et al., 2000). It has been thought that this could result from β-carotene only being effective as an antioxidant at low O₂ tension, whereas at high O₂ tensions it may even stimulate lipid peroxidation (Bast et al., 1998).

The free radical addition as depicted in Fig. 4, leads to the free radical form of the carotenoid molecule. Lipid peroxyl radicals (LOO⁺) that arise during lipid peroxidation may undergo such a radical addition reaction. Subsequent oxygenation of the radical-carotenoid adduct is possible:

\[
\text{LOO-carotenoid}^* + O_2 \rightarrow \text{LOO-carotenoid-OO}^*
\]

This LOO-carotenoid-OO⁺ might have a pro-oxidant activity. Its formation has been used to explain the stimulation of lipid peroxidation by β-carotene at relatively high oxygen tensions.

The instability of β-carotene or its metabolites (including oxidised products) in the lungs of cigarette smokers or asbestos workers have been suggested to be responsible for the carcinogenic response (Wang and Russell, 1999). The oxidised products of β-carotene facilitate carcinogenesis by promoting DNA damage or by inducing cytochrome P450 enzymes that promote carcinogen activation (Wang and Russell, 1999; Paolini et al., 1999). Carcinogens are abundantly found in cigarette smoke. The cytochrome P450 induction might also enhance the catabolism of retinoic acid decreasing its levels. Retinoic acid regulates epithelial cell growth through its nuclear receptors (RARs). RARβ, which is induced by retinoic acid, can inhibit activator protein 1 (AP-1) activity via a protein-protein interaction. In addition, oxidative stress induced by smoking enhances AP-1 expression. AP-1 binds to the AP-1 responsive element in DNA, and affects the actions of signal transduction pathways, usually resulting in cell proliferation. It is plausible that the β-carotene induced decrease in retinoic acid level in the lung, or the down-regulation of RARβ might reduce the inhibitory effect of retinoids on AP-1, so enhancing lung cell proliferation and potentially tumor formation (Wang and Russell, 1999).

The Scandanavian Alpha-tocopherol, Beta-Carotene Cancer Prevention Study (ATBC) showed that a supplementation with 20 mg β-carotene resulted in an 18% increase in lung cancer incidence. In the classical toxicological approach the ‘no-observed effect level’ (NOEL) in a lab animal is divided by a safety factor of 10 to find the NOEL in human. A second factor of 10 is used to account for interindividual differences. The 20 mg β-carotene as used in the ATBC trial is apparently not a safe dose in human. If we nevertheless (viz. it is not the NOEL) divide this dose by 10 for the interindividual differences, this would result in an as-
Lipoic acid is present in most kinds of pro- and eukaryotic cells. In the late 1940s, it received attention as a growth factor. The natural occurring enantiomer of lipoic acid is \(R\)-lipoic acid. It is linked to lysine residues of the 2-oxo acid dehydrogenase multienzyme complexes, where it acts as a cofactor (Biewenga et al., 1997b, 1998a). It binds acyl groups and transfers them from one part of the enzyme complex to another. During this process, lipoic acid is reduced to dihydrolipoic acid, which is subsequently reoxidized by lipoamide dehydrogenase with concomitant formation of NADH. Lipoic acid and dihydrolipoic acid can thus act as a redox couple, carrying electrons from the substrate of the dehydrogenase to NAD\(^+\). Racemic lipoic acid has been widely used in the treatment of patients with liver cirrhosis, mushroom poisoning and heavy metal intoxication. In recent years, emphasis has been given to the antioxidant action of lipoic acid to explain the activity of the compound. It is now evident that oxidative stress is associated with diabetes-induced tissue damage (Biewenga et al., 1997b). The antioxidant function of lipoic acid was, therefore, also used to explain its preventive activity in diabetes-induced damage. Interestingly both the reduced (dihydrolipoic acid) and the oxidised forms of lipoic acid can act as antioxidants. The reduced form has an antioxidant function because of the two SH-moieties that may result in the 1,2-thiolane group upon oxidation. The lone pair repulsion in the 1,2-dithiolane moiety of oxidised lipoic acid becomes less by reduction of the electron density in the ring system. And indeed the radical cation and the sulfoxide of lipoic acid are relatively stable. This explains the antioxidant activity of oxidised lipoic acid.

In contrast to its antioxidant action dihydrolipoic acid can also function as a pro-oxidant. Similar to vitamin E and vitamin C, this pro-oxidant action of lipoic acid is probably mediated by the reduction of transition metals (Bast and Haenen, 1988). In combination with other antioxidants, it has been shown that dihydrolipoic acid regenerates ubiquinol from ubisemiquinone, ascorbate from semidehydroascorbate and GSH from GSSG.

A remarkably diverse range of actions can be ascribed to lipoic acid (Table 1). Some new features of lipoic acid were discovered only recently. Knowledge of the multifunctional role of lipoic acid is, however, essential in order to evaluate the safety profile of this nutraceutical. Interestingly, we have reported that dihydrolipoic acid can play a role in the repair of oxidatively damaged proteins (Biewenga et al., 1998b).
In hyperglycemia, glucose-utilizing enzymes become saturated and react with a variety of oxidants. The latter is explained by the ring strain in the 1,2-dithiolane ring in lipoic acid.

(2) **Regeneration of vitamin E, vitamin C and glutathione**

This is the consequence of the relative redox potentials of the compounds.

(3) **Complexing metals**

Both the sulphur atoms and the carboxylic moiety are involved.

(4) **Inhibition of NF-κB**

Both the activation as well as nuclear translocation and action are inhibited.

(5) **Increase in glucose utilisation**

Dihydrolipoic acid has been suggested to substitute for HS-CoA in various enzymatic reactions and to decrease acetyl-CoA.

(6) **Increase in glucose uptake**

The increase in the glucose transporter GLUT-4 is not accompanied by an increase in its mRNA. It is presumed, therefore, that the degradation of GLUT-4 is decreased by lipoic acid.

(7) **Alleviate NADH surplus in diabetes**

In hyperglycemia, glucose-utilizing enzymes become saturated and glucose is irreversibly reduced to sorbitol by aldose reductase at the expense of NADPH. Sorbitol is then oxidised to fructose forming NADH. This so-called polyol pathway, therefore, shifts reducing equivalents from NADPH to NADH.

Reduction of lipoic acid by lipoamide reductase is NADH dependent.

(8) **Prevention of the formation of advanced glycation end products (AGEs)**

In this way lipoic acid prevents glucose mediated damage. Its action is explained by sequestration of reactive aldehydes. Moreover AGE-induced oxidative stress is inhibited.

(9) **R-lipoic acid is the natural form and 3-ketolipoic acid is a major metabolite**

Many studies have been performed with the racemate of lipoic acid (R,S-lipoic acid). Lipoamide dehydrogenase reduces the R-enantiomer 28 times faster than the S-enantiomer. The bioavailability and the action on glucose uptake are better for the R-enantiomer. The metabolite 3-ketolipoic acid has not been tested thus far, but is presumed to be a bio-active compound.

Destruction of oxidatively modified proteins through selective recognition and subsequent degradation by proteasomes might eventually lead to renewal of the protein. Direct repair of oxidatively damaged proteins, instead of degradation and renewal, might be of particular importance for proteins with a low turnover rate.

We could establish that dihydrolipoic acid delivers reducing equivalents through thioredoxin, to the enzyme peptide methionine sulfoxide reductase, thereby reducing and activating the oxidised α1-antiprotease (Biewenga et al., 1998b). This activation of oxidised α1-antiprotease inhibits elastase activity. In this way deterioration of elastin by elastase and subsequent lung emphysema, might be prevented. This illustrates that research on the mode of action may provide new applications of the nutraceutical.

Even of more importance is knowledge on the bio-transformation of the nutraceutical. In the case of lipoic acid it was found that the oral administration of 1 g of R-lipoic acid to a male volunteer resulted in the formation of 3-ketolipoic acid as a major metabolite (Fig. 6). It appeared in relatively high concentrations in plasma. Its structural characteristics suggest that it is still an antioxidant. This metabolite is probably formed during beta-oxidation of lipoic acid. It may largely contribute to the therapeutic activity of lipoic acid and surely needs further research (Biewenga et al., 1998a).

### 5. Recommendations

In order to avoid toxicity of nutraceuticals in the near future on the one side and to circumvent unrealistic precautionary measures at the other side, various recommendations could be given.

1. The safety evaluation of food supplements/nutraceuticals demands another approach than customary in the classical toxicology. Arbitrary safety factors (e.g. a factor of 100) to allow extrapolation from rat to human should not be used.

2. More understanding on the molecular aspects of the action and toxicity of the active ingredient(s) in food supplements is mandatory. Knowledge on the biokinetics and biotransformation of these ingredients should be extended. The role of metabolites in both the efficacy and the toxicity of the ingredients...
should be appreciated. As shown with the three antioxidants reviewed above, metabolites may play a pivotal role. The increased knowledge on action, toxicity, biokinetcis and biotransformation will enable a more accurate risk/benefit analysis.

3. There is a tendency to increase the dose of the active ingredient(s) in food supplements according to the motto 'the more, the better'. This is a violation of the basic rule in toxicology formulated by Paracelsus that every compound is toxic provided the dose is high enough. The plasma or tissue level of the active ingredient(s) reached after consumption of a food supplement should be compared with the range of normally attained levels of these ingredient(s). The further this range is exceeded, because of excessive suppletion, the more the food supplement gets the character of a nutraceutical and additional knowledge is necessary. Bio-kinetic/bio-effficacy modelling may be of help here as well.

4. All supplements have a health claim. The more explicit this presumed health claim becomes, the more the food supplement gets the character of a nutraceutical even or a drug and the health claim gradually changes into a medical claim. As supplementation changes into therapy, increase in the accuracy in the risk/benefit analyses is warranted.

References


