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Review Article

The shifting perception on antioxidants: The case of vitamin E and β-carotene

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ABSTRACT

Antioxidants are vital for aerobic life, and for decades the expectations of antioxidants as health promoting agents were very high. However, relatively recent meta-analyses of clinical studies show that supplementation of antioxidants does not result in the presumed health benefit, but is associated with increased mortality. The dilemma that still needs to be solved is: what are antioxidants in the end, healthy or toxic? We have evaluated this dilemma by examining the presumed health effects of two individual antioxidants with opposite images i.e. the "poisonous" β-carotene and the "wholesome" vitamin E and focused on one aspect, namely their role in inducing BPDE-DNA adducts. It appears that both antioxidants promote DNA adduct formation indirectly by inhibition of the protective enzyme glutathione-S-transferase π (GST π). Despite their opposite image, both antioxidants display a similar type of toxicity. It is concluded that, in the appreciation of antioxidants, first their benefits should be identified and substantiated by elucidating their molecular mechanism. Subsequently, the risks should be identified including the molecular mechanism. The optimal benefit–risk ratio has to be determined for each antioxidant and each individual separately, also considering the dose.

Contents

Introduction

From a toxicological point of view, molecular oxygen and other reactive oxygen species (ROS) are among the most reactive compounds we encounter in daily life [1,2]. Moreover, their level of exposure is relatively high and the duration of exposure is lifelong. This cumulates in the permanent threat of oxidative stress; a toxicological process implicated in the pathogenesis of virtually any disease [3,4]. Antioxidants, which protect against ROS, are therefore vital for aerobe life. For that reason, nutrients rich in antioxidants or antioxidants administered as supplement are applied on a large scale in an attempt to alleviate ROS induced damage [5]. The concept of oxidative stress emerged in the second half of the previous century. Consequently, at that time the expectations for the health benefits of antioxidants were very high and the use of all kinds of antioxidants in relative high quantities were recommended based on the ideas that all antioxidants are

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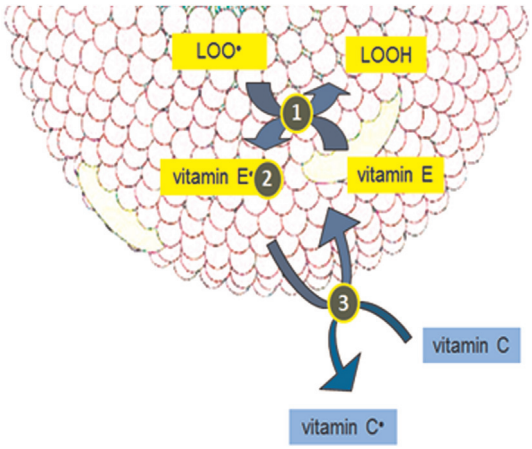
equal, and that the higher the intake, the higher the health benefits of antioxidants would be.

However, relatively recent meta-analyses of clinical studies insinuate that that the high expectations could not be met. It seems as if supplementation of antioxidants does not result in the presumed health benefit, but paradoxically a high intake of antioxidants is associated with increased mortality [6–9]. More than 68 randomized control trials were analyzed for the effects of β-carotene, vitamin A and vitamin E on mortality. All these compounds, given in a relatively high dose as a single compound or in different combinations, had no beneficial effects. The supplements even increased all-cause mortality. In this respect, it should be noted that vitamin A is considered not be an antioxidant, indicating that any compound (not only antioxidants) will be toxic when the dose is too high. In fact, this is the fundamental rule in toxicology coined five centuries ago by Paracelsus. As a consequence of the reports on the adverse effects of antioxidants given in a relatively high dose, the appreciation of antioxidants rigorously changed from healthy to toxic [10–12]. To date, the discussion on the health benefits of antioxidants continues.

The dilemma remains: what are antioxidants in the end, toxic or healthy? We evaluated this dilemma by examining the presumed health effects of two individual natural antioxidants in which the opposite images are materialized i.e. the "poisonous" β-carotene and the "healthy" vitamin E [13–17]. The interaction of β-carotene and vitamin E with physiological systems should be evaluated at a molecular level, because at this level the biological effect of a compound arises. β-carotene and vitamin E display a wide variety of effects from free radical scavenging to modulation of signal transduction [18–22]. In the present evaluation, we focused on one aspect, namely their role in inducing DNA damage by focusing on the interaction of β-carotene and vitamin E with glutathione-S-transferase π (GST π). However, our perception of antioxidants should not be limited to their risks; actually their benefits should be put in first place. A risk–benefit analysis is made and is based on the interaction of the antioxidants on the molecular level. It will be discussed if we should adjust our perception of both compounds. The benefits of antioxidants on a molecular level are neutralizing ROS and prevent oxidative damage. The health benefits are supported by a large body of epidemiological evidence. For instance, a low plasma level of β-carotene is associated with increased mortality [23–25]. Additionally, vitamin E is associated with a reduced risk of coronary heart disease and colon cancer [13,14,26,27]. Our knowledge on how these compounds act has drastically increased (Box 1). In the 1970s, it was believed that antioxidants only needed to scavenge free radicals which would totally absorb their reactivity. In the current concept on the molecular mechanism of action, scavenging is the first step in a series of at least three steps. The second step is that the radical is safeguarded in the antioxidant radical. Finally the radical has to be transferred safely into the antioxidant network. Although initially all antioxidants were considered to be equal, we now realize that each antioxidant has its own biochemical profile. Vitamin E for instance protects membranes from lipid peroxidation by scavenging lipid peroxyl radicals, while β-carotene is one of the most potent scavengers of singlet oxygen [28,29]. The molecular mechanism of action of the major antioxidants is already partially unraveled. Nevertheless, several important aspects such as the interaction with the endogenous antioxidant network, needs to be fully elucidated in order to completely exploit the health benefits of these antioxidants.



Box 1. Antioxidant protection: for a long time, it was thought that antioxidants only needed to scavenge radicals to offer protection. Currently, our knowledge on how antioxidants functions has progressed. The mechanism of action of antioxidants consists of multiple steps, as illustrated with the protection of lipoproteins against lipid peroxidation by vitamin E. During the process of lipid peroxidation lipid peroxyl radicals (LOO•) are formed. In the protection, the first step is the scavenging of LOO• by the lipophilic antioxidant vitamin E·. This is a chemical reaction in which the reactive radical (LOO•) is transformed into a non-radical (LOOH) that is relatively unreactive. The second step is that the radical is safeguarded in the antioxidant radical. The vitamin E radical is relatively unreactive due to delocalization of the radical over the antioxidant molecule. In the final step, the radical is transferred safely into the antioxidant network. In sequential reactions, the radical is transferred from one antioxidant to another antioxidant. In the example, the radical located on vitamin E in the lipoprotein is taken over by vitamin C in the plasma [62]; the vitamin C radical might react with NADH to regenerate vitamin C, a reaction catalyzed by the ascorbate free radical reductase. In the end, the reactivity of the radical is totally absorbed in the 3 steps.

β-Carotene

β-Carotene belongs to the group of naturally occurring carotenoids and has a lipophilic character containing pro-vitamin activity. β-Carotene is naturally occurring in dietary fruits and vegetables, but is also present as food supplements. The chemical, biological and physical properties of β-carotene are well-described [31]. Besides the proposed beneficial effects of β-carotene, several clinical trials do not support these effects and reported toxic effects of β-carotene supplementation. Regarding these risks, the ATBC study revealed in 1994 that β-carotene supplementation was associated with an increased incidence of lung cancer in smokers [32]. In the years that followed, other epidemiological studies corroborated this alarming effect [33–36]. The blame was put on β-carotene. However, the molecular mechanism for inducing lung cancer could not be evidently tied to β-carotene.

Especially in the combination with smoking, β-carotene supplementation is extra dangerous [37,38]. The polycyclic aromatic hydrocarbon benzyo[a]pyrene (BaP), present in tobacco smoke, has a well-known carcinogenic track record and the molecular evidence is beyond reasonable doubt [39]. BaP is activated into its highly mutagenic metabolite benzo[a]pyrene diol epoxide (BPDE). The electrophile BPDE attacks the nucleophilic regions of DNA, yielding BPDE-DNA adducts [40,41]. The carcinogenic potential of BPDE is corroborated in the present study by the presence of 153 ± 23 BPDE-DNA adducts/10^7 nucleotides (Fig. 1A) in lung epithelial cells (BEAS-2B) after exposure to BPDE. These DNA adducts cause chromosomal instability and eventually initiate tumor formation [42].

Protection against BPDE is provided by GSTs. This family of enzymes catalyzes the conjugation of BPDE to glutathione (GSH), which neutralizes the carcinogenic potential of BPDE [43]. These GSTs are divided into five major isoforms (α, μ, π, θ, and σ), of which the π isoform is found to be the most abundant isoform in erythrocytes, lung and the human skin [44–47]. In our experiments, GST π effectively decreased the number of BPDE-DNA adducts in the lung epithelial cells to 75 ± 10 BPDE-DNA adducts/10^7 nucleotides ($P < 0.01$) (Fig. 1B). The evidence on the protective role

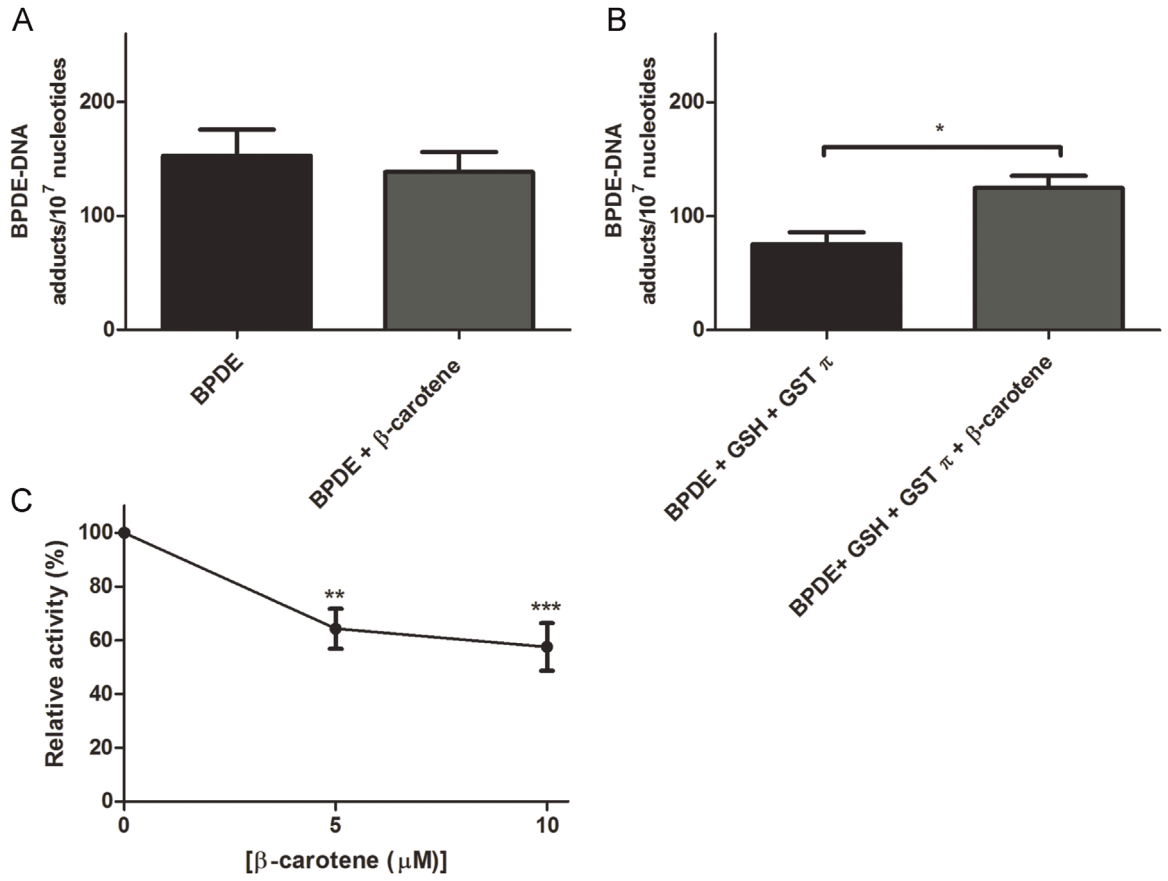


Fig. 1. The effects of β-carotene (10 μM) on BPDE-DNA adduct formation (A), on GST π induced detoxification of BPDE (B) and the inhibitory effect β-carotene on the activity of GST π. The inhibitory effect of β-carotene on GST π induced BPDE detoxification. BPDE-DNA adduct formation was measured by 32P-postlabeling assay. BEAS2B-cells were treated for 1 h with 0.1 μM BPDE in the absence or in the presence of β-carotene, the combination of GST π (50 mU/ml) and GSH (1 mM) or the combination of GST π (50 mU/ml), GSH (1 mM) and β-carotene (10 μM). After treatment, medium was removed and cells were collected and stored at − 20 °C. DNA adduct levels were determined according to the nuclease P1 enrichment technique as described by Reddy and Randerath with minor modifications [59,60]. GST π activity measurements were performed as described by Mannervik and Guthenberg with slight modifications [61]. In short, the reaction of 1 mM CDNB with 1 mM GSH in 100 mM potassium phosphate buffer (pH 6.5; 37 °C) was spectrophotometrically monitored by measuring the increase in absorbance at 340 nm. The effect of β-carotene (final concentrations: 5 and 10 μM) on GST enzyme activity (0.05 U/ml in buffer) was determined. The control activity (of 127 ± 3 nmol/min/ml) was set to 100%. Data are shown as means ± SEM ($n=6$). One way analysis of variance (ANOVA) with Bonferroni post hoc correction was used to examine differences in enzyme activities of GST π. In order to determine differences in BPDE-DNA adduct formation, student's *t*-test was used. Differences were considered to be statistically significant when $P<0.05$. $^{*}P<0.05$, $^{**}P<0.005$, and $^{***}P<0.001$.

of GST π is circumstantiated by the elevated risk for lung cancer in humans with impaired GST π activity [46,48,49].

We also observed that β-carotene attenuated the enzyme activity of GST π. Five and 10 μM β-carotene inhibited the GST-π activity with 35% and 43% respectively (Fig. 1C). Consequently, β-carotene weakens the defense against BPDE. Indeed, in lung epithelial cells exposed to BPDE, β-carotene inhibits the protective effect of GST π, by increasing the number of BPDE-DNA adducts to $125 \pm 10/10^7$ nucleotides ($P<0.05$) (Fig. 1B). This means that β-carotene indirectly increases the formation of BPDE-DNA adducts, since the conjugation of BPDE to GSH will not take place. This finding implies that inhibition of the protective activity of GST π by β-carotene is involved in the tumor promoting effect in smokers (Fig. 3) [32].

The blame the ATBC study put on β-carotene supplementation fitted in the disappointment on the unrealistically high expectations of the health effect of antioxidant supplementation of that time [3]. However, by classifying it as toxic does injustice to β-carotene, and we do not recognize its beneficial health effects. Nevertheless, we do have to be fully aware of the hazard of β-carotene in a specific group at risk, namely smokers.

Vitamin E

Vitamin E is the blanket term that covers all biological active tocopherols and tocotrienols and their derivatives. The chemical, biological and physical properties of vitamin E are well-described [27] Vitamin E is abundant in our diet and present in numerous food supplements. Also the dermal exposure has to be considered due to the inclusion of vast quantities of vitamin E in cosmetic products such as shampoos and skin creams. Numerous beneficial health effects have been proposed for vitamin E, which are attributed to its antioxidants activity. Despite its reputation of being healthy, vitamin E has a dark side that is astonishingly similar to that of β-carotene. Mitchel et al. [50] found that vitamin E had tumor promoting activity of in the skin of mice exposed to 7,12-dimethylbenz(a)anthracene (DMBA), a reference compound for inducing cancer that displays a molecular mechanism similar to that of BPDE.

In accordance to these findings, we observed that BPDE-DNA adducts were formed in skin keratinocytes (HaCaT cells) after exposure to BPDE (221 ± 28 BPDE-DNA adducts/10^7 nucleotides) (Fig. 2A). The effective protection of GST π is corroborated in these cells, evidenced by the effective decrease in the number of BPDE-

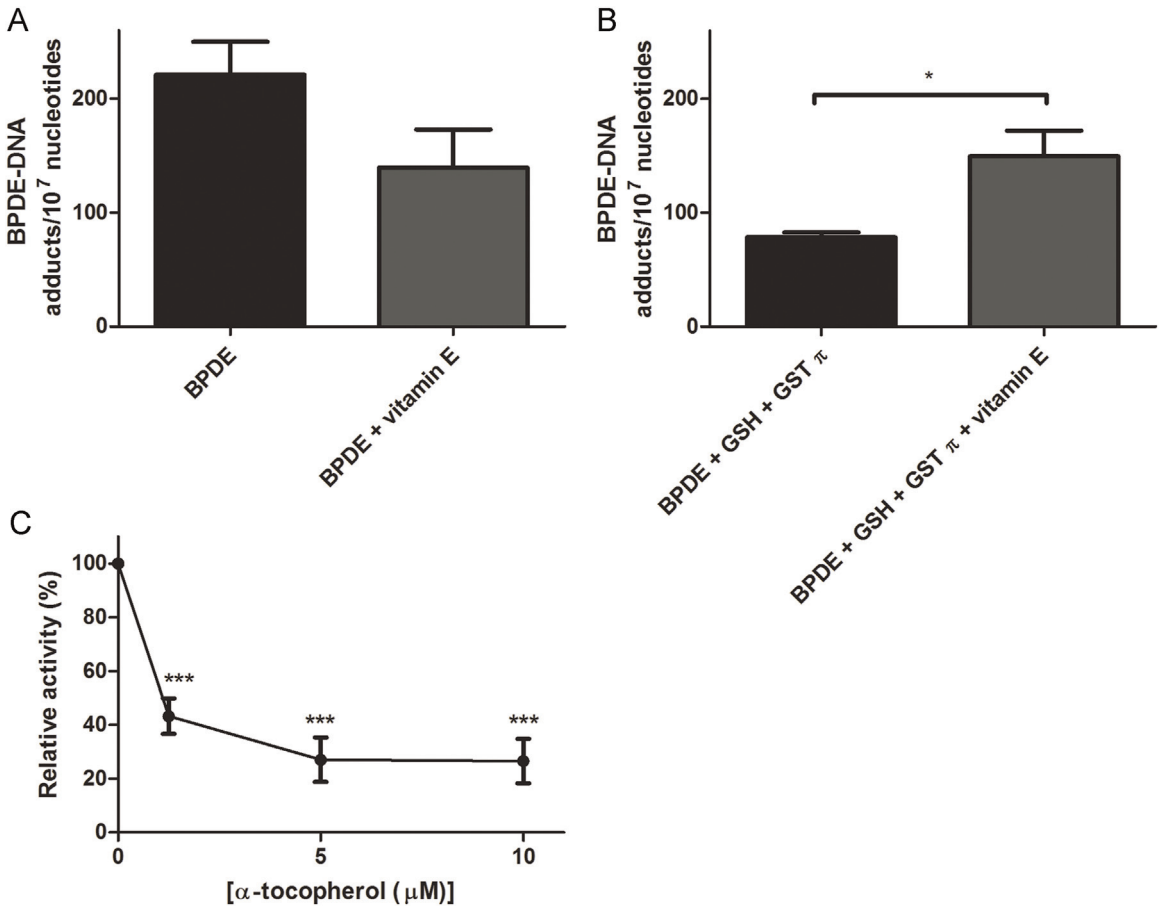


Fig. 2. The effects vitamin E (30 μM) on BPDE-DNA adduct formation (A), on GST π induced detoxification of BPDE (B) and the inhibitory effect of vitamin E on the activity of GST π. BPDE-DNA adduct formation was measured by 32P-postlabeling assay. HaCaT cells were treated for 1 hour with 0.1 μM BPDE dissolved in DMSO in the absence or in the presence of vitamin E, the combination of GST π (50 mU/ml) and GSH (1 mM) or the combination of GST π (50 mU/ml), GSH (1 mM) and vitamin E. After treatment, medium was removed and cells were collected and stored at − 20 °C. DNA adduct levels were determined according to the nuclease P1 enrichment technique as described by Reddy and Randerath with minor modifications [59,60]. The GST π activity was determined by recording the conjugation of 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) by 1 mM GSH. The reaction of 1 mM CDNB with 1 mM GSH in 100 mM potassium phosphate buffer (pH 6.5; 37 °C) was spectrophotometrically monitored by measuring the increase in absorbance at 340 nm. The effect of vitamin E (final concentrations: 1.25, 5 and 10 μM) on GST enzyme activity (0.05 U/ml in buffer) was determined. The control activity (of 127 ± 3 nmol/min/ml) was set to 100%. Data are shown as means ± SEM ($n=6$). One way analysis of variance (ANOVA) with Bonferroni post-hoc correction was used to examine differences in enzyme activities of GST π. In order to determine differences in BPDE-DNA adduct formation, student's *t*-test was used. Differences were considered to be statistically significant when $P<0.05$. $^{*}P<0.05$, and $^{***}P<0.001$.

DNA adducts, which were found to be 78 ± 4 BPDE-DNA adducts/10^7 nucleotides ($P<0.01$) (Fig. 2B). Similar to inhibition of GST π by β-carotene, we found that vitamin E also inhibited this enzyme [51]. The enzyme activity of GST π is even more potently inhibited by vitamin E in a concentration dependent manner (Fig. 2C). In a concentration of 1.25, 5 or 10 μM vitamin E significantly decreased the enzyme activity of GST π with 57%, 73% and 74% respectively ($P<0.001$). Conversely, in rats a diet deficient in vitamin E was found to increase GST activity [52]. As a consequence of GST π inhibition, vitamin E also significantly attenuated detoxification of BPDE by GST π (149 ± 22 BPDE-DNA adducts/10^7 nucleotides; $P<0.05$). The resulting net effect is an increase in BPDE-DNA adducts (Fig. 2B). This finding implies that inhibition of the protective activity of GST π might explain the tumor promoting effect of vitamin E in mice (Fig. 3) [50].

Coal tar contains polycyclic aromatic compounds (PAH) including BP (the precursor of BPDE) and dermal exposure is associated with inducing skin cancer [53]. After being banned, coal tar is currently making a surprising comeback as ingredient in medicated shampoos, soap and ointment for the treatment of numerous skin diseases as eczematous dermatitis and psoriasis [54,55]. Coal tar ointments induce PAH-DNA adducts in skin [56] and in analogy with β-carotene in smokers, vitamin E may have an additional toxic effect on people using coal tar creams. By classifying it as wholesome we have a one-sided view on the health effect of vitamin E, and we do not recognize its toxic effect. Indeed, we do have to be fully aware of the hazard of vitamin E in a specific group at risk, namely people using coal tar creams.

Conclusion and perspectives

Apparently, the appreciation of an antioxidant is a reflection of the prevailing perception of the risks and benefits at a certain point in time, and this is subject to change (Box 2). We earlier found that smokers and patients treated with coal tar ointments that are genetically deficient in GST detoxifications had higher DNA adducts in blood cells and skin [57,58]. So modulation of GSTs by antioxidants as β-carotene and vitamin E would certainly impose a risk in these groups of exposed persons. To come to an

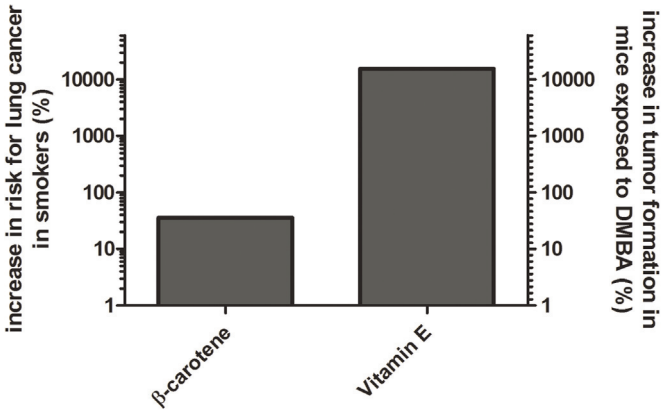
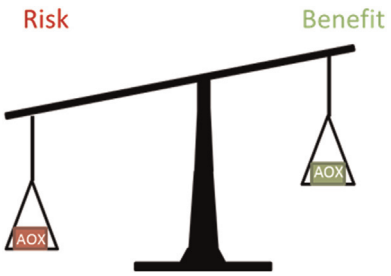


Fig. 3. Increase (%) in risk for lung cancer in smokers induced by β-carotene (left *y*-axis) and in skin tumor formation in mice treated with DMBA induced by vitamin E (right *y*-axis). β-carotene has a relative risk of lung cancer in smokers of 1.36, giving an increase of 36%. Vitamin E increased the number of skin tumors in mice from 1 tumor/25 animals in the control group to 154 tumors/26 animals in DMBA treated mice, giving an increase of 15,400%. The β-carotene data were obtained from the alpha-tocopherol, β-carotene Cancer Prevention Study Group, in which male smokers were daily supplemented with β-carotene (20 mg). Lung cancer incidence was determined during a follow-up of 5–8 years [32]. Vitamin E data were obtained from Mitchel et al. [50]. In this study, DMBA with and without vitamin E were topically applied to the dorsal skin of female SENCAR mice from which the hair was shaved. ninety-eight nd 153 days after DMBA initiation, skin tumor formation was determined.



Box 2. The health effect of antioxidants: in the perception of the health effect of an antioxidant, it is often considered that an antioxidant either only provides benefits or that it only poses risks. These opposing, one side views are the main obstacles in the accurate perception of the health effect of antioxidants. A more balanced view is mandatory as any bioactive, antioxidants included, have benefits as well as risks. In fact, the distinction between the two is not as clear-cut as generally assumed; as coined by Adrien Albert, the benefit is a form of selective toxicity. Critical in the risk–benefit analysis is that each individual balance is biased. In people that are expected to profit, the arm of the benefit is relatively long. Contrariwise, in vulnerable people, the arm of the risk is relatively long. The latter is seen for the risk of β-carotene in smokers. Apparently, it has to be determined for each individual separately whether the benefit outweighs the risk. The molecular mechanism is the pulling force that determines the weight of the benefit as well as the risk.

accurate risk–benefit analysis the following issues should be kept in mind:

1. Antioxidants are consumed to improve health and not because they are not toxic. In our appreciation on antioxidants priority is given to the risks and this needs to be corrected. The priority should be to identify groups that are likely to benefit. A challenge is that clear pharmacological endpoints still need to be defined that also includes the impact on redox signaling.
2. The molecular mechanism of the benefit is the actual fundament. Since each antioxidant has its own unique biochemical profile, antioxidants should not be treated as group. Based on the molecular pathways involved in pathology, the specific protection can be determined and the antioxidant which fits the profile best can be selected.
3. Next to groups that benefit, also groups that are at risk should be identified. Benefits are well-defined and therefore relatively easy evaluated. In contrast, risks can in principle be anything. Therefore, finding a group at risk is often a matter of chance. For example, it could not have been foreseen that smokers are at risk for β-carotene supplementation. Probably, there are additional, not yet identified, risks in smokers or other specific groups.
4. Risks should be substantiated by elucidating the molecular mechanism. This does not have to be related to the molecular mechanism for the benefit. The toxicity of the antioxidants in the present study does not arise from their antioxidant effect or their reactive products, but from the interaction with a protein. β-carotene inhibited BPDE detoxification by GST π. This molecular mechanism puts the toxicity of β-carotene in the right perspective. BPDE is the actual culprit, whereas β-carotene is more of an accomplice to BPDE, since it inhibits the detoxifying enzyme GST. By putting the toxicity in the right perspective, an overreaction (such as a general ban on β-carotene) can be prevented. On the other hand, the molecular mechanism can be used to predict other risks, such as shown in the present study, the toxicity of vitamin E in people using coal tar.

Although the accurate appreciation of antioxidants seems straight forward to do, in practice this appears to be quite awkward. This can be exemplified by the case of β-carotene in smokers. Since smoking causes oxidative stress, it is logical to supplement smokers with antioxidants. β-carotene was selected because relatively low levels of this antioxidant were found in smokers. Unpredictably, β-carotene appeared to be toxic in this group and therefore β-carotene proved to be the wrong choice. Undoubtedly, oxidative stress has a substantial contribution to smoke toxicity. Therefore, smokers are expected to benefit from antioxidant supplementation. Fully elucidating the molecular mechanism is needed to identify the antioxidant that fits smokers.

In conclusion, as seen in the case of β-carotene and vitamin E, there is a thin line between toxic and healthy. Supplementation of antioxidants does not necessarily have to be beneficial and a natural origin is no guaranty for safety. The optimal benefit–risk ratio has to be determined for each antioxidant and each individual separately, also considering the dose. The molecular mechanism is the fundament to put the benefits as well as the risks in an accurate perspective.

Conflicts of interest

None.

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