*Free Radical Research*, January 2006; 40(1): 21–30



# Chemical structure–dependent gene expression of proteasome subunits via regulation of the antioxidant response element

WAKAKO TAKABE[1,2], NAEKO MATSUKAWA[1,2], TATSUHIKO KODAMA[1], KEIJI TANAKA[3], & NORIKO NOGUCHI[1]

[1]*Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro, Tokyo 153-8904, Japan,* [2]*NEDO fellow, Chugai Pharmatheutical Co., Ltd, 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan, and* [3]*The Tokyo Metropolitan Institute of Medical Science, and CREST, Japan Science and Technology Corporation, 3-18-22 Komagome, Bunkyo, Tokyo 113-8613, Japan*

Accepted by Professor E. Niki

*(Received 16 August 2005; in revised form 13 September 2005)*



**Abstract**

Antioxidants possess potent ability to regulate gene expression beyond their specific antioxidant activity. Genomic analysis reveals that three phenolic antioxidants, probucol, BO-653, and *t*BHQ, all of which have a phenoxyl group with one or two *tert*-butyl groups at the *ortho*-position, inhibit both the mRNA and protein levels of proteasome α-subunits in human endothelial cells. The chemical structure required for the gene regulation was studied by using derivatives of BO-653 and other antioxidants. It was found that the phenoxyl group and *tert*-butyl group at the *ortho*-position of the compounds were critical for down-regulation of the proteasome gene. Two antioxidant responsive elements (AREs) were identified in the promoter region of proteasome α subunit 3 (PSMA3). Results from promoter truncation analysis revealed that the proximal ARE region was necessary for the down-regulation of the expression of PSMA3. Electrophoretic mobility shift assays revealed that BO-653-mediated induction of DNA-binding to an upstream promoter region of PSMA3 containing the ARE motif was blocked by antibody against c-Jun but not Nrf2. These results indicate that the suppression of the proteasome α subunits expression by phenolic antioxidants is strictly dependent on both their chemical structure and the ARE consensus region in the promoter, which may be negatively regulated by AP-1.

**Keywords:** *Antioxidant, proteasome, gene expression, antioxidant responsive element, AP-1, Nrf2*

**Abbreviations:** *AP-1, Activator Protein 1; BOB, 2,3-dihydro-5-hydroxy-2,2-dimethyl-4,6-di-tert-butylbenzofuran; BOM, 2,3-dihydro-5-hydroxy-2,2-dimethyl-4,6-dimethylbenzofuranand; BO-653, 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-tert-butylbenzofuran; BZ1044, 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-methylbenzofuran; GCLM, glutamate cysteine lygase modifier-1; HO-1, hemeoxygenase-1; HUVEC, Human umbilical vein endothelial cells; Met-BO, 4,6-di-tert-butyl-2,3-dihydro-5-methoxy-2,2-dipentylbenzofuran; NQO-1, NADPH quinone oxidoreductase 1; PSMAs, proteasome α-type subunits; t BHQ, tert-butyl hydroquinone*

## Introduction

Antioxidants that inhibit lipid peroxidation have been extensively characterized in terms of their chemical reactivity, but the possibility that these structurally diverse compounds regulate cell function by mechanisms unrelated to radical scavenging has been advanced for consideration [1,2]. For example, the idea that vitamin E, the most abundant lipophilic antioxidant, has a capacity similar to that of vitamins D and A to regulate transcription has recently been proposed [3–5]. However, it has proven difficult to determine the specific mechanism by which certain

Correspondence: N. Noguchi, Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro, Tokyo 153-8904, Japan. Tel: 81 3 5452 5356. Fax: 81 3 5452 5359. E-mail: noguchi@lsbm.org

ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Taylor & Francis
DOI: 10.1080/10715760500354430

antioxidants regulate gene expression and cell function beyond their antioxidant effect.

It was found that three phenolic antioxidants, probucol, 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran (BO-653) and *tert*-butyl hydroquinone (*t*BHQ) inhibit both the mRNA and protein levels of the proteasome α-type subunits (PSMAs) as well as the function of the proteasome [6,7]. These compounds are known to act as radical scavenging antioxidants by donating hydrogen atoms to the peroxyl radical. Apparently this radical scavenging activity has been shown to be needed to regulate gene expression. On the other hand, these compounds also possess common structural moieties on the aromatic ring; a phenoxyl group and one or two *tert*-butyl groups at the *ortho*-position. The relevant question is which is more important for gene regulation, the radical scavenging activity or the specific structural moiety.

The mechanisms by which the mammalian proteasome is regulated are not well understood. Kwak et al. have reported that certain indirect antioxidants such as sulforaphane and 3H-1,2-dithiole-3-thione (D3T) increase the mRNA level for 24 out of the 34 proteasome subunits and that the proteasome β-type subunit 5 (PSMB5) gene is induced through the Keap1-Nrf2 signaling pathway [8]. It is implicated that both the tandem AREs in the PSMB5 promoter are needed for the maximal response of this promoter. In contrast to this report, however, previous findings from our laboratory demonstrated that four PSMAs were down-regulated by phenolic antioxidants and the other subunits were unaffected [6,7].

There are two perfect AREs in the promoter region of PSMAs, which have been shown to be critical for the down-regulation effected by phenolic antioxidants. In the present study, we investigated the structural moieties of compounds required for the ARE dependent down-regulation of the proteasome subunit in endothelial cells, and propose a mechanism in which Activator Protein 1 (AP-1) may be responsible for the suppression of the basal PSMA expression levels maintained by Nrf2.

## Experimental procedures

### *Materials*

BO-653, methyl ether derivative of BO-653; 4,6-di-*tert*-butyl-2,3-dihydro-5-methoxy-2,2-dipentylbenzofuran (Met-BO), 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-dimethylbenzofuran (BZ1044), 2,3-dihydro-5-hydroxy-2,2-dimethyl-4,6-dimethylbenzofuran (BOM) and 2,3-dihydro-5-hydroxy-2,2-dimethyl-4,6-di-*tert*-butylbenzofuran (BOB) were a kind gift from the Chugai Pharmaceutical Co. (Shizuoka, Japan). 4,4′-Isopropylidenedithio-bis-(2,6-di-*tert*-butylphenol) (Probucol) and α-tocopherol were kindly supplied by the Daiichi Pharmaceutical Co. (Tokyo, Japan) and the Eisai Co. (Tokyo, Japan), respectively. *t*BHQ and sulforaphane were purchased from SIGMA (St. Louis, MO) and CALBIOCHEM (Darmstadt, Germany), respectively. Methyl linoleate was obtained from Sigma and purified before use as described previously [9]. The lipophilic azo compound 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) used as a peroxyl radical generator was purchased from Wako Pure Chemical Ind. and used as received. Fetal bovine serum (FBS) was obtained from Clonetics (SanDiego, CA). The antibodies raised against c-Jun, phospho c-Jun and Nrf2, were obtained from Santa Cruz (Santa Cruz, CA).

### *Cell culture*

Human umbilical vein endothelial cells (HUVEC) were obtained from a commercial source (Clonetics) and grown in endothelial growth medium-2 (EGM-2) BulletKit (Clonetics) with 2% FBS at 37°C in a 5% $CO_2$ atmosphere. All experiments were carried out within four passages. Compounds were dissolved in DMSO (SIGMA), which was diluted with endothelial cell basement medium-2 (EBM-2; Clonetics). The final concentration of DMSO was 0.01%. The control cells were cultured in EBM-2 containing 2% FBS and 0.01% DMSO in the absence of compounds.

### *Intracellular concentration of compounds*

After incubation of HUVEC with each compound, the cells were collected and washed with HBSS three times. After centrifugation at 700 *g* for 5 min, the pellet of cells was stored at −80°C until analysis. For analysis, distilled water was added to the pellet which was then subjected to three freeze–thaw cycles and sonicated using an ultrasonic processor. The compounds were extracted with two volumes of chloroform/methanol (2/1,v/v). The chloroform layer was collected and replaced with methanol after drying under $N_2$. The compounds except Met-BO were analyzed by an HPLC equipped with an electrochemical detector (Shiseido, Tokyo, Japan) set at +600 mV with an LC-18 column (4 mm × 25 cm, 5 μm particle size, Supelco, Tokyo, Japan). Met-BO was detected with a UV detector at 220 nm. Methanol containing 50 mM $NaClO_4$ was used as the mobile phase at a flow rate of 0.8 ml/min for measurement of the compounds except BOM. For measurement of BOM methanol and $H_2O$ (80/20, v/v) containing 50 mM $NaClO_4$ were used.

### *Antioxidantactivity against lipid peroxidaiton*

Methyl linoleate (15.2 mM) was incubated in acetonitrile in the presence of AMVN (0.2 mM) at 37°C with or without 5 μM of the compounds studied and formation of methyl linoleate hydroperoxides was monitored with a spectrophotometer at 234 nm [10].

*Real-time PCR analysis*

For real-time PCR, 1 μg of total RNA was reverse transcribed into cDNA using a GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA). The housekeeping genes GAPDH and cyclophilin were used as an endogenous control. The following primer sequences were used. PSMA1: forward primer, 5′- TTT TGA CTG CAG AGC CAT GTC C -3′, reverse primer, 5′- TTA AGG CAC GCA GAC CAT GTT T -3′; PSMA2: forward primer, 5′- CTG GAG CTT ACT TTG CCT GGA AA -3′, reverse primer, 5′- CCA GCT TCA TTG CAG ATT CCA A -3′; PSMA3: forward primer, 5′- TGC TGT TAG ACC TTT TGG CTG C -3′, reverse primer, 5′- CCC CAA TAA CCG TAT GAA ACA CC -3′; c-Jun: forward primer, 5′- AAC TCG GAC CTT CTC ACG TCG -3′, reverse primer, 5′- TGC TGA GGT TGG CGT AGA CC -3′; Nrf2: forward primer, 5′- ATT GCC TGT AAG TCC TGG TCA TC -3′, reverse primer, 5′- ACT GCT CTT TGG ACA TCA TTT CG -3′; HO-1: forward primer, 5′- CGG GCC AGC AAC AAA GTG -3′, reverse primer, 5′- ACT GTC GCC ACC AGA AAG CT -3′; GCLM: forward primer, 5′- CAG CCG AGG AGC TTC ATG ATT G -3′, reverse primer, 5′- TGC ATT CCA AGA CAT CTG GAA A -3′; GAPDH: forward primer, 5′-TTT GGC TAC AGC AAC AGG GTG GTG-3′, reverse primer, 5′-ATG GTA CAT GAC AAG GTG CGG CTC-3′; cyclophilin: forward primer, 5′-TGG AGA GCA CCA AGA CAG ACA-3′, reverse primer, 5′- TGC CGG AGT CGA CAA TGA T-3′. The mRNA was quantified using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

*Plasmids*

The promoter region of *PSMA3* (from −3059 to −30; NT 026437) was isolated by PCR amplification from the genomic DNA of HUVEC. The isolated PCR product was ligated into the luciferase reporter vector pGL3basic (Promega, Madison, Wis.). Deleted sequences of the *PSMA3* promoter (−698 bp-luc, −698 to −30) were produced by PCR amplification and inserted into the pGL3 basic vector. Mutated *PSMA3* promoters were generated by PCR using primers containing the mutated −46 ARE (TG*AG*CGGGC–TG*GA*CGGGC) (the mutated nucleotides are underlined) as described previously [11]. The sequence of each promoter was verified.

*Transient transfection and measurement of luciferase activity*

HUVEC were transfected by means of the FuGENE6 reagent (Roche Diagnostics, Mannheim, Germany). Briefly, $5 \times 10^4$ cells/well of HUVECs were seeded in 12-well plates 18–24 h before transfection with EGM-2-MV (Clonetics). The transfection complex containing 0.3 μg of plasmid DNA, 50 ng of a cytomegalovirus enhancer/promoter (pRL-CMV) (Promega), and the transfection reagents were added to each well, and cells were incubated for 22 h. Cells were washed with phosphate-buffered saline two times and recovered in EBM-2 containing 2% FBS. The cells were then incubated in the presence or absence of drugs for 4 or 8 h, cells were lysed and assayed for luciferase activity using a dual luciferase reporter assay system (Promega) and a Lumat LB 9507 luminometer (Berthold, Gaithersburg, MD).

*Nuclear extracts*

HUVEC were cultured in 150 mm culture dishes. After reaching sub-confluency cells were deprived of growth factors for 2 h before the addition of BO-653. Nuclear protein was isolated by the method of Dignam et al. [12].

*Electrophoretic mobility shift assay*

HUVEC were stimulated with 50 μM BO-653 for 30 min and nuclear extracts were prepared as described above. Double-stranded oligonucleotides were labeled with [α-$^{32}$P]dCTP and Klenow fragment and purified by using Sephadex™ G-50 Medium (Amersham Pharmacia Biotech, Buckinghamshire, UK). Binding reactions were carried out in a total volume of 30 μl containing up to 10 μg of nuclear protein and were incubated with 10 fmol of $^{32}$P-labeled probe, 1 μg of poly(dI-dC), and 3 μl of 10 × binding buffer (100 mM Tris–HCl (pH 7.5), 50% glycerol, 10 mM dithiothreitol, 10 mM EDTA) for 20 min at room temperature, followed by 30 min at 4°C. The following oligonucleotides sequences were used for probes: PSMA3 ARE/AP1 motifs, 5′- GTT-CAGCCAATGAGCGGGCCTGTTAC -3′. To test the effect of antibodies on DNA-protein binding, nuclear extracts were pre-incubated with 10 μl of antibodies of Nrf2, and c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA) for 10 min at room temperature. In competition studies, nuclear extracts were pre-incubated with a 100-fold molar excess of unlabeled oligonucleotides for 10 min at room temperature, then added to the reaction mixture. DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel containing 5% glycerol in 0.5 × TBE (50 mM Tris, 50 mM boric acid, and 1 mM EDTA). The loaded gel were dried, and then the gels were autoradiographed and analyzed by a Fuji Bioimage Analyzer BAS-1800 (Fuji Photo Film Co. Ltd, Tokyo, Japan).

*Western blot analysis*

HUVEC were stimulated with 50 μM BO-653 for up to 4 h, and nuclear extracts were prepared as described above. A measure of 10 μg of nuclear protein were fractionated on 12% polyacrylamide gel with SDS and

electroblotted onto Hybond™ ECL™ nylon membranes (Amersham pharmacia bioteck) for 2 h at 80 V. The membranes were blocked overnight at 4°C in $H_2O$ containing 2% fat-free dried milk powder and was then incubated with anti-phosho c-Jun and c-Jun antibody (diluted 1:200) in $H_2O$ containing 2% fat-free dried milk powder for 1 h at room temperature. After treatment with peroxidase-conjugated goat anti-mouse IgG antibody (SIGMA) (diluted 1:10 000) or anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) (diluted 1:10 000) in $H_2O$ containing 2% milk powder for 1 h at room temperature, labeled bands from washed blots were detected by ECL plus a Western Blotting Detection System (Amersham Biosciences). Membranes were exposed to FUJI MEDICAL X-RAY FILM (Fuji Photo Film Co. Ltd) at room temperature.

### *Statistical study*

All experiments were repeated at least four times and the results are given as the means ± standard deviation, and Student's *t*-test was used to analyze the statistical significance.

## Results

### *Identification of the structural moiety in phenolic antioxidants required for decreasing mRNA levels of proteasome subunits*

In a previous study, we have shown that treatment of HUVEC with phenolic antioxidants results in decreased expression of the mRNA for the α-type proteasome subunits PSMA1, PSMA2, PSMA3, and PSMA4 [6,7]. Using the series of compounds shown in Figure 1, the effects on the mRNA levels of PSMA1, PSMA2, and PSMA3 were determined by real-time PCR. Prior to analysis of their ability to affect mRNA levels, we measured the intracellular concentrations of the compounds used (Figure 2). When HUVEC were treated with 50 μM of each compound for 4 h, BO-653 and its derivatives except for Met-BO reached a similar level of intracellular concentration. To gain a similar intracellular concentration of Met-BO, it was necessary to treat HUVEC with a 10 times higher concentration of Met-BO than other compounds. Real time PCR analysis clearly showed that the mRNA levels of all three proteasome α-subunits were decreased by BO-653 or BOB but not by Met-BO or BZ1044 (Figure 3). All of the data normalized by GAPDH and cyclophilin yielded essentially the same results. Although BOB has a di-methyl instead of di-penthyl form of BO-653, it still decreased the mRNA levels of the α-subunit, implying that the side chain was not a determinant of the effect. Methylation of the phenoxyl group and replacement of the *tert*-butyl group with the methyl group on the aromatic ring at the *ortho*-positions of the phenoxyl group of BO-653 resulted in a disappearance of its suppressive effects on the α-type subunit of the proteasome, which was clearly shown by using MetBO and BZ1044, respectively. The importance of the *tert*-butyl group at the *ortho*-position was also shown in a comparison of BOB and BOM. These compounds differ only in the substituents at the *ortho*-positions flanking the phenoxyl group; with a *tert*-butyl group in the

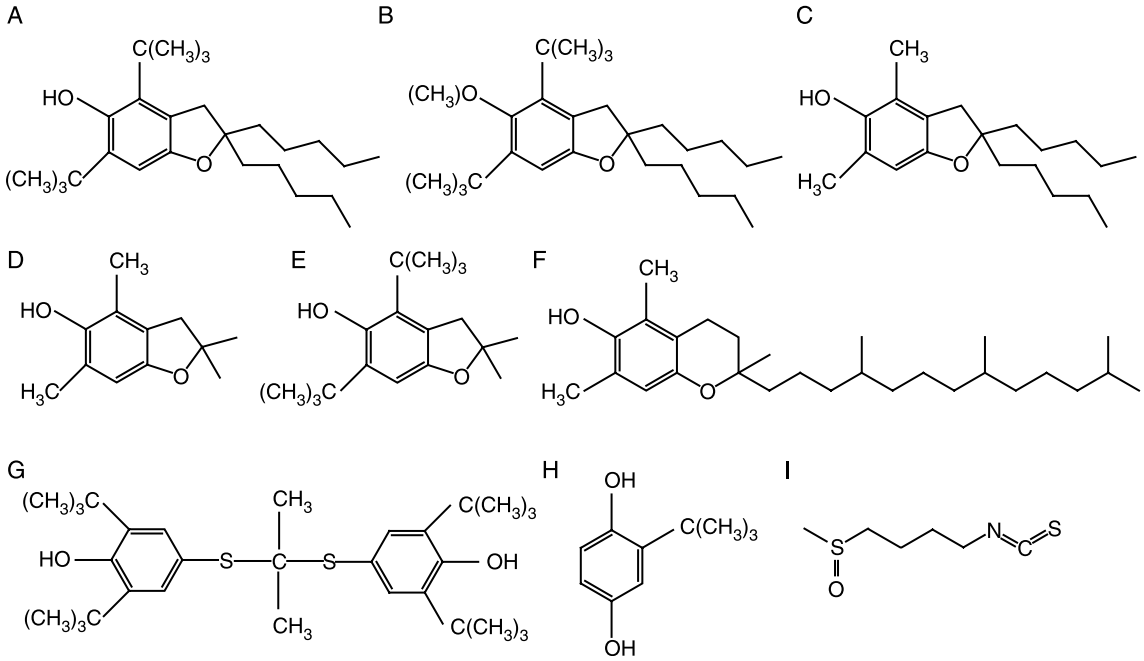


Figure 1. Chemical structures of (A) BO-653, (B) Met-BO, (C) BZ1044 (D) BOM, (E) BOB, (F) α-tocopherol, (G) probucol, (H) *t*BHQ, and (I) sulforaphane.

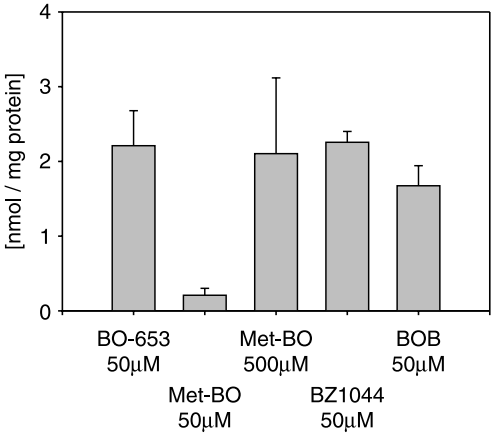


Figure 2. Intracellular concentrations of compounds in HUVECs. HUVECs were exposed to 50 μM BO-653, Met-BO, BZ1044, BOB, and 500 μM Met-BO for 4 h, and the intracellular concentrations of these compounds were analyzed as described in Materials and Methods. Values represent mean ± SD of three independent experiments in triplicate.

case of BOB and a methyl group for BOM. It was found that the mRNA level of PSMA1 in HUVEC decreased upon exposure to BOB but not to BOM (data not shown). α-Tocopherol also did not affect the mRNA levels of PSMA1 (data not shown).

*Radical scavenging antioxidant activity of BO-653 and its derivatives*

To test the importance of antioxidant activity for the regulation of gene expression, we examined radical scavenging activity of the compounds studied (Figure 4). Methyl linoleate was incubated in acetonitrile in the presence of the radical initiator AMVN at 37°C, and formation of methyl linoleate hydroperoxides was monitored with a spectrophotometer at 234 nm. As expected, MetBO, which does not have a hydrogen/electron donating phenoxyl group, did not inhibit free radical mediated lipid peroxidation. In contrast, BZ1044 inhibited lipid peroxidation to the same extent as BO-653, indicating BZ1044 and BO-653 have similar radical scavenging activity regardless of the substituents at the *ortho*-position of the phenoxyl group. We have already demonstrated a similar radical scavenging activity of BO-653, BOM and BOB in homogeneous solution [13].

*ARE-dependent regulation of PSMA3 expression by antioxidants*

To analyze the regulation of PSMA, the promoter region (689 and 3059 bp) of PSMA3 was isolated from human genomic DNA by PCR amplification and ligated into a luciferase reporter pGL3 basic vector. Two AREs were identified 46 and 2586 bp upstream of the PSMA3 gene coding region. Both AREs were found to be perfect forms and promoter truncation analysis was performed to identify the functional

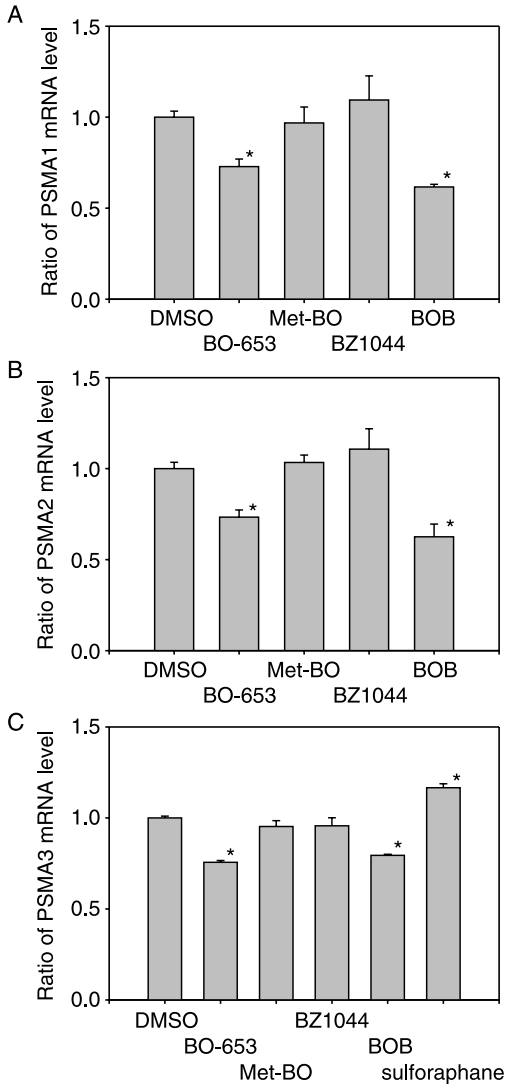


Figure 3. Effect of phenolic compounds on expression of PSMA mRNA. Real-time PCR analyses were performed for (A) PSMA1, (B) PSMA2, and (C) PSMA3 expression in HUVEC following treatment with BO-653 (50 μM), its derivatives (50 or 500 μM), and sulforaphane (10 μM) for 6 h. All data obtained were normalized by GAPDH values and are shown as the mean ± SD ($n = 3$) of the ratio against the value of vehicle (DMSO) treatment. *$p < 0.005$ compared with vehicle (DMSO)-treated control.

AREs from this promoter. The luciferase activities from three constructs containing a different promoter (Figure 5A) in HUVEC following treatment with the compounds studied in this paper were measured. Sulforaphane, which is known to increase the mRNA level of PSMB5 through ARE, was used for comparison [8]. While sulforaphane treatment significantly induced the 3059 bp promoter activity, BO-653 and its derivatives did not exert any effect on this activity (Figure 5B). The 698 bp promoter containing only the proximal ARE was also activated by sulforaphane treatment less extensively than the 3059 bp promotor (Figure 5C). BO-653 significantly decreased luciferase activity for the 698 bp promoter, but neither Met-BO nor BZ1044 had any effect.

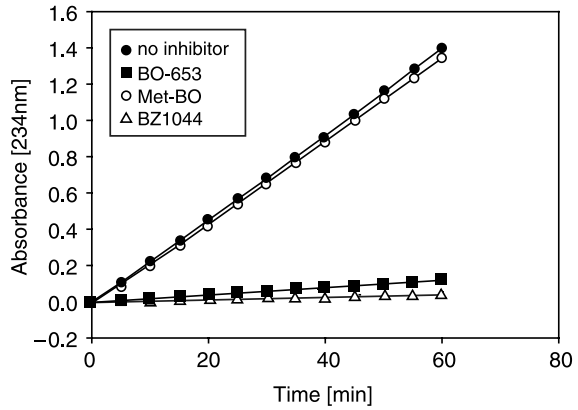


Figure 4. Radical scavenging activity of BO-653 and its derivatives. Radical scavenging antioxidant activity of each compound (5 μM) was measured in oxidation of methyl linoleate (15.2 mM) induced by radical initiator, AMVN (0.2 mM) at 37°C in acetonitrile. The representative data of three independent experiments is shown.

To confirm the promoter truncation result, the ARE was mutated. When the construct containing the mutated-ARE was transfected, the basal promoter activity level was decreased (Figure 5D). Mutation in the proximal ARE largely abolished promoter activation and inactivation by sulforaphane and BO-653, respectively (Figure 5D). Taken together, these data suggest that compounds with at least one *tert*-butyl group at the *ortho*-position of the phenoxyl group decrease the expression of PSMA3 through the proximal ARE.

### *Effects of BO-653 on mRNA expression levels of Nrf2 and AP-1*

The consensus sequences of ARE in the PSMA3 promoter region contains not only a Nrf2 but also a AP-1 like binding sequence. We examined the effects of BO-653 on the mRNA expression levels of Nrf2 and c-Jun by real time PCR (Figure 6A,B). Treatment of HUVEC with BO-653 induced mRNA expression of c-Jun but not Nrf2 over time. In addition, the expression of Nrf2-regulated genes such as hemeoxygenase-1(HO-1) and glutamate cysteine lygase modifier-1 (GCLM) [14] was decreased (Figure 6C,D).

### *BO-653-mediated induction of DNA-binding levels on ARE motif in PSMA3 promoter*

Since it is reported that AP-1 proteins negatively regulate the human ARE [15], we monitored nuclear phosphorylated c-Jun during the treatment of cells with BO-653. The phosphorylation of nuclear c-Jun was observed at an early time point after treatment of HUVEC with BO-653 (Figure 7A).

To determine the effect of BO-653 on DNA-binding activity, electrophoretic mobility shift assays were carried out. A radio-labeled probe containing the proximal ARE site was incubated with nuclear extracts derived from untreated and BO-653-treated HUVEC, resulting in specific DNA-protein complexes (arrow, Figure 7B, lane 2 and 3). The formation of these DNA-protein complexes was inhibited by addition of a 100 fold molar excess of unlabeled self-competitor (Figure 7B, lane 4).

We next performed supershift experiments with specific antibodies against Nrf2 and c-Jun to find the identity of proteins in the BO-653-induced ARE binding complex. The addition of an anti-c-Jun antibody failed to result in a shifted band but did induce a decrease in the binding complex (Figure 7B, lane 6). On the other hand, the binding complex was not affected by the addition of an antibody against Nrf2 (Figure 7B, lane 5).

## Discussion

Phenolic antioxidant compounds such as *t*BHQ and tBHA are known to induce gene expression through the binding of Nrf2 to ARE [2]. The induction of genes encoding detoxification phase II enzymes is important for cell protection, especially in the liver. The present study was initiated as an investigation of the potency of the endothelial cell's response to phenolic compounds using a DNA microarray. In contrast to expectations, few Nrf2 regulating genes were induced in HUVEC by treatment with three phenolic antioxidants: probucol, BO-653, and *t*BHQ [6]. This was not due to a reduced potency of HUVEC in inducing the Nrf2 regulating genes, since many of those genes were induced more than ten fold under laminar flow at a low shear stress [16] and 4-hydroxy -2-nonenal level (unpublished data).

Microarray analysis, instead, revealed a novel ability of phenolic antioxidants to decrease the expression of proteasome α-type subunits in HUVEC [6,7]. The regulation of the mammalian proteasome remains poorly understood. The enhancement of PSMBs and PSMEs by lipopolysaccharide in mouse liver has been reported [17]. Foss et al. found that γ-interferon induced three PSMBs in several human cell lines in a cell type-dependent manner [18]. Kwak et al. studied the mechanisms underlying the finding that expression of PSMB5 was enhanced and clearly showed transcription factor Nrf2 dependent regulation of two tandem AREs in the promoter by the indirect antioxidants sulforaphane and D3T in mouse liver and embryonic fibroblast cells [8]. The present study has found ARE-dependent down regulation of PSMA3 by phenolic radical scavenging antioxidants in HUVEC. The AREs from PSMA3 contain the complete consensus sequence of the ARE, i.e. TGAGCGGGC and TGACAGAGC at −46 and −2586 bp in the 5′-flanking region. From the results of promoter truncation and mutation analyses

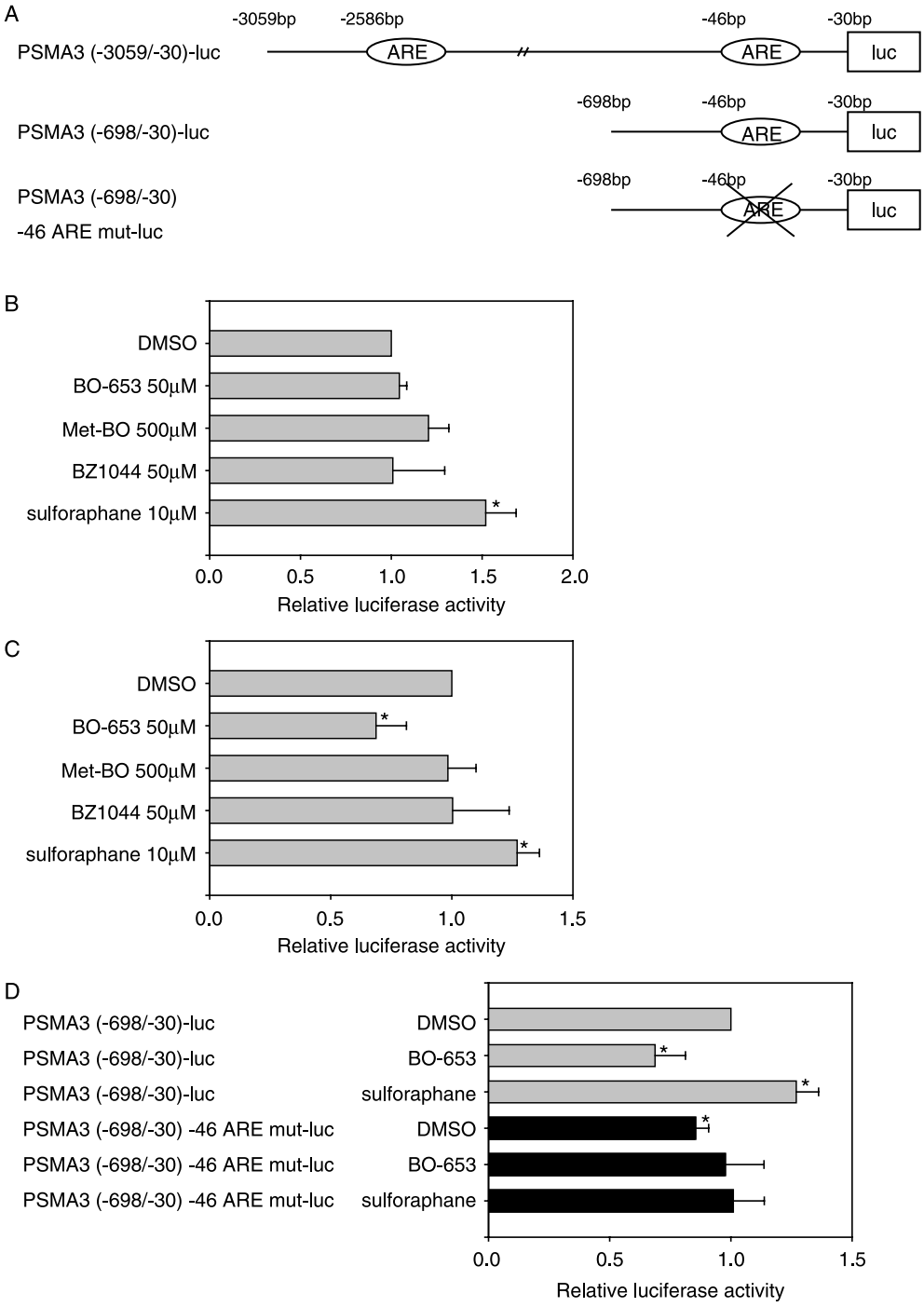


Figure 5. ARE-dependent regulation of the promoter of PSMA3 by compounds. The human PSMA3 promoter constructs (A) and luciferase activities derived from these truncated promoters following treatment with BO-653 (50 μM), its derivatives (50 or 500 μM), and sulforaphane (10 μM) (B-D) for 8 or 4 h, respectively, are shown. Two AREs were identified 2859 and 46 bp upstream of the PSMA3 gene coding region. The 3059 bp promoter contained both of the two AREs (B) but the 689 bp promoter (-689bp-luc) had just one proximal ARE (C). Mutated 689 bp promoter (−46 ARE mut-luc) was generated at the proximal ARE (D). *$p < 0.05$ compared with plasmid, (-3059/-30)-luc or (-698/-30)-luc-transfected, vehicle (DMSO)-treated control.

(Figure 5), the proximal ARE in the PSMA3 promoter appears to be important for both basal expression and the negative response to phenolic antioxidants.

Nrf2 is an essential element in the ARE transcription complex in regulation of a number of cytoprotective genes such as NADPH quinone oxidoreductase 1(NQO-1), HO-1 and GCLM [19–22]. The expression levels of Nrf2 itself were not changed in HUVEC treated with BO-653. However, the expression levels of HO-1 and GCLM were decreased, indicating that Nrf2 can be the modulator but yet negatively regulated

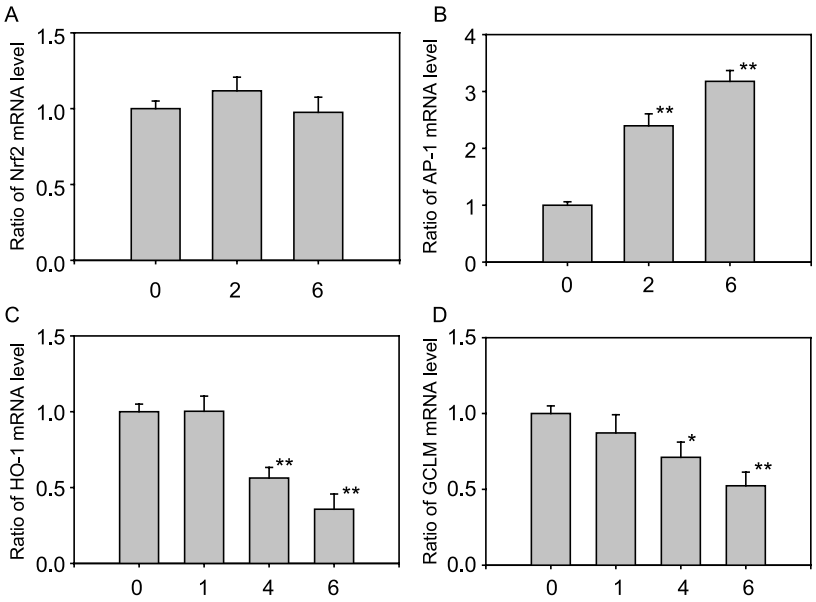


Figure 6. Effect of BO-653 on mRNA expression of Nrf2, c-Jun and Nrf2-regulated genes. Real-time PCR analyses were performed for (A) Nrf2, (B) c-Jun, (C) HO-1 and (D) GCLM expression in HUVEC following treatment with BO-653 (50 μM) for up to 6 h. All data obtained were normalized by GAPDH values and are shown as the mean ± SD ($n = 3$). $*p < 0.05$, $**p < 0.005$ compared with time 0.

by other transcription factors. It is reported that AP-1 negatively regulates ARE [15] and that the ARE in the PSMA promoter region contains a consensus AP-1 sequence. The data showing an increase in mRNA levels of c-Jun (Figure 6) and phosphorylation of the c-Jun protein (Figure 7 A) in BO-653-treated HUVEC suggested the possibility of negative regulation by AP-1. The EMSA experiments for PSMA3 in which an antibody against c-Jun reduces the DNA-protein complex support the proposed mechanisms. BACH1 may be another interesting candidate in the ARE-dependent negative response by antioxidants in HUVEC [23]. The specific mechanisms of the repressive response of the ARE to antioxidants is an interesting issue which will require further study.

In a previous paper, DNA microarray and northern blot analyses revealed that phenolic antioxidants changed the mRNA levels of PSMA1 ~ 4 but not PSMBs, PSMCs, PSMDs, or PSMEs [7]. PSMA1, 2 and 4 have the following AREs. PSMA1: TGAGGAAGC at −868; PSMA2: TGAGAAAGC at −1066 and TGAGGCCGC at −448; PSMA4: TGAGGATGC at −1422. Kwak et al. showed the up-regulation of PSMA1 and PSMA4 by D3T in mouse liver [8]. There is an ARE-like sequence, TGGGCGAGC at −36 bp in the 5′-flanking region of mouse PSMA3, corresponding to TGAGCGGGC at −46 of the human form, but the response to chemicals in mouse is not known.

The present study sheds some light on the chemical structural moieties of compounds which suppress the expression of PSMA mRNA in HUVEC. The phenoxyl group and *tert*-butyl group at the *ortho*-position on the aromatic ring are strictly required for a reduced change in the mRNA levels. Interestingly, radical scavenging activity is critical but is not a determinant. It was reported that *ortho*- or *meta*-diphenol has a high potency in alteration of gene expression [24]. BO653 and BOB may be converted to diphenol by hydroxylation of *t*-butyl group at the *ortho*-position with an action of some enzymes. Sulforaphane increased PSMA3 expression in the same analyzing system, implying different mechanisms underlying ARE-dependent gene expression are regulated by the phenolic antioxidants and the isothiocyanates (e.g. sulforaphane). These results suggest that regulation of the proteasome is dependent on the subunit types, cell types, species and the kinds of inducing chemicals.

## Acknowledgements

We thank Chugai Pharmatheutical Co., Ltd for kind giving us BO-653 and its derivatives. We thank Dr Masayuki Yamamoto for his kind and instructive discussion. This study was carried out as a part of The Project (FY2003-FY2005) for Bio-nanochip Analysis of Protein Interaction (Focus 21) of Health Assurance Program which was performed by Ministry of Economy, Trade & Industry, and entrusted by New Energy and Industrial Technology Development Organization (NEDO). Pacific Edit reviewed the manuscript prior to submission.

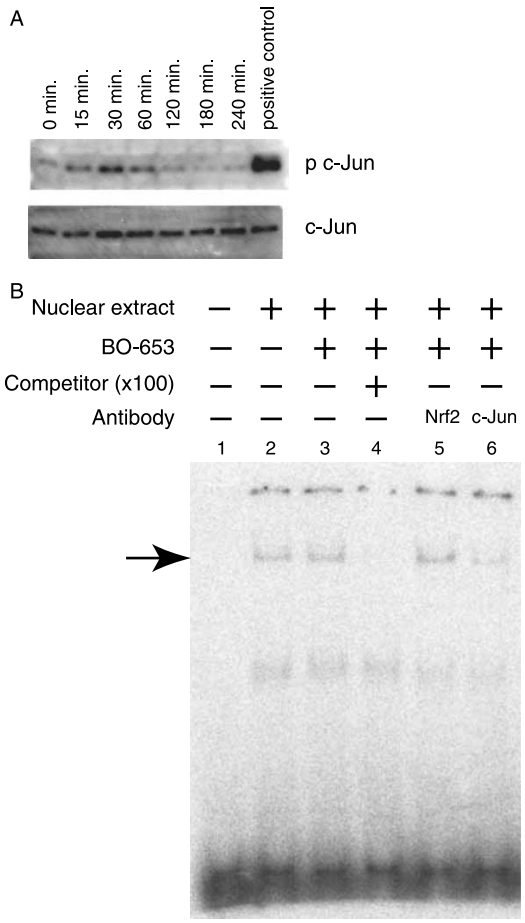


Figure 7. Western blot analysis for AP-1 and Electrophoretic mobility shift assays for ARE of PSMA3. (A) Western blot analysis were performed for the nuclear extract (10 μg of protein) prepared from HUVECs after treatment with 50 μM BO-653 for up to 4 h using a mouse monoclonal antibody against phosphorylated-c-Jun and a rabbit polyclonal antibody against c-Jun. (B) Electrophoretic mobility shift assays were performed with a $^{32}$P-labeled ARE probe for PSMA3and 10 μg of nuclear extract from HUVECs treated with or without 50 μM BO-653 for 30 min (lane 2 and 3). In competition assays, a 100-fold molar excess of unlabeled ARE oligo probe (lane 4) and polyclonal antibodies directed to Nrf2 (lane 5) and c-Jun (lane 6) were incubated in the nuclear extracts 10 min before the addition of $^{32}$P-labeled ARE probe.

## References


[1] Vershinin A. Biological functions of carotenoids—diversity and evolution. Biofactors 1999;10:99–104.

[2] Rushmore TH, Kong AN. Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes. Curr Drug Metab 2002;3:481–490.

[3] Zimmer S, Stocker A, Sarbolouki MN, Spycher SE, Sassoon J, Azzi A. A novel human tocopherol-associated protein: Cloning, *in vitro* expression, and characterization. J Biol Chem 2000;275:25672–25680.

[4] Azzi A, Gysin R, Kempna P, Munteanu A, Negis Y, Villacorta L, Visarius T, Zingg JM. Vitamin E mediates cell signaling and regulation of gene expression. Ann N Y Acad Sci 2004;1031:86–95.

[5] Yamauchi J, Iwamoto T, Kida S, Masushige S, Yamada K, Esashi T. Tocopherol-associated protein is a ligand-dependent transcriptional activator. Biochem Biophys Res Commun 2001;285:295–299.

[6] Takabe W, Mataki C, Wada Y, Ishii M, Izumi A, Aburatani H, Hamakubo T, Niki E, Kodama T, Noguchi N. Gene expression induced by BO-653, probucol and BHQ in human endothelial cells. J Atheroscler Thromb 2000;7:223–230.

[7] Takabe W, Kodama T, Hamakubo T, Tanaka K, Suzuki T, Aburatani H, Matsukawa N, Noguchi N. Anti-atherogenic antioxidants regulate the expression and function of proteasome alpha-type subunits in human endothelial cells. J Biol Chem 2001;276:40497–40501.

[8] Kwak MK, Wakabayashi N, Greenlaw JL, Yamamoto M, Kensler TW. Antioxidants enhance mammalian proteasome expression through the Keap1-Nrf2 signaling pathway. Mol Cell Biol 2003;23:8786–8794.

[9] Komuro E, Takahashi M, Morita T, Tsuchiya J, Arakawa Y, Yamamoto Y, Niki E, Sugioka K, Nakano M. Inhibition of peroxidations of lipids and membranes by estrogens. J Phys Org Chem 1990;3:309–315.

[10] Noguchi N, Iwaki Y, Takahashi M, Komuro E, Kato Y, Tamura K, Cynshi O, Kodama T, Niki E. 2,3-Dihydro-5-hydroxy-2,2-dipentyl-4,6-di-tert-butylbenzofuran: Design and evaluation as a novel radical-scavenging antioxidant against lipid peroxidation. Arch Biochem Biophys 1997;342:236–243.

[11] Kwak MK, Itoh K, Yamamoto M, Kensler TW. Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: Role of antioxidant response element-like sequences in the nrf2 promoter. Mol Cell Biol 2002;22:2883–2892.

[12] Dignam JD, Martin PL, Shastry BS, Roeder RG. Eukaryotic gene transcription with purified components. Methods Enzymol 1983;101:582–598.

[13] Watanabe A, Noguchi N, Fujisawa S, Kodama T, Tamura K, Cynshi O, Niki E. Stability and reactivity of aryloxyl radicals derived from a novel antioxidant BO-653 and related compounds. Effects of substituent and side chain in solution and membranes. J Am Chem Soc 2000;122:5438–5442.

[14] Chen XL, Varner SE, Rao AS, Grey JY, Thomas S, Cook CK, Wasserman MA, Medford RM, Jaiswal AK, Kunsch C. Laminar flow induction of antioxidant response element-mediated genes in endothelial cells. A novel anti-inflammatory mechanism. J Biol Chem 2003;278:703–711.

[15] Ng D, Kokot N, Hiura T, Faris M, Saxon A, Nel A. Macrophage activation by polycyclic aromatic hydrocarbons: Evidence for the involvement of stress-activated protein kinases, activator protein-1, and antioxidant response elements. J Immunol 1998;161:942–951.

[16] Warabi E, Wada Y, Kajiwara H, Kobayashi M, Koshiba N, Hisada T, Shibata M, Ando J, Tsuchiya M, Kodama T, Noguchi N. Effect on endothelial cell gene expression of shear stress, oxygen concentration, and low-density lipoprotein as studied by a novel flow cell culture system. Free Radic Biol Med 2004;37:682–694.

[17] Yoo JY, Desiderio S. Innate and acquired immunity intersect in a global view of the acute-phase response. Proc Natl Acad Sci U S A 2003;100:1157–1162.

[18] Foss GS, Larsen F, Solheim J, Prydz H. Constitutive and interferon-gamma-induced expression of the human proteasome subunit multicatalytic endopeptidase complex-like 1. Biochim Biophys Acta 1998;1402:17–28.

[19] Wasserman WW, Fahl WE. Functional antioxidant responsive elements. Proc Natl Acad Sci USA 1997;94:5361–5366.

[20] Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 1997;236:313–322.

[21] Kwak MK, Itoh K, Yamamoto M, Sutter TR, Kensler TW. Role of transcription factor Nrf2 in the induction of hepatic phase 2 and antioxidative enzymes *in vivo* by the cancer chemoprotective agent, 3H-1, 2-dimethiole-3-thione. Mol Med 2001;7:135–145.

[22] Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer Res 2002;62:5196–5203.

[23] Dhakshinamoorthy S, Jain AK, Bloom DA, Jaiswal AK. Bach1 competes with Nrf2 leading to negative regulation of the antioxidant response element (ARE)-mediated NAD(P)H: Quinone oxidoreductase 1 gene expression and induction in response to antioxidants. J Biol Chem 2005;280: 16891–16900.

[24] Prochaska HJ, De Long MJ, Talalay P. On the mechanisms of induction of cancer-protective enzymes: A unifying proposal. Proc Natl Acad Sci U S A 1985;82:8232–8236.