Anti-atherogenic antioxidants regulate the expression and function of proteasome α-type subunits in human endothelial cells

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Running title: Regulation of proteasome subunits by antioxidants
Summary

It has been proposed that phenolic antioxidants such as probucol exert their anti-atherogenic effects through scavenging lipid-derived radicals. In this study the potential for genomics to reveal unanticipated pharmacological properties of phenolic antioxidants is explored. It was found that two anti-atherogenic compounds, BO-653 and probucol, inhibited the expression of three α-type proteasome subunits, PMSA2, PMSA3, and PMSA4 in human umbilical vein endothelial cells. Here we report that both BO-653 and probucol caused not only inhibition of the mRNA levels of these three subunits but also inhibition of both the gene expression and protein synthesis of the α-type subunit, PMSA1. Other subunit components of the proteasome such as the β-type subunits (PMSB1, PMSB7), the ATP-ase subunit of 19S (PMSC6), the non-ATP-ase subunit of 19S (PMSD1), and PA28 (PMSE2) were not significantly affected by treatment with these compounds. The specific inhibition of α-type subunit expression in response to these antioxidants resulted in functional alterations of the proteasome with suppression of degradation of multi-ubiquitinated proteins and I-κBα. These results suggest that certain compounds previously classified solely as antioxidants are able to exert potentially important modulatory effects on proteasome function.
**Introduction**

Genomics and proteomics offer powerful tools for discovering molecular mechanisms underlying complex biological responses. For example, these methods enable comparison of the levels of gene and protein expression between diseased and normal cells or cells treated with pharmacological agents. Atherosclerosis is an interesting example in this respect since it is a complex chronic inflammatory condition resulting from the interaction between modified lipoproteins, monocyte-derived macrophages, and other cellular elements of artery wall in which numerous pharmacological agents have been tested for efficacy (1,2). Indeed, one important class of molecules assessed in these models are generically classified as “antioxidants”, and the anti-atherogenic effects of these compounds have contributed to the evolution of the LDL oxidation hypothesis of atherosclerosis. However, the detailed mechanisms of antioxidant compounds, such as probucol, remain complex and elusive with a number of biological responses identified which are not easily reconciled with a unique mechanism depending solely on free radical scavenging. Recently, we have applied the oligonucleotide microarrays analysis (HuGene human FL array, Affimetrix, Inc., Santa Clara, California) to determine the effects of two anti-atherogenic phenolic compounds, 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-tert-butyldifuran (BO-653) and 4,4’-isopropylidenedithio-bis-(2,6-di-tert-butyldifuran) (probucol) on gene expression in human umbilical vein endothelial cells (HUVEC).

Probucol has been used clinically as an anti-atherogenic compound due to its lipid lowering properties, while it’s antioxidant properties subsequently emerged from a
combination of \textit{n vitro} (3,4) and \textit{in vivo} models of the atherosclerotic processes (5,6). With the advent of effective lipid lowering therapies probucol’s use as an anti-atherogenic agent has become limited. However, the mechanisms underlying probucol’s action remain important. For example, recent studies have shown that probucol is effective in reducing the incidence of restenosis after percutaneous transluminal coronary angioplasty (PTCA) (7,8). It has been also reported that probucol inhibits expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) both in animal models (9,10) and in HUVEC (11,12). Notwithstanding the continuing interest in probucol molecular mechanisms remain obscure with several controversial results with probucol observed in different animal models. For example, probucol effectively inhibited development of atherosclerosis in Watanabe heritable hyperlipidemia (WHHL) rabbits (5,6) while it exacerbated the development of atherosclerosis in apolipoprotein E (apoE) knockout mice (13) and LDL receptor knockout mice (14). The responses in apoE knockout mice were particularly interesting with a recent report showing that lesion development was increased by probucol in the aortic root yet decreased in the aortic arch (15). These studies implicate mechanisms that are more complex than simple scavenging of lipid peroxyl radicals and encompass processes that involve site specific modulation of gene transcription in the endothelium. It remains uncertain whether the antioxidant properties of probucol and its lipid lowering effects are related or critical for the anti-atherogenic effects in humans.

The LDL oxidation hypothesis of atherosclerosis also prompted a series of initiatives to elaborate and improve on the antioxidant component of probucol. The
compound, BO-653, is such an example and shares the structural motif for scavenging peroxyl radicals, the phenol group, while the rest of the molecule is structurally distinct from probucol (Figure 1). It was found that BO-653 exerted a potent antioxidant activity against lipid peroxidation (16) and oxidative modification of LDL (17) and also exhibited anti-atherogenic effects in three different animal models (14). Surprisingly, it is about 10 times more effective than probucol as an antioxidant yet still requires similar concentrations to exert anti-atherogenic properties. We hypothesized that properties other than the antioxidant activity of these compounds could contribute to inhibition of atherosclerosis.

The use of genomic analysis to elucidate the mechanisms of pharmacological agents is an important application of this emerging technology. Indeed, a novel and unexpected finding derived from gene chip analysis (18) indicated that regulation of proteasome function could occur on exposure of HUVEC to phenolic antioxidants.

The ubiquitin-proteasome pathway has been shown to be involved in various biologically important processes, such as the cell cycle, cellular metabolism, apoptosis, signal transduction, immune response, and protein quality control (19-23). Consequently, this proteolytic machinery is capable of catalyzing the turnover of proteins in a regulated fashion. The potential of a pharmacological agent to modulate proteasome function through transcriptional regulation has not been reported previously. The complete catalytic complex, the 20S proteasome, is a barrel-like particle appearing as a stack of four rings made up of two outer α-rings and two inner β-rings. The α- and β-rings are each made up of seven structurally similar α- and β-subunits, respectively.
(23). The regulatory complex termed PA700 (also called the “19S complex”) associates with 20S proteasome to form the 26S proteasome with a molecular mass of ~2500 kDa (24). Moreover, another proteasome activator PA28 (11S REG), consisting of α and β subunits, is known to bind to α-ring of the 20S proteasome (19). In this report we show that the selective inhibition of the α-type subunits of the 20S proteasome in HUVEC treated with either probucol or BO-653 results in suppression of proteasome activity.

Experimental procedures

Chemicals. 2,3-Dihydro-5-hydroxy-2,2-dipentyl-4,6-di-tert-butylbenzofuran (BO-653) was a kind gift from the Chugai Pharmaceutical Co (Shizuoka, Japan). 4,4’-Isopropylidenedithio-bis-(2,6-di-tert-butylphenol) (Probucol) was kindly supplied by the Daiichi Pharmaceutical Co (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KA). Monoclonal antibody against multi-ubiquitinated chains and polyclonal antibody against I-κBα were obtained from MBL (Nagoya, Japan) and ROCKLAND (Gilbertsville, PA), respectively.

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were obtained from a commercial source (Clonetics Corp, Walkevsville, MD) and grown in endothelial cell growth factor containing medium-2 (EGM-2, Clonetics Corp, Walkevsville, MD) with 2% FBS at 37°C in a 5% CO₂ atmosphere. All experiments were completed within 4 passages. After reaching confluency, the medium was replaced by endothelial basement medium (EBM) 2 hours before the addition of the antioxidants. The final concentration
of BO-653 and probucol was 50 µM, except for the concentration-dependent experiments and selected on the basis of the plasma levels in animals and humans (25). Antioxidants were dissolved in dimethylsulfoxide (DMSO, SIGMA, St. Louis, MO), which was diluted with EBM resulting in a final DMSO concentration of 0.01%. The control cells were cultured in EBM containing 0.01% DMSO in the absence of antioxidant.

**Gene Chip™ analysis.** Oligonucleotide microarray analysis was performed by using gene chip, the HuGene human FL array (Affymetrix Inc., Santa Clara, CA) as described (18).

**Northern blot analysis (RNA Blot Hybridization).** Total RNA was extracted by the method of Chomczynski et al. (26). Samples of 5 µg of total RNA were denatured and separated by electrophoresis on a 1% agarose gel containing 2.2% formaldehyde. Total RNA was then transferred to Hybond-XL nylon membrane (Amersham pharmacia biotech, Buckinghamshire, UK) and hybridized with 32P-labeled probes as described previously (27). For the Northern blot analysis of proteasomes, the cDNAs for the subunits PMSA1 (HC2), PMSA2 (HC3), PMSA3 (HC8) and PMSA4 (HC9) of human proteasomes were constructed according to the literature (28) and the cDNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a standard. These probes were labeled with a random primer labeling kit, the BCA BEST™ Labeling kit (Takara, Shizuoka, Japan), using [α-32P] dCTP. The membranes were washed as described previously (29) and autoradiographed with a BAS-1800 (Fuji Photo Film Co., Tokyo, Japan). To measure the stability of proteasome subunit mRNA, cells were
treated with Actinomycin D (5 µg / ml), an inhibitor of transcription of mRNA at the same time as antioxidant treatment as described (30).

**Western blot analysis.** Cells were harvested and pelleted at 1000 x g for 3 min. Cells were washed with PBS and disrupted by sonication in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM dithiothreitol, 0.25 M sucrose and 0.5 mM phenylmethyl sulfonoyl fluoride. The homogenates were centrifuged at 20,000 x g for 30 min at 4°C, and the resulting supernatants were used for analysis. Samples (5 µg) separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 10 % gel were transferred electrophoretically to Hybond™ ECL™ nylon membranes (Amersham pharmacia biotech, Buckinghamshire, UK) for 2 hours at 80 V. The membranes were pretreated with Block Ace (Yukijirushi Co., Sapporo, Japan) and then treated with monoclonal antibodies which were raised against human PMSA1 (HC2), PMSB7 (Z), PMSC6 (p42), PMSD1 (p112) and PMSE2 (PA28β) (28). After treatment with peroxidase-conjugated goat anti-mouse IgG monoclonal antibody (SIGMA, St. Louis, MO) or anti-rabbit IgG monoclonal antibody (Amersham pharmacia biotech, Buckinghamshire, UK), labeled bands from washed blots were detected by Super Signal West Dura Extended Duration Substrate (Pierce, Rockford, IL). Membranes were exposed to FUJI MEDICAL X-RAY FILM (Fuji Photo Film Co., Tokyo, Japan) at room temperature. To detect the multi-ubiquitinated proteins and I-κBα, after treatment of cells with 100 µg/ml cycloheximide dissolved in PBS for 2 hours the cells were treated with either 50 µM BO-653 or probucol for 12 hours. Control cells were treated with vehicle (DMSO 0.01%). After electrophoresis under the same conditions to detect
the proteasome subunits, the membrane was treated with monoclonal antibody against the multi-ubiquitinated proteins or polyclonal antibody against I-κBα. Protein concentrations were measured with a BCA protein assay kit (Pierce, Rockford, IL).

Results

Phenolic Antioxidant-dependent modulation of the level of mRNAs encoding proteasomal α-type subunits: Gene chip analysis revealed a decrease in the levels of mRNAs encoding proteasome α-type subunits, PMSA2(HC3), PMSA3(HC8) and PMSA4(HC9) in HUVEC exposed to the phenolic antioxidants, probucol (50 µM) and BO-653 (50 µM) for 6 hours (Table1). These concentrations are equivalent to those achieved in both human and animal studies (14,25). The gene chip analysis can result in false positives, and thus the responses were carefully validated and extended in the first series of experiment using Northern blot analysis. It is clear that treatment of HUVEC with either BO-653 or probucol results in a decreased expression of these three genes at 6 hours (Fig. 2 A, B and C). The gene chip used in the present study contains only a subset of the proteasome subunits (Table 1). To test for changes in the mRNA for other α-type subunits not included on this analysis the message for PMSA1 (HC2) was determined. In this case, the mRNA level decreased to its minimum over 3-6 hours on exposure to BO-653 or probucol and recovered by 12 hours (Fig. 2 D and E). To test for the possibility that the antioxidants may enhance degradation of mRNA, we investigated the effect of BO-653 and probucol on mRNA levels observed in cells treated with Actinomysin D, an inhibitor of transcription. neither BO-653 nor probucol affected the
rate of mRNA degradation as shown for PMSA2 (Fig. 2 F). The decreased mRNA for PMSA1 on exposure to the antioxidants was also found to result in decreased protein levels of PMSA1 after a 12 hour exposure, with levels making a partial recovery to control values at 24 hours (Fig. 3). To determine the concentration dependence of the antioxidants, mRNA levels and protein levels of PMSA1 in cells treated with different concentrations (1, 10, 50 µM) of either BO-653 or probucol were measured. These antioxidants inhibited both levels of message and protein of PMSA1 in a dose-dependent manner with maximal effects achieved with the 1µM BO-653 and 50 µM probucol (Table 2).

**Specificity of regulation of proteasome subunits by BO-653 and probucol:** From the data obtained from the gene analysis (Table 1) it was evident that only the α-type proteasome subunits were down-regulated significantly in HUVEC treated with BO-653 or probucol. To further assess the specificity of this response, we investigated changes in the β-type subunits (PMSB1 (HC5) and PMSB7 (Z)) in response to BO-653 and probucol by means of Northern and Western blotting. Neither subunit exhibited significant changes. The data of mRNA and protein levels for PMSB7 are shown after exposure to either BO-653 or probucol for 6 hours and 12 hours, respectively (Fig. 4 A and B). In addition, the effects of BO-653 and probucol on protein levels of PMSC6 (ATP-ase subunit of 19S), PMSD1 (non-ATP-ase subunit of 19S), and PMSE2 (PA28) were determined and found to be unchanged by treatment of cells with these antioxidants over a period of 12 hours (Fig. 4 C, D and E). These results are consistent with those of the gene chip experiment (Table 1). These data support the hypothesis that
both BO-653 and probucol show selectivity in modulating the expression of the $\alpha$-type subunits.

**Accumulation of the proteasome target multi-ubiquitinated proteins and I-\(\kappa\)B\(\alpha\) in HUVEC treated with antioxidants:** These data suggest that BO-653 and probucol have a functional impact on proteasome activity. We investigated this further by determining the accumulation of multi-ubiquitinated proteins and I-\(\kappa\)B\(\alpha\), well-known target proteins of the proteasome. HUVECs were pre-incubated with the protein synthesis inhibitor cycloheximide for 2 hours, followed by additional incubation with or without either BO-653 or probucol to determine if the stability of ubiquitinated proteins and I-\(\kappa\)B\(\alpha\) was increased. Incubation of HUVEC with cycloheximide alone significantly decreased levels of both multi-ubiquitinated proteins and I-\(\kappa\)B\(\alpha\) compared to non-treated cells (Fig. 5, lane 2 vs lane 1). This decrease is thought to be due to degradation of these proteins in the absence of new protein synthesis. In contrast, co-incubation of the cells with either BO-653 or probucol in the presence of cycloheximide maintained the levels of multi-ubiquitinated proteins and I-\(\kappa\)B\(\alpha\) at control levels. (Fig. 5, lane 3 and 4 vs lane 1). This increase is consistent with inhibition of degradation of these proteins by these antioxidants, but cannot be due to increased protein synthesis.

**Discussion**

Taken together these data provide a novel and interesting perspective on the biological properties of phenolic lipid peroxyl radical scavengers. The two compounds selected for study have both been shown to be anti-atherogenic in animal models, yet differ
markedly in radical scavenging ability, with probucol being about 10 times less effective than BO-653 (4,14,16). The unforeseen property revealed by genomic analysis was an apparent modulatory effect on the transcriptional regulation of subunits of the proteasome. Such an effect would be particularly significant in atherosclerosis, since nuclear factor κB (NF-κB) expression is increased in human atherosclerotic lesions (31), and is thought to make a significant contribution to the development of the inflammatory process. The ubiquitin-proteasome pathway degrades the inhibitory binding protein, I-κBα, which ordinarily keeps NF-κB sequestered in the cytoplasm. The degradation of I-κBα by proteasomes allows nuclear translocation of NF-κB in cells treated with various external stimuli including tumor necrosis factor (TNF)-α. Inhibition of I-κBα degradation by a proteasome inhibitor blocks NF-κB activation. Accordingly, the expression of the endothelial cell adhesion molecules (ECAMs) such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin induced by TNF-α (32,33) or interleukin 1-β (IL-1 β) (34) was suppressed. It is generally accepted that these adhesion molecules play important roles in the development of atherosclerotic lesions (1,2). The induction of VCAM-1 and ICAM-1 by oxidized LDL has been reported (11). Progesterone is known to inhibit the expression of VCAM-1 in the aortic wall of rabbits (9,10) and in HUVEC (11,12). Our findings provide the key elements for a molecular mechanism that contributes to the inhibition of VCAM-1 expression and contributes to the anti-atherosclerotic properties of phenolic antioxidants such as probucol and BO-653.

With respect to the implications for the role of the proteasome in endothelial cell...
responses to atherosclerosis these data are intriguing. Thus far most reports have focussed on the biological importance of the 26S proteasome that selectively degrades a multitude of ubiquitinated cellular proteins. In contrast, little is known of the direct role of the 20S proteasome in regulating cellular processes. However, it was recently reported that interactions with the 20S proteasome was a critical determinant in p21WAF1/CIP1 turnover (35). The inhibitory effect of probucol and BO-653 on 20S proteasome activity demonstrated herein is the first example of a pharmacological agent mediating its effects through this mechanism.

The mechanisms by which these phenolic compounds regulate gene expression of proteasome subunits are not clear. Several possibilities exist with perhaps the most likely involving the effects on the cell signaling mediated by low level reactive oxygen and nitrogen species that are now known to control transcriptional events through activation of proteins such as the MAP kinases. If so then the effects of phenolic compounds may have some interesting implications for the role of low levels of lipid mediators acting as intermediaries in these cell signaling cascades. Interesting examples include 4-hydroxynonenal (36) or acrolein as we have recently demonstrated in mouse embryo fibroblast cells (37). Other possibilities include antioxidant independent mechanism in which these compounds may act as direct modulators of transcription factors or ligands for nuclear receptors. Current investigations are directed at analysis for the promotor for the proteasome subunits which should reveal deeper insights into these interesting responses.
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Foot note

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Abbreviations: BO-653, 2,3-Dihydro-5-hydroxy-2,2-dipentyl-4,6-di-tert-butylbenzofuran; DMSO, dimethylsulfoxide; EBM, Endothelial basement medium; ECAMs, endothelial cell adhesion molecules; EGM-2, Endothelial cell growth factor containing medium-2; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; I-κBα, inhibitory binding protein κBα; IL-1β, interleukin-1β; NF-κB, nuclear factor κB; PBS, phosphate buffered saline; probucol, 4,4’-isopropylidenedithio-bis-(2,6-di-tert-butylphenol); SDS, sodium dodecyl sulfate; VCAM-1, vascular cell adhesion molecule-1; TNF-α, tumor necrosis factor-α
**Figure legends**

Fig. 1 Chemical structure of (A) BO-653 and (B) probucol

Fig. 2 Decreased expression of mRNA encoding proteasome α-type subunits by phenolic antioxidants. Northern blot analysis was performed for the mRNA of (A) PMSA2 (HC3), (B) PMSA3 (HC8) and (C) PMSA4 (HC9) prepared from HUVEC treated with DMSO (D), 50 µM BO-653 (B) and 50 µM probucol (P) for 6 hours. The changes in mRNA levels of PMSA1 (HC2) in HUVEC treated with either (D) 50 µM BO-653 or (e) 50 µM probucol were followed for up to 12 hours. (F) After HUVECs were treated with actinomycin D (5 µg / ml) in the absence (open mark) or presence of either BO-653 (closed circle) or probucol (closed triangle), mRNA of PMSA2 was analyzed by Northern blotting. All data obtained were normalized for GAPDH values and shown as the mean +/- S.D. (n=4) of the ratio against DMSO-treated control obtained from densitometric quantitation of subunit band.

Fig. 3 Changes in protein levels of proteasome α-type subunit, PMSA1 (HC2). Western blot analysis of PMSA1 (HC2) was performed for the protein obtained from HUVEC treated with (A) 50 µM BO-653 or (B) 50 µM probucol for up to 24 hours. The numbers show the mean +/- S.D. (n=4) of the ratio against time 0 obtained from densitometric quantitation of subunit band.
Fig. 4 Effect of antioxidants on the proteasome β-type subunit, ATP-ase subunit of 19S, non-ATP-ase subunit of 19S, and PA28. Northern blot analysis for PMSB7 (Z, β-type subunit) was performed for total RNA extracted from HUVEC treated with DMSO (D), 50 µM BO-653 (B) or 50 µM probucol (P) for 6 hours. Western blot analysis for PMSB7 (Z), PMSC6 (p42, ATP-ase subunit of 19S), PMSD1 (p112, non-ATP-ase subunit of 19S), and PMSE2 (PA28β, PA28) was performed. Proteins (5 µg) were obtained from HUVECs treated with the same concentration of antioxidants for 12 hours. The experiments were repeated 4 times and showed the same results.

Fig. 5 Accumulation of multi-ubiquitinated proteins and I-κBα induced by antioxidants in cycloheximide pretreated HUVEC. HUVECs were preincubated without (lane 1) and with 100 µg/ml cycloheximide for 2 hours, followed by co-incubation with DMSO (lane 2), 50 µM BO-653 (lane 3) or 50 µM probucol (lane 4) for 12 hours. Samples (5 µg of proteins) of the crude extract were used for Western blot analysis with a corresponding monoclonal antibody against multi-ubiquitinated proteins and I-κBα. The numbers show the mean +/- S.D. (n=4) of the ratio against non-treated (lane 1) obtained from densitometric quantitation of subunit.
Table 1 Effect of BO-653 and probucol on proteasome subunits studied by DNA microarray

| Subunits of proteasome complexes | Gene No. | Name               | average difference | foldchange (ranking No.) |
|----------------------------------|----------|--------------------|--------------------|--------------------------|
|                                  |          |        | DMSO    | BO-653 | probucol | BO-653 | probucol |
| α-type subunits                  | D00762   | PSMA3 (HC8)**     | 5573               | 1411   | 2492     | -4.5 (1) * | -2.2 (4) * |
|                                  | D00763   | PSMA4 (HC9)**     | 7425               | 1973   | 3619     | -3.8 (2) * | -2.1 (5) * |
|                                  | D00760   | PSMA2 (HC3)**     | 6568               | 1792   | 3396     | -3.2 (4) * | -1.7 (17) * |
| β-type subunits                  | D00761   | PMSB1 (HC5)**     | 9996               | 5378   | 7274     | -1.9      | -1.4      |
|                                  | D26599   | PMSB2 (HC7)       | 8136               | 7316   | 8006     | -1.0      | -1.0      |
|                                  | D26598   | PMSB3 (HC10)      | 7296               | 7729   | 7136     | -1.0      | -1.0      |
|                                  | D26600   | PMSB4             | 5907               | 6246   | 6215     | -1.0      | -1.0      |
|                                  | D29012   | PMSB6 (Y)         | 16463              | 9310   | 12064    | -1.8      | -1.4      |
|                                  | D38048   | PMSB7 (Z) **      | 9586               | 6365   | 8009     | -1.5      | -1.2      |
|                                  | Z14982   | PMSB8             | -159               | 115    | -192     | --------  | --------  |
| ATP ase subunits of 19S          | D78275   | PMSC6**           | 1242               | 568    | 920      | -1.9      | -1.4      |
| non-ATP ase subunits of 19S      | D44466   | PMSD1**           | 4252               | 2080   | 2140     | -1.9      | -1.6      |
|                                  | D78151   | PMSD2             | 5774               | 7430   | 6900     | 1.2       | 1.2       |
|                                  | D50063   | PMSD7             | 4346               | 2826   | 3554     | -1.5      | -1.2      |
|                                  | D38047   | PMSD8             | 12384              | 9133   | 10604    | -1.4      | -1.2      |
|                                  | AB003177 | PMSD9             | 2259               | 1637   | 1715     | -1.4      | -1.3      |
|                                  | AB003102 | PMSD11            | 1611               | 1740   | 1587     | 1.1       | -1.0      |
|                                  | AB003103 | PMSD12            | 263                | 202    | 244      | -1.3      | -1.1      |
| PA28 (11S regulator)             | D45248   | PMSE2**           | 4309               | 3055   | 3200     | -1.4      | -1.3      |

*The fold change was calculated as previously described (18) and ranking No. was given to significantly down-regulated genes.

**The effects of BO-653 and probucol were investigated by Northern and/or Western blot analysis.
Table 2 The concentration dependency of antioxidants on mRNA and protein levels of PMSA1

|        | BO-653     | probucol  |
|--------|------------|-----------|
|        | mRNA levels | protein levels |
| 1µM    | 0.62+/-0.09 | 0.80+/-0.04 |
| 10µM   | 0.62+/-0.10 | 0.68+/-0.07 |
| 50µM   | 0.69+/-0.11 | 0.51+/-0.14 |
| probucol | 1µM 0.67+/-0.07 0.70+/-0.14 | 0.56+/-0.08 0.47+/-0.13 |
| probucol | 50µM 0.29+/-0.04 0.43+/-0.09 |

The numbers (mean +/- SD ) show a ratio against control (DMSO-treatment).
Fig. 1

A

B
Fig. 3

A

BO-653

0 1 3 6 12 24 hr

B

probucol

0 1 3 6 12 24 hr

Ratio of protein level

PMSA1

Time [hr]

- BO-653
- probucol

Fig. 3
Fig. 5

A  multi-Ubiquitinated protein

B  I-κBα

Ratio of protein level

1  2  3  4

Ratio of protein level

1  2  3  4
Anti-atherogenic antioxidants regulate the expression and function of proteasome α-type subunits in human endothelial cells
Wakako Takabe, Tatsuhiko Kodama, Takao Hamakubo, Keiji Tanaka, Toshiaki Suzuki, Hiroyuki Aburatani, Naeko Matsukawa and Noriko Noguchi

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