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# Review

# Is there a future for antioxidants in atherogenesis?

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Antioxidants, preferentially those of dietary origin, have for a long time been considered to help against diseases that are presumably aggravated by oxidative stress, such as cardiovascular diseases, cancer, and neurodegenerative disorders. The outcome of clinical trials undertaken to corroborate this hypothesis, however, remained largely inconclusive. Evidence is now emerging that some dietary "antioxidants" influence signaling pathways and the expression of genes relevant in atherosclerosis by mechanisms other than antioxidative ones. By concrete examples we show that (1) vitamin E has gene regulatory functions which might be more important than acting as an antioxidant *in vivo*, (2) selenium itself is not an antioxidant at all, and even not in general when incorporated into glutathione peroxidases, and (3) a moderate oxidative stress is beneficial rather than detrimental since it can induce defense mechanisms counteracting xenobiotic and oxidative stress. Thus, there is only a future for antioxidants in the prevention of any disease if their real mechanism of action is considered and suitable read-outs and biomarkers are established.

**Keywords:** Glutathione peroxidase / Heme oxygenase / Nrf2 / Selenium / Vitamin E

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## 1 Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall in which lipid accumulation is accompanied by thickening and hardening of the vessel wall combined with a lowered elasticity thereof, which together result in impaired blood flow. Atherosclerosis is the leading cause of cardiovascular death in the developed world. The "oxidative modification hypothesis" considers the oxidation of low-density lipoproteins (LDL) to be an important event in the development of atherosclerosis [1, 2]. Accordingly, every antioxidant that can directly or indirectly prevent the oxidation of LDL possibly reduces the risk of atherosclerosis or the progression of the disease. However, large clinical trials undertaken to prove the postulated preventive effect of antioxidants on cardiovascular diseases ended with a disappointing outcome, which substantially tempered the initial enthusiasm for antioxidant therapy. Only a few studies showed a positive effect, most of them did not find any correlation between the intake of antioxidants and cardiovascular diseases, and some even observed negative effects. Since the related state-of-the-art has been amply reviewed [3], mentioning some recent meta-analyses and studies with high statistic power may here suffice: The MRC/BHF heart protection study, *e.g.*, stated that antioxidant vitamins appear to be safe, however, do not significantly reduce the 5-year mortality from cardiovascular events [4]. The Washington County, MD, prospective study, which involved 6151 CLUE participants, observed that a greater intake of fruits and vegetables decreased the risk of deaths from cancer or cardiovascular diseases, whereas vitamins E and C or β-carotene did not show any effect [5]. A meta-analysis of randomized trials testing the intake of single compounds revealed a lack of benefit of vitamin E and β-carotene and concluded that such lack of clinical evidence together with the lack of mechanistic data cannot support the recommendation to routinely use vitamin E [6]. Another meta-analysis even reported an increase in all-cause mortality by dosages of vitamin E exceeding 400 IU *per* day [7]. Of course one

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**Abbreviations: ARE**, antioxidant response element; **CEHC**, carboxyethyl hydroxychroman, **CYP**, cytochrome $P_{450}$ enzyme; **GI-GPx**, gastrointestinal glutathione peroxidase; **GPx**, glutathione peroxidase; **HO-1**, heme oxygenase-1, **Keap1**, Kelch-like ECH-associated protein-1; **LDL**, low-density lipoprotein; **15-LOX**, 15-lipoxygenase, **Nrf2**, NF-E2 related factor-2; **PHGPx**, phospholipid hydroperoxide GPx; **PXR**, pregnane X receptor; **SMC**, smooth muscle cell; **VCAM-1**, vascular cell adhesion molecule-1

can argue that meta-analyses might have been biased and might not have used an appropriate statistical approach. Similarly, it is being argued that the large-scale trials might suffer from poor monitoring, inadequate dosage, or selection of the suitable antioxidant. In short, however, mega-studies and meta-analyses critically challenge the validity of the general belief that antioxidant supplementation prevents cardiovascular diseases. Accordingly, the antioxidant hypothesis of atherogenesis is to be reconsidered.

An antioxidant, in its proper definition, is a compound that scavenges radicals, is itself transformed into a rather inert radical, and thereby terminates a radical-driven chain reaction. However, not everything that acts this way in a chemically defined test tube setting will necessarily react the same way in a complex biological system. Evidence is now emerging that some dietary antioxidants influence signaling pathways and the expression of genes, be they relevant to atherosclerosis or not, by mechanisms other than antioxidant ones. In this context we will here discuss two examples of antioxidant micronutrients, vitamin E and selenium.

## 2 Vitamin E

Vitamin E has for a long time been categorized as the most important lipophilic chain-breaking antioxidant [8] and, for this reason only, was extensively tested for its ability to prevent atherosclerosis. Indeed, vitamin E reduced the susceptibility of LDL to become oxidatively modified *ex vivo* [9], but, as outlined above, it did not prevent cardiovascular diseases in most studies [3] and did not prevent the progression of intima-media thickening in healthy individuals [9]. Furthermore, it turns out that vitamin E has novel, unexpected, and important roles in cellular signaling and gene expression [10, 11] indicating that, in order to understand the essentiality of vitamin E for mammals and its putative beneficial effects, we have to look beyond free radical biochemistry.

### 2.1 Metabolism and new functions

Vitamin E covers a group of tocopherols and tocotrienols, which have different bioavailability and bioefficacy. Moreover, they are metabolized at different rates. The form, concentration, and source of vitamin E used in the different trials are often not comparable and variations thereof may have contributed to the inconclusive outcome. These aspects have been discussed in detail recently [12]. We will here discuss an aspect, which so far has not been considered: vitamin E is metabolized by the xenobiotic/drug metabolizing system and might thereby interfere with drug metabolism.

Vitamin E is not metabolically inert. All forms of vitamin E are degraded by the same mechanism, an initial ω-hydroxylation followed by β-oxidation [13, 14]. ω-Hydroxylation is catalyzed by cytochrome $P_{450}$ enzymes (CYP), of which CYP3A4 [15, 16] and CYP4F2 [17] are the most likely candidates. The final products of all forms are the respective carboxyethyl hydroxychromans (CEHCs). CEHCs are conjugated with glucuronic acid or sulfate and eliminated *via* the urine. Considering this pathway of degradation, vitamin E is metabolized like xenobiotics (Fig. 1). First, a functional group is introduced *via* the phase 1 enzyme(s) CYP3A4 or CYP4F2, followed by β-oxidation, then the degradation product is conjugated by phase 2 enzymes like UDP-glucuronosyltransferases or sulfotransferases, and the conjugate finally eliminated *via* the bile or urine. Whether elimination requires phase 3 transporters such as MRP-2 remains to be investigated. Despite the identical metabolic fate of vitamin E forms, the rate of metabolism varies substantially between the individual forms. Forms of vitamin E distinct from α-tocopherol are preferentially degraded, a fact made responsible for the exceptionally high biological activity of α-tocopherol.

The metabolism of a vitamin by CYPs, which are usually engaged in the detoxification of xenobiotics, was intriguing and provoked the question whether there are conditions under which vitamin E is considered to be "foreign" [18, 19]. Since many xenobiotics induce their own metabolizing enzymes, we asked whether this also holds true for vitamin E. Induction of CYP3As is mediated by the nuclear pregnane X receptor (PXR) [20]. The endogenous ligand of PXR has not yet been identified, and therefore PXR is still regarded as an "orphan receptor." Tentatively, PXR is considered to be a xenobiotic sensor for lipophilic compounds with discrete polarity. Vitamin E fulfils these requirements [21] and, indeed, the activation of a PXR-driven reporter gene by different forms of vitamin E could be demonstrated [22]. Tocotrienols displayed the highest activity followed by δ- and α-tocopherol. Direct binding of vitamin E to PXR has been demonstrated recently [23]. In HepG2 cells, endogenous CYP3A4 and CYP3A5 were also substantially enhanced in HepG2 cells after treatment with γ-tocotrienol [22]. The induction of CYPs was checked for *in vivo* relevance in an animal study, in which mice were fed an α-tocopherol-deficient, -adequate, and -supranutritional diet for 3 months [24]. Half of each group was fed γ-tocotrienol for the last 7 days. Cyp3a11 mRNA, the murine analog to human CYP3A4, was quantitated by real-time PCR. The study revealed that Cyp3a11 mRNA was up-regulated by γ-tocopherol but, in contrast to the *in vitro* observations, not by γ-tocotrienol [24]. This failure of γ-tocotrienol to induce CYPs *in vivo* could easily be explained by its high metabolic rate, as was demonstrated by the excretion of large amounts of γ-CEHC and low γ-tocotrienol levels in liver and plasma after γ-tocotrienol application [24]. Thus,

 

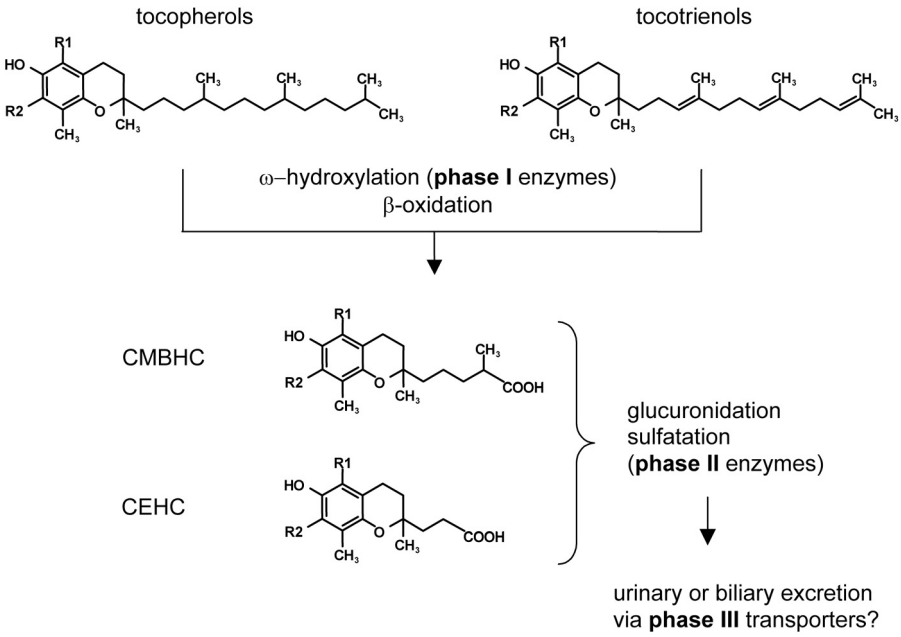


**Figure 1.** Metabolism of vitamin E. Tocopherols and tocotrienols are degraded by side-chain degradation starting with an initial ω-hydroxylation, followed by five steps of β-oxidation cycles. Final products CEHCs and the precursors carboxymethylbutyl hydroxychromans (CMBHCs) of the respective tocopherols and tocotrienols are identical. They are glucuronidated or sulfated and excreted in the urine and probably in the bile. α-, β-, γ-, and δ-Forms of tocopherols and tocotrienols differ only in the number and position of methyl groups at the chroman ring, α-form (R1 = $CH_3$, R2 = $CH_3$), β-form (R1 = $CH_3$, R2 = H), γ-form (R1 = H, R2 = $CH_3$), and δ-form (R1 = H, R2 = H).

degradation prevented an accumulation of γ-tocotrienol to the extent required for gene activation.

The physiological function of vitamin E, similar to that of other lipophilic vitamins, is more likely due to its ability to interfere with signaling cascades that ultimately trigger expression of specific genes than to its antioxidant potential. The interaction of certain forms of vitamin E with the xenobiotic sensor PXR has been clearly demonstrated and this might not be the only nuclear receptor which responds to vitamin E. Whether vitamin E also affects drug metabolizing enzymes in humans remains to be investigated. However, since high-dose vitamin E trials, being related to atherosclerosis, cancer, or other diseases, were commonly performed in risk patients who most probably were under drug therapy, it is worth considering whether the negative outcome of these trials might have been caused by a lowered efficacy of essential drugs due to a vitamin E-induced drug metabolism.

## 3 Selenium

Selenium is an element and, as such, it is present in many chemical compounds. Neither selenium nor selenium compounds are antioxidants *per se*. Selenium, in mammals, bacteria, and protozoa, acts as an integral part of selenoproteins [25], from which only the classical glutathione peroxidase (cGPx, GPx-1) has been proven to counteract oxidative stress; cGPx (–/–) mice were substantially more susceptible to oxidative challenge and a treatment with redox-cycling herbicides or LPS [26–29]. None of the other selenoproteins could replace cGPx which underlines that cGPx is the only selenoprotein acting as a general antioxidant device. Protective cellular effects of selenium-dependent GPxs against atherosclerosis-relevant processes have been reported, *e.g.*, the inhibition of oxidative modification of LDL [26], the inhibition of the putatively proatherogenic 15-lipoxygenase (15-LOX) by phospholipid hydroperoxide GPx (PHGPx)-catalyzed removal of hydroperoxides [31], or modification of cytokine-induced expression of adhesion molecules [33, 34], as summarized in [32].

Clinical trials investigating an effect of selenium supplementation on cardiovascular diseases are rare, and epidemiological studies remain controversial [35]. An early epidemiologic study observed an association between cardiovascular death or myocardial infarction and low serum selenium concentration [36]. The risk was significantly elevated when serum levels had fallen below 45 μg Se/L. However, other studies failed to show such a correlation [37–39]. In a recent reevaluation of a subgroup of the Linxian Study, plasma selenium concentrations were correlated

 

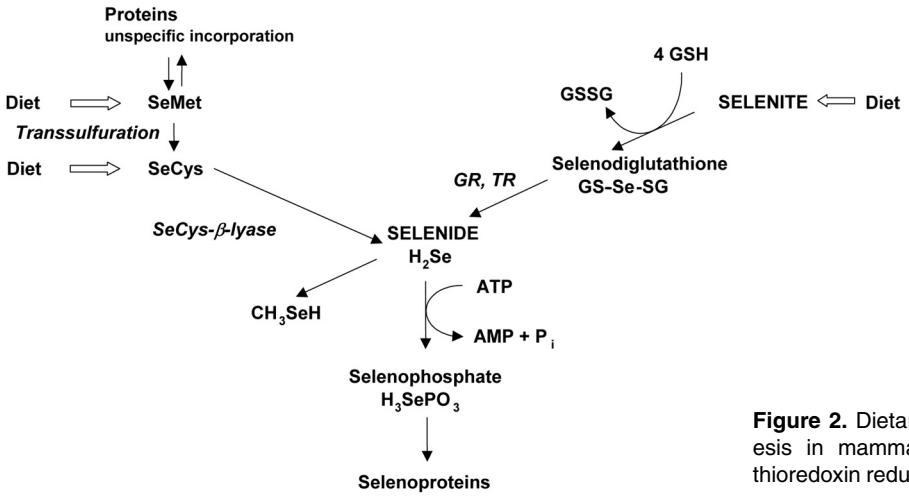


**Figure 2.** Dietary sources for selenoprotein biosynthesis in mammals. GR, glutathione reductase; TR, thioredoxin reductase. For further details see text.

with esophageal and gastric cancer, heart disease, stroke, and total death [40]. There was a significant inverse correlation for cancer, an effect close to significance for death from heart disease, but no association was found for total death or stroke. Also here, a threshold was obvious. Serum selenium levels should be above 58 μg/L for becoming protective [40]. Obviously, there is an optimum level of selenium in plasma and exceeding this does not offer any relevant benefit. This kind of dose-effect relationship does evidently not comply with a functioning of selenium as an antioxidant. As such according to mass law, it would display an unsaturated dose-effect response.

As stated above, selenium has to be incorporated into selenoproteins. For incorporation into selenoproteins, selenide has to be phosphorylated into selenophosphate which is then used to convert serine into selenocysteine at the level of the selenocysteyl-specific tRNA [41, 42]. Only selenium compounds which can be metabolized to selenide can act as selenoprotein precursors (Fig. 2). Selenite is one of them. It has to be reduced by means of glutathione. The intermediary formed selenodiglutathione is reduced by thioredoxin reductase or glutathione reductase. The reduction equivalents come from NADPH. Thus, conversion of selenite into selenide, by consuming essential cellular reduction equivalents, is an oxidative challenge. Organic sources are selenomethionine, which has to be transformed into selenocysteine *via* the transsulfuration pathway, and selenocysteine itself. Selenocysteine is cleaved by the selenocysteine-β-lyase to produce selenide and alanine [43]. Thus, none of these most commonly used selenocompounds exerts any antioxidant function.

Selenoproteins are supplied with selenium according to a certain hierarchy. The term describes the observation that the individual selenoproteins specifically respond to selenium deprivation. While some of the selenoproteins disappear rapidly upon selenium deprivation and are resynthesized with delay upon supplementation, others decline slowly at limited selenium supply and are rebuilt immediately upon resupplementation [44]. The major regulatory principle determining this hierarchy appears to be the selenium dependency of the mRNA stability [45, 46]. For obvious reasons, selenoproteins ranking high in the hierarchy are believed to have more important functions than those ranking low.

The selenoproteins we have investigated are GPxs. So far, four selenium-dependent GPxs were cloned, sequenced, and functionally at least in part elucidated [46]. A fifth has been identified by *in silico* analyses [47]. PHGPx and gastrointestinal GPx (GI-GPx) rank high in the hierarchy [44, 48], whereas cGPx disappears fast when selenium becomes limiting.

### 3.1 GI-GPx

GI-GPx is up-regulated in human colon cancer [49], skin preneoplasia [50], Barrett's esophageal mucosa [51], and pulmonary fibrosis [52]. Since in none of these diseases an increased selenium supply is obvious, we investigated whether GI-GPx can be up-regulated by alternative mechanisms. Surprisingly, a promoter analysis of the presumably antioxidant enzyme revealed two putative antioxidant response elements (ARE), from which one consisted of the correct consensus sequence and was functional in the experiments described below. AREs are activated by NF-E2 related factor-2 (Nrf2), a cap "n" collar (CNC) member of the family of basic leucine zipper transcription factors [53]. Nrf2 is sequestered in the cytosol by Kelch-like ECH-associated protein-1 (Keap1). The commonly accepted mechanism of activation includes a (oxidative) modification of SH groups in Keap1 (Fig. 3). The resulting alteration in the conformation liberates Nrf2 which then can be transferred into the nucleus and activate gene expression [54]. Recently a

 

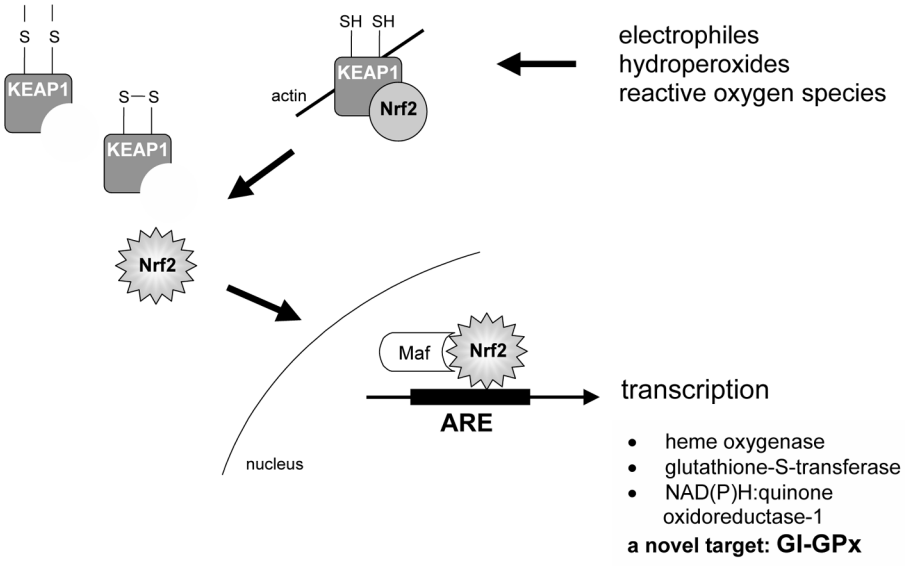


**Figure 3.** Keap1/Nrf2-system. After modification of protein thiols in Keap1 by either oxidation or alkylation/arylation, Nrf2 dissociates from Keap1 and translocates into the nucleus. Nrf2 forms a heterodimer with other transcription factor (*e.g.*, small Maf, c-Jun), binds to the antioxidant responsive element in the promoter of target genes (ARE), and induces transcriptional activation. Some target genes coding for antioxidant/detoxifying proteins are listed and the novel target GI-GPx highlighted. For further details see text.

novel mechanism has been proposed: Keap1 acts as a shuttle for Nrf2 between cytosol and nucleus [55]. According to the first mechanism, Keap1 is modified by electrophiles able to react with protein thiols, like curcumin, sulforaphane, or *t*-butylhydroquinone [56, 57]. The promoter of GI-GPx could be activated by these compounds as tested in reporter gene assays, and endogenous GI-GPx was increased as shown at the mRNA and protein levels [58]. Thus, GI-GPx was identified as a target for Nrf2. This demonstrates that selenoproteins not responding to physiological variations of selenium supply can be up-regulated by (dietary) compounds, so far considered to be antioxidants but rather acting as modifiers of protein thiols *in vivo*.

### 3.2 PHGPx

The expression of cellular adhesion molecules (CAMs) is a key event in inflammatory responses of the vascular system and, in consequence, in atherogenesis [59, 60]. CAMs can be induced by hydroperoxides not only in endothelial cells but also in smooth muscle cells (SMC) where they contribute to leukocyte recruitment [61]. Accordingly, we hypothesized that SMC overexpressing PHGPx would express only low amounts of vascular cell adhesion molecule-1 (VCAM-1), whereas in SMC overexpressing 15-LOX VCAM-1 expression would be facilitated. Surprisingly, however, basal and IL-1-induced VCAM-1 expression, basal VCAM-1 promoter activity, NFκB transactivation activity, and proliferation of cells were inhibited in both cells, with a more pronounced effect on all parameters in 15-LOX-transfected cells [34].

This indicates that both enzymes supported antiinflammatory and antiatherogenic cellular events, which was expected for PHGPx but not for 15-LOX. Reconsidering the initial hypothesis we suggested thiol modification to be the common mechanism exerted by both enzymes. PHGPx not only reduces hydroperoxides but also prevents the suggested oxidative enhancement of NFκB activation and subsequent VCAM-1 expression. However, it can also use hydroperoxides to modify protein thiols [62, 63]. 15-LOX produces hydroperoxides which might oxidize protein thiols directly. In line with this new hypothesis, cellular protein thiols were found to be reduced in PHGPx- and 15-LOX-overexpressing cells. Protein thiols could be completely restored by thiol reduction in PHGPx cells and to about half of the controls in 15-LOX cells. As mentioned above, the Nrf2/Keap1 transcription factor system can be activated by thiol modification of Keap1. We indeed found a slightly activated Nrf2 in PHGPx cells and a distinctly activated Nrf2 in 15-LOX cells. Overexpression of Nrf2 inhibited the basal and IL-1-induced expression of a reporter gene driven by the VCAM-1 promoter [64]. Coexpression with Keap1 partially reversed the Nrf2 effect. These observations link the inhibition of VCAM-1 expression by PHGPx and 15-LOX to the Nrf2/Keap1 system. Obviously, activation of Nrf2 led to the production of a protein inhibiting the VCAM-1 promoter and, thus, VCAM-1 expression.

A prototype of Nrf2-induced genes is the gene encoding heme oxygenase-1 (HO-1) [65]. HO-1 has been shown to inhibit growth of SMCs [66], NFκB activation, and VCAM-1 expression [67]. HO-1 was indeed up-regulated

 

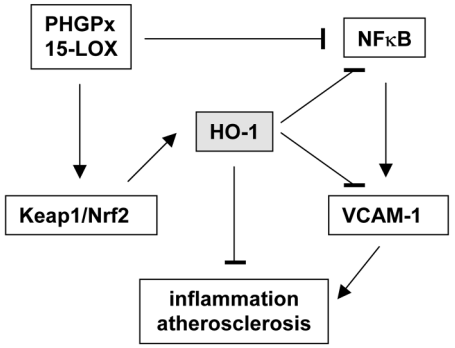


**Figure 4.** HO-1 is the common denominator of the inhibition of VCAM-1 expression by PHGPx and 15-LOX. Both enzymes inhibit NFκB activity and up-regulate HO-1 expression *via* Nrf2. Since HO-1 has been shown to also inhibit NFκB and VCAM-1 expression, it might be the mediator of the effects of two enzymes believed to exert contradictory functions in the metabolism of hydroperoxides. For further details see text.

in PHGPx- and 15-LOX-overexpressing SMC [64]. Furthermore, induction of HO-1 by heme, which is a potent inducer of HO-1 [68], abrogated VCAM-1 expression in untransfected control SMC. Thus, the common denominator of PHGPx- and 15-LOX-mediated inhibition of VCAM-1 expression might be HO-1, the defense system of which is up-regulated by the moderate oxidative stress initiated by both enzymes and which contributes to the prevention of further proinflammatory stimulation (Fig. 4).

## 4 Conclusion

We present here a few examples showing that antioxidants do not always act as such in biological systems. These observations might help to finally fill the gaps in our understanding of how antioxidants act *in vivo*. Vitamin E as example of an antioxidant vitamin is treated as foreign if concentrations become too high. Selenium has diverse effects that may counteract each other depending on the enzyme in which it is incorporated. The selenoenzyme PHGPx presumed to be an antioxidant enzyme acts as a protein thiol oxidant *in vivo*, and 15-LOX, an oxidizing enzyme, activates the "antioxidant responsing element," while the antioxidant selenoenzyme GI-GPx is up-regulated by the same ARE.

Evidently, nature does not follow the simplistic scheme that oxidants are devils and antioxidants are angels. Available evidence argues against the view that nutritive antioxidants act as such *in vivo*. As far as mechanistically understood, their actions are those of specific ligands, cofactors, or integral part of proteins. This implies that we need these redox-active micronutrients at a dosage that guarantees optimum physiological function but not at "supranutritional" dosages that might unbalance physiology. In this context, the mislabeling of redox-active micronutrients as antioxidants is misleading, since it promises their benefit to increase with the dosage.

The answer to the question whether there is room for antioxidants in atherosclerosis is clearly "no" if their primary pharmacodynamic action, *i.e.* the action observed at the lowest concentration, is simply an antioxidant one. There is room for antioxidants in atherogenesis under two provisions: (i) if the hypothesis is correct that inflammation is linked to atherosclerosis and there is evidence in support of this assumption and (ii) if the primary pharmacodynamic action of the redox-active compounds by modulating specific cellular processes is an antiinflammatory one.

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