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Diverse Functions of Antioxidants

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All biological organisms have developed a defense system against oxidative stress, which is comprised of many kinds of antioxidants. Antioxidants are classified by function into four categories; preventive antioxidants; radical scavenging antioxidants; repair and de novo antioxidants; and adaptation. Radical scavenging antioxidants have the greatest advantage. Although the activities of radical scavenging antioxidant are determined by several factors, their chemical structure is of key importance. Furthermore, radical scavenging antioxidants have been explored to have a novel function by which they regulate gene expression of cell.

Keywords: free radical, oxidative stress, defense system, antioxidant, gene expression

INTRODUCTION

Life on earth started with anaerobic organisms. With increasing oxygen molecules by photosynthesis, aerobic organisms became superior due to their extremely efficient energy production. At the same time, however, oxygen radicals and other reactive species are continuously being generated under physiological conditions. Biological organisms controlled this free radical generation and developed efficient defense systems against oxidative stress. It could be assumed that the history of evolution of aerobic organisms is namely the history of development of a defense system with diverse functions of antioxidants. The functions of antioxidants will be summarized and especially, the diversity of functions of radical scavenging antioxidants will be discussed.

ANTIOXIDANT DEFENSE SYSTEM IN EUKARIOTIC CELLS

As outlined by Noguchi and Niki [1], an antioxidant defense system against oxidative stress is composed of several lines. The antioxidants which act defense systems are classified into 4 categories, based on function. The first line of defense is the preventive antioxidants, which suppress the formation of free radicals. The second line of defense is the radical scavenging antioxidants suppressing chain initiation and/or breaking chain propagation reactions. Repair and de novo antioxidants act as a third defence system. The fourth line is an adaptation where

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the signal for the production and reactions of free radicals induces formation and transport of the appropriate antioxidant to the right site.

Preventive Antioxidants

Alkoxyl radicals and peroxyl radicals are generated via the decomposition of hydroperoxides (hydrogen peroxide) by transition metal ions such as iron and copper. Numerous enzymes, such as glutathione peroxidase [2], phospholipid hydroperoxide glutathione peroxidase [3], selenoprotein [4] and catalase reduce hydroperoxide and hydrogen peroxide, preventing the formation of free radicals. Ebselen, a synthetic selenium containing compound, is also classified into this group [5]. On the other hand, proteins such as transferrin, ferritin, lactoferrin, and ceruloplasmin are known to prevent formation of free radicals by sequestering transition metal ion. Carotenoids act as a quencher of singlet oxygen which oxidizes unsaturated lipids to give hydroperoxides. Some inhibitors of lipoxygenase that also oxidizes unsaturated lipids specifically could have a similar effect.

Superoxide is one of the active oxygen species. Although its chemical reactivity is not high, it induces release of metal ions from their binding protein [6]. Superoxide reacts with nitric oxide at diffusion limited rate to give peroxynitrite which has a high oxidizing potency [7]. Superoxide is converted to hydrogen peroxide by superoxide dismutase (SOD) [8]. Some inhibitors of nitric oxide synthetase have the same preventive effect on generation of peroxynitrite.

Antioxidants classified in this line have specificity for the target molecule or enzyme, which gives the antioxidants a limitation in their antioxidant action against oxidative stress.

The antioxidants in the third line of defense have the same restriction since they specifically act upon the substrate. For example, phospholipase A2 hydrolyzes phospholipid hydroperoxides to give lysophospholipids and free fatty acid hydroperoxides [9]. Some proteolytic enzymes degrade oxidatively modified proteins [10]. Several kinds of repair enzymes of oxidized DNA have been discovered [11]. Radical Scavenging Antioxidant

The radical scavenging antioxidants overcome the disadvantage of preventive antioxidants and repair enzymes because they are able to break free radical mediated chain propagation, regardless of initiating species. Antioxidant activities are determined not only by their inherent chemical reactivities toward radicals, but also by several factors shown in Table I. Since a number of studies on the chemical structure of compounds and their antioxidant activities have been published and comprehensive information about antioxidant activities has been accumulated, that enabled us to design an ideal antioxidant. Actually we designed and synthesized 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran (BO-653) as a novel antioxidant by taking these factors into consideration [12]. The structure of BO-653 was based on α-tocopherol (α-TOH)(Fig. 1). To have high reactivity toward radical by resonance-stabilization, we chose a five-membered ring instead of a six-membered ring of α-TOH. *tert*-Butyl groups were chosen because the substituents at the 4 and 6 positions on the aromatic ring should be sufficiently bulky to stabilize phenoxyl radical but should also be small to minimize a steric hindrance for approach of radical to the phenolic hydrogen. *tert*-Butyl groups are also effective in increasing lipophilicity and facilitating the incorporation of antioxidants into the core of low density lipoproteins (LDL). 2,2-Dipentyl groups were selected as a side chain for incorporation, retention, and elimination of asymmetric carbon.

TABLE I Evaluation of antioxidant activites against oxidative stress

Oxidation systems	*Factors which determine antioxidant activity*
Homogeneous solution	Intrinsic chemical reactivity toward radical
Micelles	Fate of antioxidant-derived radical
Liposomal membranes	Location of antioxidant
Lipoproteins	Concentration and mobility at the microenvironment
Tissue homogenate	
Subcellular fractions	Site of generation and reactivity of the radical
Cells	Interaction with other antioxidants
In vivo	Absorption, distribution, retention, metabolism, and safety

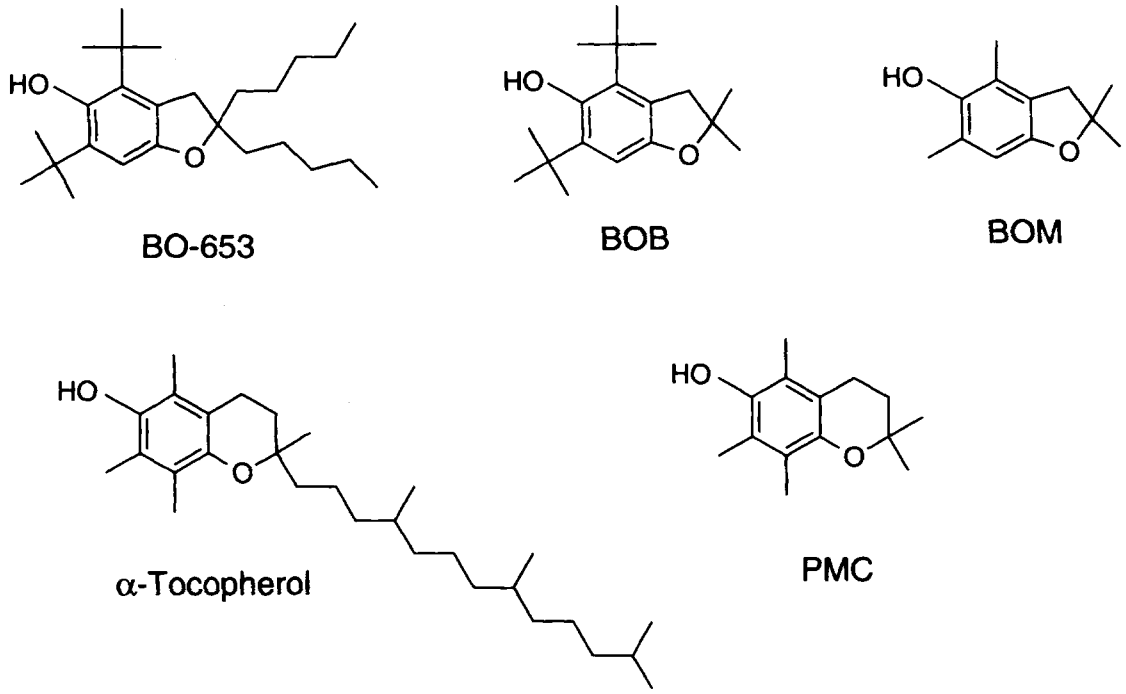

FIGURE 1 Chemical structure of phenolic antioxidants PMC. contains a dimethyl group instead of the phytyl group of α-tocopherol as a side chain. BOB has dimethyl group instead of dipentyl group of BO-653 as a side chain. BOM has methyl groups instead of *tert*-butyl groups of BOB as substituents on aromatic ring. BOM has a five-membered ring instead of a six-membered ring of PMC

We investigated antioxidant activities along with those factors in several reaction systems as shown in Table I. The antioxidant activities of BO-653 were studied extensively by comparing it to α-TOH and it was shown that BO-653 was very effective antioxidant *in vitro* and in animal

models [12–14]. α -TOH and BO-653 have similarity in their chemical structures but they are still too different to compare directly. To have more precise information about the relationship between the chemical structure of an antioxidant and its activity, we studied antioxidant activity of analogues of α -TOH and BO-653 [15]. Chemical structures of compounds used in this study are shown in Fig. 1. Reactivity of Antioxidant toward Radicals.

The reactivity of antioxidant toward galvinoxyl radical, a stable phenoxyl radical, was studied by following a decrease in absorbance of galvinoxyl radical at λ max in ethanol. Pseudo first-order rate constants were obtained using Guggenheim plot and were plotted against concentration of antioxidant, whose slope gives the second-order rate constant of reaction between antioxidant and galvinoxyl radical. The rate constants of α-TOH, PMC, BOM, BOB and BO-653 were 4.13×10^3, 3.36×10^3, 3.53×10^3, 3.48×10^3, and 3.62×10^3, respectively. They all have the same order of the rate constant as 10^3, showing that they have similar reactivities toward a stable phenoxyl radical.

Fate of Antioxidant Derived Aryloxyl Radical

The stability of antioxidant-derived aryloxyl radical was studied by following a decay of its electron spin resonance (ESR) signal that appeared from the reaction of the excess amount of antioxidant with galvinoxyl in ethanol under nitrogen using a rapid-mixing stopped-flow ESR. Furthermore, in order to understand the fate of antioxidant-derived radical, the rates of reaction of the aryloxyl radicals with lipid and hydroperoxide were also measured by following the decay of ESR signal of the antioxidant-derived radical. The results are summarized in Table II. The rate constants for hydrogen atom abstraction from these substrates by antioxidant-derived radicals decreased in the order of α-TOH~PMC>BOM>BO-653~BOB, but the differences in the reactivities varied significantly with the substrates. These results show that BOB and BO-653 having *tert*-butyl groups at both ortho positions of phenoxyl group have higher stability.

The lipophilic antioxidants are localized *in vivo* in the lipophilic domain of membranes and lipoproteins, where the antioxidant efficacy is affected by physical factors such as fluidity and mobility of the microenvironment. It is generally thought that the side chain of the antioxidants has a significant effect on those factors. The action of antioxidant-derived aryloxyl radicals was also studied in liposomal membranes. The antioxidant was incorporated into phosphatidylcholine liposomal membranes and reacted with galvinoxyl to generate the aryloxyl radical. The rates of spontaneous decay of α-TOH and PMC radicals were too fast to permit a measurement of the rate constant accurately. The rate constants for bimolecular interactions of aryloxyl radicals of BOM, BOB and BO-653 were obtained as 3.8×10^2, 0.52, and 0.28 $M^{-1}s^{-1}$, respectively. The difference of the decay of antioxidant-derived radicals in homogeneous solution and liposomal membranes are shown in Table III for comparison. While BOB and BO-653 showed little difference in stability of their radicals in homogeneous solution, BO-653 radical was more stable as much as twice than BOB radical in liposomal membranes. These results show that dipentyl group, rather than dimethyl group, restricts the mobility of the compound in membranes.

TABLE II Rate constants of antioxidant-derived radicals for spontaneous decay and hydrogen atom abstraction from methyl linoleate and *tert*-butyl hydroperoxide in ethanol at 37 °C under nitrogen

	Spontaneous decay ($M^{-1}\ s^{-1}$)[a]	*Hydrogen atom abstraction* ($M^{-1}\ s^{-1}$)	
		methyl linoleate[b]	*tert-butyl hydroperoxide*[c]
α-TOH	$1.03\ (\pm 0.03) \times 10^{3}$	$2.7\ (\pm 0.4) \times 10^{-2}$	$4.1\ (\pm 0.1) \times 10^{-1}$
PMC	$1.10\ (\pm 0.12) \times 10^{3}$	$2.3\ (\pm 0.4) \times 10^{-2}$	$3.7\ (\pm 0.2) \times 10^{-1}$
BOM	$7.03\ (\pm 0.02) \times 10^{2}$	$2.4\ (\pm 0.3) \times 10^{-2}$	$2.6\ (\pm 0.5) \times 10^{-1}$
BOB	$1.08\ (\pm 0.10)$	$2.5\ (\pm 0.2) \times 10^{-3}$	$1.1\ (\pm 0.4) \times 10^{-1}$
BO-653	$1.13\ (\pm 0.15)$	$2.8\ (\pm 0.2) \times 10^{-3}$	$1.4\ (\pm 0.2) \times 10^{-1}$

a. Data are mean ± SD of four independent determinations.
b. Methyl linoleate (367 mM) was reacted with antioxidant-derived radical.
c. *tert*-Butyl hydroperoxide (69.6 mM) was reacted with antioxidant-derived radical.

TABLE III Effect of substituent and side chain on spontaneous decay in ethanol and liposomal membranes

	Rate Constant ($M^{-1}\ s^{-1}$)	
	in solution	*in membranes*[a]
BOM	7.03×10^{2}	3.80×10^{2}
BOB	1.08	0.52
BO-653	1.13	0.28

a. The antioxidant was incorporated into dimyristoyl phosphatidylcholine liposomal membranes.

Interaction of Antioxidant with Other Antioxidants

When we evaluate antioxidant activity, it is important to consider the interaction of antioxidant with other antioxidants. We studied the reactivity of antioxidant-derived radical with ascorbic acid in solution and membranes. Table IV shows the rate constants for the reaction of aryloxyl radical with ascorbate in ethanol and liposomal suspensions. It can be seen that bulky *tert*-butyl substituents markedly reduce the reactivties of aryloxyl radicals toward ascorbate. The rate constants of interaction of BO-653 radical and BOB radical with ascorbate in liposomal membranes were 16 and 2 times smaller than those in ethanol solution, respectively. These results suggest that side chains with different lengths affect the apparent reactivities of aryloxyl radicals in the membranes.

TABLE IV Rate constants of antioxidant-derived radicals for hydrogen atom abstraction from ascorbate in ethanol and in liposomal membranes at 37 °C under nitrogen

	Hydrogen atom abstraction from ascorbate (500 μM) ($M^{-1}\ s^{-1}$)[a]	
	in solution	*in membranes*[b]
α-TOH	$1.1\ (\pm 0.2) \times 10^{5}$	c
PMC	$9.5\ (\pm 0.0) \times 10^{4}$	c
BOM	$8.3\ (\pm 0.1) \times 10^{4}$	c
BOB	29 ± 8	14
BO-653	33 ± 5	2

a. Data are mean ± SD of four independent determinations.
b. The antioxidant was incorporated into dimyristoyl phosphatidylcholine liposomal membranes.
c. The aryloxyl radicals decayed too fast to be measured accurately.

ANTIOXIDANT ACTIVITY OF VITAMIN E

Vitamin E, a major lipophilic antioxidant *in vivo*, is composed of homologues such as α-, β-, γ-, and δ-TOH. It is known that α-tocopherol transfer protein binds α-TOH selectively and eliminates other forms of tocopherols, with the result that α-TOH is the most abundant TOH *in vivo* [16]. The antioxidant activities of α-TOH have been studied extensively comparing to other TOHs. It has been shown that α-TOH has the highest activity against lipid peroxidation induced by peroxyl radical [17].

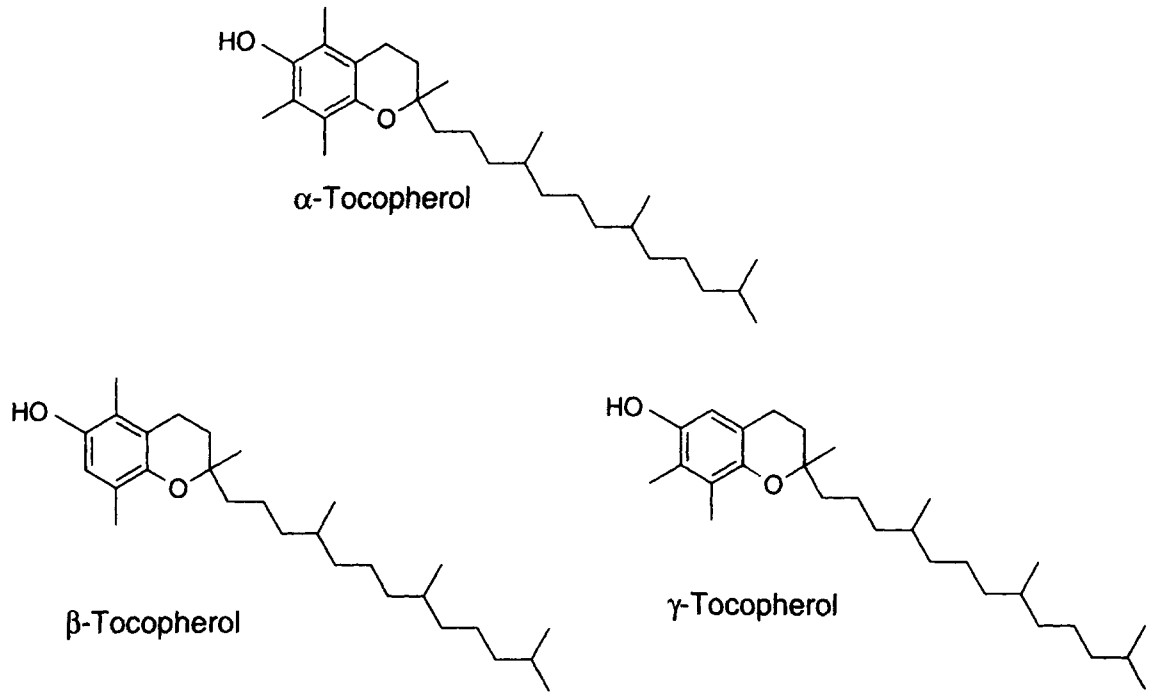

FIGURE 2 Chemical structure of tocopherols

Recently, however, reactive nitrogen species have received much attention since their stable end products are detected in both animals and human with on going inflammation. Chronic inflammation is a major contributor to the development of several kinds of diseases. Even under physiological condition, the reactive nitrogen species are generated. Christen et al reported that γ-TOH is superior antioxidant than α-TOH against lipid peroxidation of phosphatidylcholine liposome and lipoprotein induced by peroxynitrite and that supplementation with α-TOH should be reconsidered so as not to avoid the antioxidant effect of γ-TOH [18]. On the other hand, Goss et al [19]showed that α-TOH alone acted as a potent antioxidant against lipid peroxidation induced by peroxynitrite. The action of α-TOH and γ-TOH is still controversial issue.

As shown in Figure 2, the chemical structures of β-TOH and γ-TOH differ from that of α-TOH only by a methyl group substitution at 7-position or 5-position, respectively. The reactivity of these TOHs toward peroxyl radicals was measured with a spectrophotometer. The actual rate of free radical flux was measured by using *N,N'*-diphenyl-1,4-phenylenediamine (DPPD) as a radical scavenger (Fig. 3). One molecule of DPPD scavenges two radicals rapidly to give *N,N'*-diphenyl-*p*-benzoquinone diimine (DPBQ) [20,21], which has a strong absorption at 440 nm. The absorption at 440 nm increased with time due to the formation of DPBQ from DPPD by the reaction with the radicals generated from methoxy-AMVN. These radical initiators induced a linear increase in the absorption at 440 nm with time in the absence of antioxidant and then

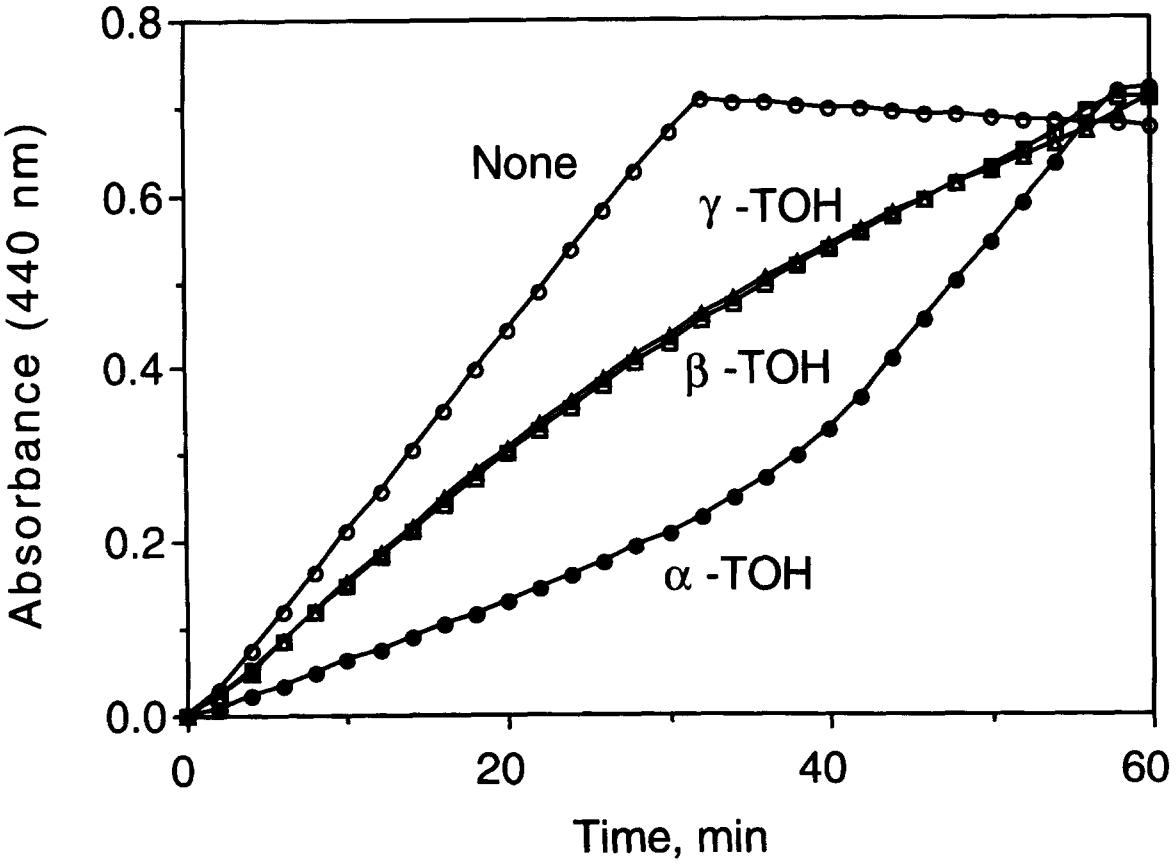

FIGURE 3 Reactivity of tocopherol toward peroxyl radical. The formation of DBPQ due to reaction of DPPD (100 μM) with peroxyl radical derived from AMVN (30 mM) was observed in acetonitrile at 37°C. Tocopherols (100 μM) suppressed it by competition with DPPD, in terms of reacting with peroxyl radicals

became constant when all the DPPD was converted to DPBQ. The plateau absorbance was directly proportional to the initial DPPD concentration independent of the radical initiator concentration. The addition of TOH to this mixture suppressed the formation of DPBQ due to competition with DPPD in scavenging peroxyl radicals. α-TOH showed the most effective inhibition, while β-TOH and γ-TOH had similar inhibitory effect.

α-TOH also was a more effective antioxidant than γ-TOH against lipid peroxidation induced by peroxyl radical in homogeneous solution and micelles of methyl linoleate. Furthermore, α-TOH spared γ-TOH in the oxidation of liposomal membranes and LDL induced by either peroxyl radical or peroxynitrite when they were present simultaneously. These results are consistent with that of Goss et al. [19] but not that of Christine et al. [18]. Interestingly, however, different results were obtained in the oxidation of brain homogenate induced by peroxynitrite not by peroxyl radical. γ-TOH inhibited the formation of hydroperoxides of phosphatidylcholine and phosphatidylethanolamine more efficiently than α-TOH. The reason for this stronger inhibitory effect of γ-TOH is not known at present but some of the oxidation products or metabolites of

γ-TOH in the homogenate may have potent antioxidant activities. These results suggest that the antioxidant activities are affected by initiating radical species and also by the oxidation products of antioxidant.

OTHER FUNCTIONS OF ANTIOXIDANTS

In addition to the radical scavenging activity of α-TOH, it was found that α-TOH regulates cell functions such as adhesion [20] and proliferation [21]. In vascular smooth muscle cells α-TOH, but not β-TOH, negatively regulates proliferation by preventing PKC activation [22].

In 1978, evidence that the dietary administration of antioxidants induced phase II xenobiotic metabolizing enzymes was first presented by Benson *et al.* [23] and Cha *et al* [24]. These results suggested that antioxidants act not only to directly inhibit free radical-mediated reaction but also to increase the activity of enzymes which readily metabolize cytotoxic chemicals. Transcriptional activation by antioxidants has been demonstrated to be the result of enhanced transcription factor binding to a *cis*-acting element known as antioxidant responsive element (ARE) [25] or electrophile responsive element (EpRE) [26], associated with target genes. It is assumed that the antioxidant itself and/or antioxidant-derived radical affect redox state of transcription factors. The studies using the Gene Chip system have explored regulation of gene expression by phenolic antioxidants. It was found that some of phenolic antioxidants including BO-653 induced consistently up-regulation and down-regulation of certain genes in human umbilical vein endothelial cell. However, it is not clear how the gene expression is controlled, for example, whether specific structure is required or electrophilicity is a determining factor. The molecular mechanism of the regulation of gene expression by antioxidants is one of the most interesting subjects to be proved in the future.

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