in vivo *32*: 255-264 (2018)
doi:10.21873/invivo.11232

Anti-inflammatory Activity of β-Carotene, Lycopene and Tri-*n*-butylborane, a Scavenger of Reactive Oxygen Species

AKIFUMI KAWATA, YUKIO MURAKAMI, SEIJI SUZUKI and SEIICHIRO FUJISAWA

Division of Oral Diagnosis and General Dentistry, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Japan

Abstract. *Background/Aim: The polyene carotenoids β-carotene and lycopene are antioxidants that not only quench singlet oxygen but also inhibit lipid peroxidation. Tri-n-butyl borane (TBB) is used as an initiator for dental resin materials and is extremely reactive with oxygen and reactive oxygen species (ROS). This reactionability of TBB may be analogous to that of carotenoids with ROS. To clarify the biological activity of such ROS scavengers, we investigated the anti-inflammatory activity of β-carotene, lycopene and TBB in terms of the expression of RNA for lipopolysaccharide (LPS)-induced cyclooxygenase-2 (Cox2), nitric oxide synthase 2 (Nos2) and tumor necrosis factor-alpha (Tnfa), and mRNA expression and up-regulation of heme oxygenase 1 (Hmox1) mRNA in RAW264.7 cells. Materials and Methods: mRNA expression was investigated using real-time reverse transcriptase-polymerase chain reaction (PCR). The antioxidant activity of carotenoids was evaluated using the induction period method in the azobisisobutyronitrile or benzoyl peroxide–methyl methacrylate system. Results: Hmox1 mRNA, but not Cox2 and Nos2 mRNA, was up-regulated by 100 μM β-carotene and lycopene, and by 0.125% TBB. LPS-stimulated Cox2, Nos2 and Tnfa gene expression was inhibited by 50 μM β-carotene and lycopene, and by 0.5-1% TBB. Both β-carotene and lycopene had weak antioxidant activity, but β-carotene showed pro-oxidant activity at higher concentrations. Conclusion: The anti-inflammatory activity of β-carotene, lycopene and TBB may be related to their ROS-scavenging activity. Additionally, the activity of carotenoids and TBB may be attributed to the electrophilicity of ROS-induced carotenoid intermediates and boranes, respectively. Their anti-inflammatory activity may be attributable to enhancement of the potency of the electrophile/antioxidant response element transcription system in view of their up-regulation of Hmox1 mRNA expression.*

Correspondence to: Dr. Yukio Murakami, Division of Oral Diagnosis and General Dentistry, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, 1-1 Keyakidai, Sakado City, Saitama 350-0283, Japan. Tel: +81 492855511, Fax: +81 492876657, e-mail: ymura@dent.meikai.ac.jp

Key Words: β-Carotene, lycopene, tri-*n*-butyl borane, LPS, RAW264.7 cells, COX2, NOS2, HMOX1.

It has been shown that β-carotene (Figure 1) acts as an immune modulator, quenching singlet oxygen, and scavenging free radicals at lower partial oxygen pressures than the pressure of oxygen in normal air (1). This compound can also be enzymatically cleaved by lipoxygenases, and thus prevent radical-oxidation and photo-oxidation. Compounds related to β-carotene provide photoprotection to photosynthetic organisms, and to the eye and the skin, and their intake may be linked to prevention of cancer, heart diseases, and age-related macular degeneration (2).

Burton and Ingold reported the antioxidant activity of β-carotene: this polyene compound specifically exhibits good radical-trapping antioxidant behavior only at partial pressures of oxygen significantly less than 150 torr (3). Such low oxygen partial pressures are found in most tissues under physiological conditions. At higher oxygen pressures, β-carotene loses its antioxidant activity and has an autocatalytic, pro-oxidant effect, particularly at relatively high concentrations. Moreover, the authors have suggested that the ability of β-carotene to react with or scavenge reactive oxygen species (ROS) is analogous to that of organobolones, such as tri-*n*-butyl borane {TBB: $[CH_3(CH_2)_3]_3B$} (3). TBB is used as an initiator for dental resin materials (4-6) and is extremely reactive with oxygen (O-O, bi-radical) in air. Oxygen transforms TBB into butoxydibutylborane (R_2BOR, alkoxyalkylborane), a borinic ester or borate, which in turn is oxidized in the presence of air (7, 8). As TBB auto-oxidation requires oxygen, TBB acts like an ROS scavenger.

Dietary carotenoids have been reported to have beneficial effects in preventing several types of cancer and cardiovascular disease, as well as photosensitivity disorders and eye-related disorders (9). However, a major

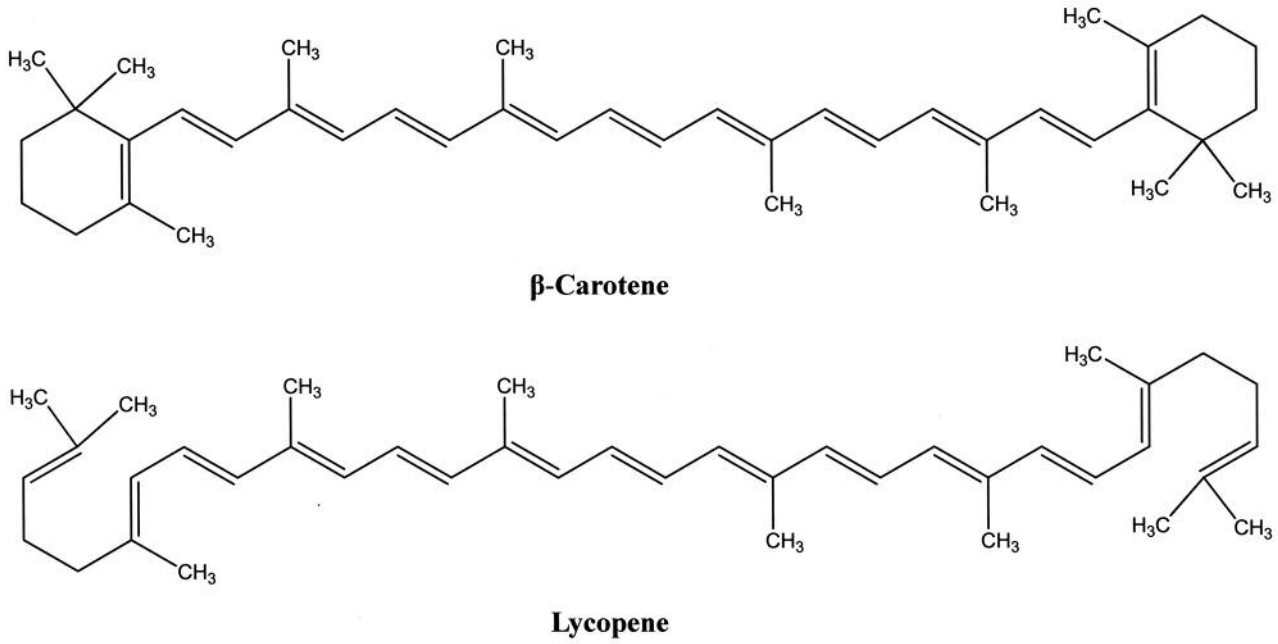

Figure 1. *The chemical structures of β-carotene and lycopene.*

chemoprevention trial, the β-carotene and Retinol Efficacy Trial (CARET) Study [Alpha-Tocopherol β-carotene Cancer (ATBC) Prevention group] previously found no health benefit, since β-carotene acts as a promotor of pre-existing latent lung cancer in smokers, possibly through the formation of β-carotene metabolites that may be responsible for the carcinogenic response in this context (10). The anti-inflammatory and pro-inflammatory activities of carotinoids remain to be clarified. Moreover, as far as we are aware, no studies have investigated the anti-inflammatory activity of organoboranes such as TBB.

Macrophages are important components of the mammalian immune system, playing a key role in immediate defense against foreign agents prior to leukocyte migration and production of various pro-inflammatory mediators such as cyclooxygenase-2 (COX2) and nitric oxide synthase 2 (NOS2) protein, the short-lived free radical nitric oxide (NO), and cytokines such as tumor necrosis factor-alpha (TNFA) (11). Lipopolysaccharide (LPS), a component of the cell walls of gram-negative bacteria, acts as a powerful activator of macrophages through the production of pro-inflammatory cytokines (12). Therefore, inhibition of COX2, NOS2 and TNFA protein or gene expression in LPS-stimulated RAW264.7 cells may be one way to screen various anti-inflammatory antioxidant drugs (13).

Macrophages are resistant to relatively high concentrations of heme in comparison to endothelial cells, and have constitutively high expression of heme oxygenase 1 (HMOX1) to protect against heme toxicity (14, 15). As an antioxidant stress protein, HMOX1 is expressed in almost all cells and tissues, and is highly up-regulated by heme or other stress stimuli to provide protection against oxidative damage and apoptosis (16). This is a protective and adaptive response inherent to most tissues (14). The cytoprotective effects of HMOX1 have been attributed to its enzymatic action in heme degradation and also its catalytic byproducts such as carbon monoxide and biliverdin (17). HMOX1 has anti-inflammatory, antioxidant, and antiproliferative effects (18). Because of its promising cytoprotective features, many researchers have pursued the targeting of Hmox1 as an attractive cellular pathway for drug discovery (19).

In the present study, we investigated whether β-carotene, lycopene, and TBB inhibit the expression of mRNAs for *Nos2, Cox2* and *Tnfa* in LPS-stimulated RAW264.7 cells. We also investigated the stimulating effects of these compounds on *Hmox1* mRNA expression in this cell line. Furthermore, we evaluated the antioxidant activity of β-carotene and lycopene according to the induction period (IP) and propagation rate constant (Rp) values based on previous raw data obtained by differential scanning calorimetry (DSC) monitoring of the polymerization of methyl methacrylate (MMA) initiated by thermal decomposition of azo*bis*isobutyronitrile (AIBN) and benzoyl peroxide (BPO) under nearly anaerobic conditions (20). The inhibition rate constant (k_{inh}) of β-carotene and lycopene is also discussed.

Materials and Methods

Materials. β-Carotene and lycopene were obtained from Wako Pure Chemical Industries (Osaka, Japan). TBB was obtained from Sun Medical Co. Ltd. (Moriyama, Japan). The structures of β-carotene and lycopene are shown in Figure 1.

Cell culture. The murine macrophage-like cell line RAW264.7, obtained from Dainippon Sumitomo Pharma Biomedical Co. Ltd. (Osaka, Japan), was used. The cells were cultured to a subconfluent state in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37˚C and 5% CO_2 in air, washed, and then incubated overnight in serum-free RPMI-1640. They were then washed again and treated with the test samples.

Preparation of total RNA and real-time polymerase chain reaction (PCR). The preparation of total RNA and the procedure for real-time PCR have been described previously (21). In brief, RAW264.7 cells in NUNC 96-flat-well-type microculture plates (10^5 cells per well) were treated with the test samples as previously shown by us (21). Total RNA was isolated using an RNeasy Plus Micro Kit (Qiagen Japan Co. Ltd., Tokyo, Japan) in accordance with the instruction manual. cDNA was synthesized from total RNA (2 μg) of each sample by random priming using a High Capacity RNA-to-cDNA Kit (Life Technologies Japan, Tokyo, Japan). Reaction mixtures without the reverse transcriptase were used as a negative control. An aliquot of each cDNA synthesis reaction mixture was diluted and used for real-time PCR quantification. An equal-volume aliquot of each cDNA was mixed, serially diluted, and used as a standard. TaqMan probes/primers for *Cox2, Nos2, Hmox1* and *18s* rRNA and the PCR enzyme mix for real-time PCR were purchased from Life Technologies Japan. Real-time PCR quantification was performed in triplicate using the GeneAmp Sequence Detection System 5700 software (Life Technologies Japan) in accordance with the instruction manual. The relative amount of target was calculated from standard curves generated in each PCR, and quantitative data with a coefficient of variance of less than 10% were used for further analyses. Each calculated amount of mRNA was standardized by reference to that for *18s* rRNA. Data are expressed as means of three independent experiments. Statistical analyses were performed using Student's *t*-test and one-way ANOVA.

Antioxidant activity. The experiments to determine the antioxidant activity of carotenoids were previously performed using a DSC (20, 22). Briefly, about 10 μl (MMA: 9.12-9.96 g) of experimental resin with 1.0 mol% AIBN or BPO and 0-0.5 mol% carotenoid was loaded into an aluminum sample container and sealed by applying pressure. The container was then placed in a DSC (model DSC 3100; Mac Science Co., Tokyo, Japan) kept at 70˚C, and the thermal changes induced by polymerization were recorded for the appropriate periods. The heat due to the polymerization of MMA was 13.0 kcal mol^{-1}. The conversion of all samples (%) was calculated from the DSC thermograms using the integrated heat evoked by MMA polymerization.

Measurement of the stoichiometric factor (n) and k_{inh}. The *n* value can be calculated from the IP in the presence of inhibitors (antioxidants) as:

$$n=R_i[IP]/[IH] \quad \text{(eq.1)}$$

where IP is the induction period in the presence of a carotenoid inhibitor (IH). The number of moles of BPO or AIBN radicals trapped by the antioxidant was calculated with respect to 1 mole of the inhibitor moiety. The initiation rate (R_i) for AIBN and BPO at 70˚C was 5.56×10^{-6} mol $l^{-1}s^{-1}$ and 2.28×10^{-6} mol $l^{-1}s^{-1}$, respectively, calculated on the basis of n=2.0 for 2,6-di-*tert*-butyl-4-methoxyphenol (20, 22, 23).

The k_{inh} is given by: $k_{inh}/k_p=([MMA]/[IP] \times [Rp])$ (eq.2)

where k_p is the propagation rate constant, and MMA, IP and the rate of propagation (Rp) are defined above. A k_p value of 930 1 $mol^{-1}s^{-1}$ was used in this work (23).

Results

Cox2, Nos2, TNFalpha, and Hmox1 mRNA expression. Firstly, we examined the expression of mRNAs for *Cox2* and *Nos2* in RAW264.7 cells after treatment with β-carotene or lycopene within the concentration range 0.1-1000 μM. β-Carotene and lycopene did not elicit any expression of *Cox2* and *Nos2* mRNA, similar to the situation in the control (data not shown). Similarly, TBB within the concentration range 0.06-1% had no influence on the expression of *Cox2* and *Nos2* mRNA (data not shown).

Next, we investigated the effects of β-carotene, lycopene and TBB on expression of *Hmox1* mRNA. The results are shown in Figs. 2 and 3, respectively. β-Carotene and lycopene up-regulated the expression of *Hmox1* mRNA at 100 μM (Figure 2). TBB also up-regulated the expression of *Hmox1* mRNA at 0.125% (Figure 3). Furthermore, we investigated the effects of β-carotene, lycopene and TBB on LPS-stimulated *Cox2, Nos2* and *Tnfa* mRNA expression in RAW264.7 cells. β-Carotene and lycopene suppressed *Cox2* and *Nos2* mRNA expression at 50 μM. β-Carotene also suppressed *Tnfa* mRNA expression at 50 μM, whereas lycopene had no effect at the same concentration (Figure 4). Interestingly, β-carotene elicited overexpression of *Cox2* mRNA at a low concentration of 0.1 μM but further studies to confirm this will be necessary. TBB suppressed LPS-stimulated *Cox2* and *Nos2* mRNA expression considerably within a concentration range of 0.5-1% (Figure 5).

Antioxidant activity. We evaluated the antioxidant activity of β-carotene and lycopene based on the previous DSC data (20) in order to clarify their essential features or the mechanism of interaction between dietary carotenoids and the AIBN (cyano-isopropyl, $R^{\bullet}$) and BPO (benzencarboxy, $PhCOO^{\bullet}$) radicals. The results are shown in Table I.

As there was a very small amount of air in the DSC sample container, this was considered responsible for the induction time in the controls, since oxygen in the DSC sample container acts as an inhibitor. The kinetics of carotenoid chemical oxidation suggested that carotenoids are

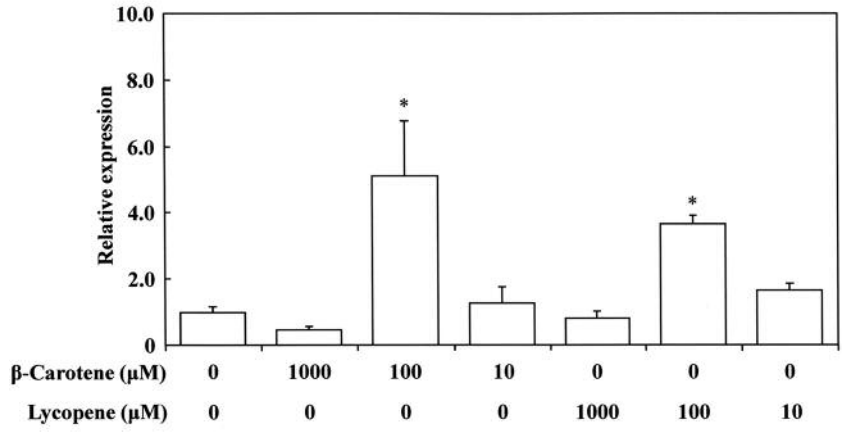

Figure 2. *Stimulation of heme oxygenase 1 (Hmox1) gene expression by β-carotene and lycopene in RAW264.7 cells. The cells were incubated for 3.5 h with the indicated doses of β-carotene or lycopene, and then their total RNAs were prepared. Each cDNA was synthesized, and the expression level of Hmox1 mRNA was determined by real-time polymerase chain reaction and standardized against the expression of 18s rRNA. The results are presented as means±standard error (SE) of three independent experiments, SE<15%. Significant differences between samples for β-carotene and lycopene were observed for stimulation of Hmox1 gene expression. *Significantly different at p<0.01 vs. control group.*

weak inhibitors of MMA polymerization. Within the concentration range 5-10 mM, the IP value of β-carotene for both the AIBN and BPO systems increased gradually but was markedly decreased at 20 mM. In contrast, that of lycopene increased gradually but reproducibly up to 20 mM. The IP value for β-carotene in the BPO system within a concentration range of 5-10 mM was greater than that of lycopene. The *n* value for β-carotene was about 0.1. In contrast, the IP value for lycopene in the AIBN system was greater than that for β-carotene. The *n* value for β-carotene and lycopene was within the range 0.2-0.3. The antioxidant activity of carotenoids was dependent on the radical species. As β-carotene was sensitive to $PhCOO^{\bullet}$ radicals, it was thought to be more sensitive to ROS than lycopene. β-Carotene also showed dual antioxidant and pro-oxidant properties, whereas lycopene showed antioxidant activity alone, as judged from the IP value. However, for both the AIBN and BPO systems, β-carotene and lycopene at 50 mM suppressed the exothermic DSC peak completely. For the BPO system, the Rp value for β-carotene at 20 mM was about 40% lower than the corresponding value for lycopene, indicating that BPO-mediated β-carotene suppressed more effectively the increase of poly-MMA radicals than lycopene at the same concentration. It was assumed from the small Rp value that β-carotene was a more effective suppressor of unsaturated phospholipid peroxidation in biological systems. The antioxidant activity of carotenoids should be evaluated by the k_{inh} value. In the AIBN system, the k_{inh} values for β-carotene and lycopene were 1.6-5.5×10^7 and 1.0-1.3×10^7 l $mol^{-1}s^{-1}$, respectively. In contrast, the corresponding values in the BPO system were 4.2×10^7-4.7×10^8 l $mol^{-1}s^{-1}$ and 2.4-8.0×10^7 l $mol^{-1}s^{-1}$, respectively. The k_{inh} value for β-carotene was greater than that for lycopene. In the present study, antioxidant kinetics were examined under nearly anaerobic conditions, since carotenoids exhibit radical-trapping antioxidant behavior only at a partial oxygen pressure significantly lower than 150 torr (3). The present result demonstrated that β-carotene exerted pro-oxidant activity at a high concentration of 20 mM in both the AIBN and BPO systems.

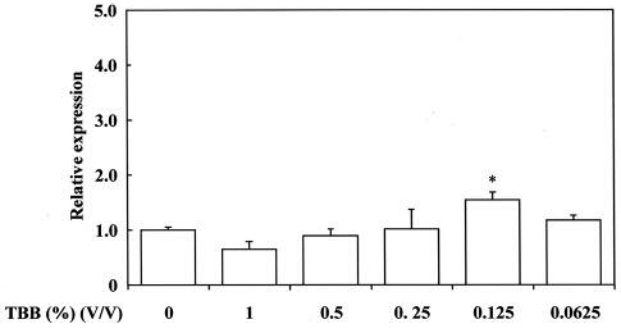

Figure 3. *Stimulation of heme oxygenase 1 (Hmox1) gene expression by tri-n-butyl borane (TBB) in RAW264.7 cells. The cells were incubated for 3.5 h with the indicated doses of TBB, and then their total RNAs were prepared. Each cDNA was synthesized, and the expression level of Hmox1 mRNA was determined by real-time polymerase chain reaction and standardized against the expression of 18s rRNA. The results are presented as means±standard error (SE) of three independent experiments, SE<15%. Significant differences between samples for each TBB were observed for stimulation of Hmox1 gene expression. *Significantly different at p<0.01 vs. control group.*

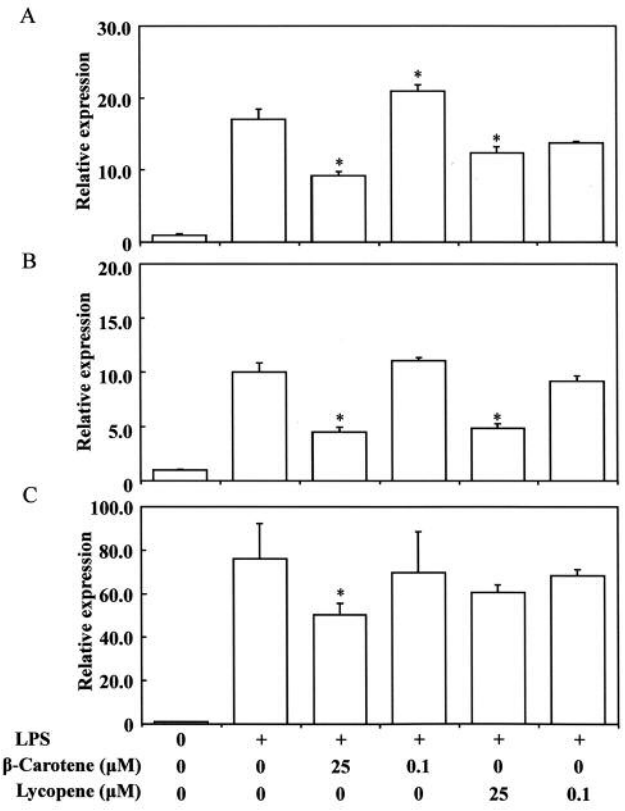

Figure 4. *Inhibitory effects of β-carotene and lycopene on lipopolysaccharide (LPS)-stimulated cyclooxygenase-2 (Cox2) (A), nitric oxide synthase 2 (Nos2) (B) and tumor necrosis factor-alpha (Tnfa) (C) gene expression in RAW264.7 cells. The cells were pretreated for 30 min with the indicated doses of β-carotene or lycopene. They were then incubated for 3 h with or without LPS at 100 ng/ml, and their total RNAs were prepared. Each cDNA was synthesized, and the expression level of Cox2, Nos2 and Tnfa mRNAs were determined by real-time polymerase chain reaction and standardized against the expression of 18s rRNA. The results are presented as means±standard error (SE) of three independent experiments, SE<15%. Significant differences between samples for β-carotene and lycopene were observed for inhibition of Cox2, Nos2 and Tnfa gene expression. *Significantly different at p<0.01 vs. control group.*

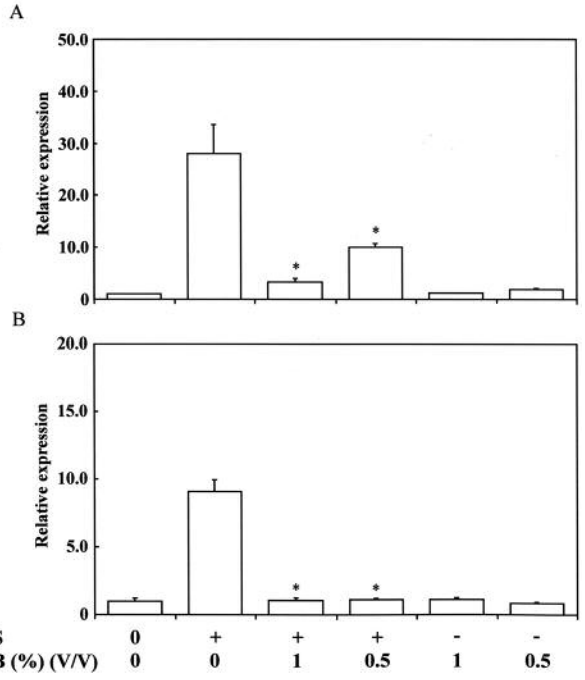

Figure 5. *Inhibitory effects of tri-n-butyl borane (TBB) on lipopolysaccharide (LPS)-stimulated cyclooxygenase-2 (Cox2) (A), nitric oxide synthase 2 (Nos2) (B) gene expression in RAW264.7 cells. The cells were pretreated for 30 min with the indicated doses of TBB. They were then incubated for 3 h with or without LPS at 100 ng/ml, and their total RNAs were prepared. Each cDNA was synthesized, and the expression level of Cox2 and Nos2 mRNAs were determined by real-time polymerase chain reaction and standardized against the expression of 18s rRNA. The results are presented as means±standard error (SE) of three independent experiments, SE<15%. Significant differences between samples for each TBB were observed for inhibition of Cox2 and Nos2 gene expression. *Significantly different at p<0.01 vs. control group.*

Discussion

Firstly, we discuss the antioxidant activity of carotenoids. β-Carotene scavenges various ROS, including singlet oxygen ($^1O_2$), the superoxide anion radical ($^{\bullet}O_2^-$), the hydroxy radical ($^{\bullet}OH$), the peroxyl radical ($RCOO^{\bullet}$) and nitric oxide (NO) (24-26). The antioxidant activity of β-carotene as well as lycopene may be attributable to their high number of conjugated dienes, which act as potent ROS quenchers. β-Carotene exhibits good radical-trapping behavior only at a low partial pressure of oxygen (3) and behaves as an interceptor of free radical species regardless of the oxygen pressure in the environment (27). The present results indicate that the k_{inh} value of carotenoids for $PhCOO^{\bullet}$ radicals, 4×10^7-5×10^8 l/mol s, was markedly greater than that of quercetin, *i.e.* approximately 1×10^3 l/mol s (23). The stoichiometric factor, *n* (*i.e.* the number of radicals trapped by each inhibitor molecule), for carotenoids is 0.1-0.3, whereas that for quercetin is 1.8 (23). The antioxidant activity of carotenoids was thought to be much smaller than that of the chain-breaking antioxidant quercetin, which has phenolic OH groups. The radical-scavenging activity of carotenoids, characterized by a small *n* value and a large k_{inh} value, may be attributable to their high number of conjugated dienes (Figure 1), which act as potent singlet oxygen quenchers and ROS scavengers; radicals are trapped by the unsaturated polyene chain of carotenoids (20). β-Carotene also yields reaction products such as epoxide or endoperoxide when it reacts with $^{\bullet}O_2^-$ and $^{\bullet}OH$ (24, 28). Epoxides of lycopene have

Table I. *Antioxidant activity of β-carotene and lycopene determined using the induction period method. The values were determined based on the DSC data reported previously (20) using differential scanning calorimetry (DSC) monitoring of the polymerization of methyl methacrylate (MMA) (9.4 mol) initiated by thermal decomposition of azobisisobutyronitrile (AIBN) or benzoyl peroxide (BPO) under nearly anaerobic conditions at 70˚C. The procedure is described in the text. Conversion, 84.9-96.9%. The values are the means of two or three independent experiments. Standard errors were <15%.*

Carotenoid	Con. mM	IP min	Max. DSC peak mcal/min	Time at max. peak min	Rp %/min
AIBN system					
β-Carotene	0	3.5	205.4	43.4	1.4
	5	6.7	46.1	57.9	1.1
	10	8.4	19.7	84.7	0.7
	20	6.7	9.4	170.0	0.3
	50	-	np	-	-
Lycopene	0	3.0	196.6	41.8	1.3
	5	8.1	49.4	55.8	1.0
	10	10.0	22.2	85.9	0.6
	20	16.5	8.4	88.0	0.3
	50	-	np	-	-
BPO system					
β-Carotene	0	8.3	199.8	61.8	0.8
	5	10.2	131.1	74.9	0.7
	10	11.5	101.2	91.3	0.5
	20	8.6	43.2	202.4	0.2
	50	-	np	-	-
Lycopene	0	7.8	192.4	58.7	0.7
	5	8.5	148.3	68.5	0.7
	10	9.0	115.1	82.5	0.5
	20	13.3	52.6	149.1	0.3
	50	-	np	-	-

IP: Induction period; Rp: propagation rate constant; np, no DSC peak for 440 min.

been found after photochemical and chemical reactions with ROS (29); the initiator radicals induce newly formed β-carotene radical products, which in turn undergo further transformation leading to a variety of secondary β-carotene derivatives. These compounds may no longer act as antioxidants, and in fact may become harmful products (9). These findings could explain the intriguing pro-oxidant and cytotoxic activity of β-carotene (26). On the other hand, we previously investigated the radical-scavenging activity of carotenoids against the eugenol phenoxyl ($PhO^{\bullet}$) radical in buffered solution at pH 9.5 (100 mM eugenol) using electron spin resonance (ESR) spectroscopy. This revealed that β-carotene efficiently and dose-dependently scavenged the radicals completely at 3 mM, new radical peaks then becoming slightly but reproducibly evident at concentrations over 10 mM. This suggested the formation of β-carotene radicals *via* eugenol $PhO^{\bullet}$ radical scavenging by β-carotene and subsequently the regeneration of eugenol. In contrast, lycopene only slightly scavenges eugenol radicals; the radical-scavenging activity of lycopene is much smaller than that of β-carotene (30). Interestingly, the regeneration of β-carotene by eugenol from the β-carotene radical cation, an initial bleaching product of β-carotene, has been demonstrated by laser flash photolysis and transient absorption spectroscopy. Eugenol is a potential protector of β-carotene for use in red palm oil (31).

The pro-oxidant action of carotenoids in biological systems seems to form part of cellular reduction-oxidation (redox) reactions in which they can act as either antioxidants (electron donors) or pro-oxidants (electron acceptors), depending on the physiological environment and general oxidative state. The difference in antioxidant properties between β-carotene and lycopene may be related to the chemical structure of carotenoids (Figure 1). β-Carotene, also known as pro-vitamin A, has nine conjugated double bonds forming a polyene chain plus two double bonds of a β-ring at both ends of the molecule, although this is not coplanar with the polyene chain. In contrast, lycopene has an extended chromophore with thirteen conjugated double bonds without beta-rings. The difference in their structures may affect their antioxidant activity and adduct formation with ROS. Adducts are formed by reaction of β-carotene with alkoxy and alkylperoxy free radicals through thermolysis of azo-initiators that are utilized in industrial processes such as benzene polymer synthesis (32). The products of the reaction between β-carotene and ROS, such as epoxides and endoperoxides, may be produced more preferentially than is the case for lycopene (24). Our data for the pro-oxidant activity of 20 mM β-carotene with both the AIBN and BPO radicals were strongly suggestive of the molecular difference between β-carotene and lycopene.

Next, we discuss the anti-inflammatory activity of carotenoids and TBB in terms of *Cox2, Nos2, Tnfa* and *Hmox1* gene expression. ROS have many interactions with the nuclear factor κB (Nf-κB) signaling pathway. The transcription of Nf-κB-dependent genes influences the cellular level of ROS, which in turn regulates the level of Nf-κB activity. ROS can both activate and inhibit Nf-κB signaling (33). Nf-κB is a transcription factor implicated in both inflammation and immune activation. It is well known that signal transduction in the Nf-κB transcription factor pathway is inhibited by NOS2 activity. Stimuli that enhance NOS2 and NO formation also may induce COX2 expression.

A high level of ROS induced by treatment with LPS modulates a number of cell signaling pathways, thus regulating the expression of multiple genes such as those for COX2 and NOS2 *in vitro* and *in vivo* (34). In general, inflammatory activity is accompanied by inducible nitric oxide synthase (iNOS), leading to production of nitric oxide,

which enhances the catalytic activity of COX2 *via* formation of the peroxinitrite anion (35). COX2 is a downstream target of NOS2. The present study showed that the concentration of carotenoids required for 50% inhibition of LPS-induced *Cox2* and *Nos2* mRNA expression in RAW264.7 cells was approximately 25 μM. β-Carotene appeared to exert stronger anti-inflammatory activity than lycopene. Lycopene inhibits the LPS-induced pro-inflammatory mediator-inducible nitric oxide system in mouse macrophage cells (36). Carotenoids such as astaxanthin inhibit the protein levels of iNOS and COX2 in LPS-stimulated microglial cells (37). Studies of the inhibitory effects of major dietary antioxidants such as β-carotene and quercetin on LPS-induced Cox2 and iNOS expression in RAW264.7 macrophages have also indicated that quercetin exerts the most potent activity at 5 μM (38). Our previous study demonstrated that the 50% inhibitory concentration of quercetin for induction of LPS-induced *Cox2* gene expression was 10 μM (39). This result, together with that obtained in the present study, suggests that quercetin possesses much more potent anti-inflammatory activity than β-carotene, possibly as a result of the former's higher antioxidant activity. As shown in Figure 4, 0.1 μM β-carotene slightly enhanced the LPS-induced overexpression of *Cox2* in RAW264.7 cells. This may have been due to its pro-oxidant activity elicited by ROS-mediated β-carotene intermediates. In contrast, lycopene at 0.1 μM had no such effect. ROS generated by RAW264.7 cells in response to LPS treatment may attack the conjugated double bonds in β-carotene at relatively low concentrations, thus leading to the formation of β-carotene radicals, *i.e.* unsaturated polyene chain-trapped radicals and their intermediates. Intermediates formed by β-carotene oxidation might exert strong, potentially harmful, oxidizing effects. Other processes might also be triggered, such as formation of adducts between β-carotene and $NO^{\bullet}$, $RSO_2^{\bullet}$ and various $ROO^{\bullet}$ radicals. In another context, carotenoids are polyenes that lack an electrophilic moiety. However, when carotenoids are oxidized and their intermediates are consequently formed, they may acquire electrophilicity. Carotenoid intermediates may have the capacity to up-regulate the nuclear factor erythroid 2-related factor 2 (NRF2)/antioxidant response element (ARE) pathway (40). Lycopene upregulates *NRF2*, resulting in a two-fold increase of nicotinamide adenine dinucleotide phospho (NAD(P)H) quinone oxidoreductase 1 (*NQO1*) mRNA and a nearly 2.5-fold increase in germ cell-less (*GCL*) mRNA, corresponding to a two-fold increase in NQO1 protein and a 1.5-fold increase in GCL (40, 41).

Our results have demonstrated that β-carotene and lycopene up-regulate the expression of the *Hmox1* gene, even though the β-carotene and lycopene molecules lack any electrophilic groups (Figure 1). Therefore, the potent *Hmox1* gene expression elicited by these polyene compounds may be attributable to the formation of electrophilic intermediates of carotenoid oxidation. Such intermediates may enhance the potency of the electrophile (EpRE)/ARE transcription system, and induce phase II enzymes and NRF2-targeting enzymes (superoxide dismutase, catalase, glutathione peroxidase, HMOX1) (40). In general, the antioxidant behavior of carotenoids can protect against the peroxidation of phospholipids as well as proteins or nucleic acids (mRNA, tRNA and DNA), but in some cases at relatively high concentrations and relatively high partial oxygen pressures, oxidation of carotenoids has an autocatalytic, pro-oxidant effect (3). Accordingly, some of their unwanted effects *in vivo* might be caused by free radical-mediated carotenoid intermediates with pro-oxidant properties, which may attack lipids, proteins or nucleic acids (mRNA, tRNA, DNA) in the vicinity due to the lipophilicity of this compound. In the present study, β-carotene and lycopene exerted potent anti-inflammatory activity by suppressing *Cox2, Nos2*, and *Tnfa* gene expression and also by up-regulating the gene expression of *Hmox1*. Appropriate intake of carotenoids may offer some protection against cancer, heart disease, and age-related macular degeneration (42, 43). Lycopene and β-carotene induce cell-cycle arrest and apoptosis in human breast cancer (43).

In the present study, LPS-induced expression of *Cox2* and *Nos2* mRNA was inhibited by TBB, and TBB stimulation also up-regulated *Hmox1* mRNA expression. TBB exerts anti-inflammatory activity and is extremely reactive with the oxygen O-O biradical and $ROO^{\bullet}$ radical. TBB possesses borane–carbon bonds that are polarized toward carbon, thus rendering the carbon attached to borane nucleophilic (44). However, TBB is electrophilic because boranes alone are generally not sufficiently nucleophilic to transfer an alkyl group to an electrophilic center. However, after nucleophilic attack by oxygen, the resulting borate is highly nucleophilic (45).

The anti-inflammatory activity of TBB in LPS-treated RAW264.7 cells may be attributable to scavenging of highly cytotoxic ROS derived from them. Additionally, electrophilic TBB may have the capacity to upregulate the NRF2/ARE pathway (40) and, consequently, *Hmox1* mRNA expression. In the present study, partially oxidized TBB, TBB-O (a mixture of TBB and TBB-O at a 1:4 weight-ratio), was used because pure TBB ignites spontaneously in air (Sun Medical Co. Ltd., Material Safety Datasheet) (46). The 50% cytotoxicity concentration (LC_{50}) of TBB-O was 0.84 wt% for RAW264.7 cells in the present study, while that for human pulp fibroblasts is approximately 0.5 wt% (6). TBB showed anti-inflammatory activity at concentrations below the LC_{50}. The oral LD_{50} toxicity of TBB (borane) in rats and that of TBB-O (borate) in mice are 1,125 mg/kg and 2,150 mg/kg, respectively (46). The toxicity of borates is half that of borane; the acute toxicity of TBB (Callery Chemical Co., PA, USA) in terms of the intravenous LD_{50} in male Donryu rats is 104 μl/kg (approx.77 mg/kg), whereas the corresponding

oral LD_{50} is 1,125 μl/kg (approx. 833 mg/kg) (47). In the early stages of the polymerization reaction, initiation involves the direct reaction between TBB (borane) and oxygen, but as the reaction proceeds, the unimolecular decomposition of alkylperoxyborane, or its bimolecular reaction with alkylborane, becomes more important in MMA polymerization. Such decomposed TBB radicals attack the beta-carbon of (meth)acrylate monomers (α, β-unsaturated carbonyl compounds) as Michael-reaction acceptors, and polymerization of vinyl monomers is initiated. On the other hand, organoborane derivatives such as amine-carboxyboranes have been reported previously to be effective anti-inflammatory agents in mice. These derivatives at 10^{-6} M are effective inhibitors of hydrolytic lysosomal and proteolytic enzymes in mouse macrophages, human leukocytes, and Be Sal osteofibrolytic cells, and in the same cell lines this compound blocks cyclooxygenase activity with an LD_{50} value of 10^{-6} M (48). Tetrahydroborates have also been shown to reduce inflammation in a rat model of chronic and granulomatous inflammation (49). These findings indicate that TBB derivatives have anti-inflammatory activity. We also previously investigated the cytotoxicity of the TBB-ethylenediamine complex/*p*-toluensulfonyl chloride-MMA resin system in dog tooth pulp, and found that this system has good pulp biocompatibility (50). Similarly, the TBB/glycerin complex/trimethyl barbituric acid-MMA resin system had been used clinically in MMA composite restorative resins, indicating that such resins have good pulp biocompatibility (4). These findings, together with those of the present study, suggest that the good biocompatibility of TBB resin systems might be attributable to the anti-inflammatory activity of the TBB catalyst.

In conclusion, β-carotene, lycopene, and TBB showed good anti-inflammatory activity in RAW264.7 cells, possibly as a result of their ROS-scavenging activity. Additionally, the electrophilic properties of intermediates of carotenoid oxidation and TBB might have caused up-regulation of the *Hmox1* gene, since electrophilic compounds induce EpRE/ARE system by disrupting the inhibitory activity of Kelch-like ECH-associated protein 1 (Keap1) on NRF2, the major EpRE/ARE-activating transcription factor (40). Curcumin and quercetin are dietary and phytochemical compounds that are known to up-regulate *Hmox1* (51, 52). These compounds show high electrophilicity, as determined by density function theory calculations (53). Although they are potent electrophiles, these dietary and phytochemical antioxidants could be intrinsically affected by oxidation and might be transformed to cytotoxic intermediates/metabolites such as pro-oxidants in some cases. The therapeutic anti-inflammatory potential of carotenoids and TBB, particularly the latter, currently remains unclear. Further studies are needed to clarify the mechanisms involved in the anti-inflammatory and antioxidant activities of β-carotene, lycopene and TBB.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Number 15K11266 from the Ministry of Education, Science, and Culture of Japan. The Authors also thank Y. Kadoma for induction period measurements using DSC.

References

1 Wang XD and Russell RM: Procarcinogenic and anticarcinogenic effects of β-carotene. Nutr Rev *57*: 263-272, 1999.
2 Nita M and Grzybowski A: The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults. Oxid Med Cell Longev. 2016; *2016*: 3164734.
3 Burton GW and Ingold KU: β-Carotene: an unusual type of lipid antioxidant. Science *224*: 569-573, 1984.
4 Fischer CH, Strassburg M and Knolle G: Tests with a new synthetic filling material over a period of three years (Palakav). Int Dent J *20*: 679-689, 1970.
5 Nakagawa K, Saita M, Ikeda T, Hirota M, Park W, Lee MC and Ogawa T: Biocompatibility of 4-META/MMA-TBB resin used as a dental luting agent. J Prosthet Dent *114*: 114-121, 2015.
6 Fujisawa S and Atsumi T: Cytotoxicity of a 4-META,MMA-TBBO resin against human pulp fibroblasts. Dent Mater J *22*: 106-108, 2004.
7 Mirviss SB: Mechanism of the oxidation of trialkylboranes. J Org Chem *32*: 1713-1717, 1967
8 Fujisawa S and Kadoma Y: Tri-*n*-butylborane/Water complex-mediated copolymerization of methyl methacrylate with proteinaceous materials and proteins: Review. Polymers *2*: 575-595, 2010.
9 Fiedor J and Burda K: Potential role of carotenoids as antioxidants in human health and disease. Nutrients *6*: 466-488, 2014.
10 Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL Jr, Valanis B, Williams JH Jr., Barnhart S, Cherniack MG, Brodkin CA and Hammar S: Risk factors for lung cancer and for intervention effects in CARET, the β-carotene and retinol efficacy trial. J Natl Cancer Inst *88*: 1550-1559, 1996.
11 Moncada S, Palmer RM and Higgs EA: Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev *43*: 109-142, 1991.
12 Nicholas C, Batra S, Vargo MA, Voss OH, Gavrilin MA, Wewers MD, Guttridge DC, Grotewold E and Doseff AI: Apigenin blocks lipopolysaccharide-induced lethality *in vivo* and proinflammatory cytokines expression by inactivating NF-κB through the suppression of p65 phosphorylation. J Immunol. *179*: 7121-7127, 2007.
13 Fujisawa S and Murakami Y: Eugenol and its role in chronic diseases. Adv Exp Med Biol *929*: 45-66, 2016.
14 Biswas C, Shah N, Muthu M, La P, Fernando AP, Sengupta S, Yang G and Dennery PA: Nuclear heme oxygenase-1 (Hmox1) modulates subcellular distribution and activation of Nrf2, impacting metabolic and anti-oxidant defenses. J Biol Chem *289*: 26882-26894, 2014.
15 Immenschuh S, Baumgart-Vogt E, Tan M, Iwahara S, Ramadori G and Fahimi HD: Differential cellular and subcellular localization of heme-binding protein 23/peroxiredoxin I and heme oxygenase-1 in rat liver. J Histochem Cytochem *51*: 1621-1631, 2003.

16 Ryter SW, Alam J and Choi AM: Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol Rev *86*: 583-650. 2006.
17 Agarwal A and Bolisetty S: Adaptive responses to tissue injury: role of heme oxygenase-1. Trans Am Clin Climatol Assoc *124*: 111-122, 2013.
18 Salvemini D, Kim SF and Mollace V: Reciprocal regulation of the nitric oxide and cyclooxygenase pathway in pathophysiology: relevance and clinical implications. Am J Physiol Regul Integr Comp Physiol *304*: R473-487, 2013.
19 Motterlini R and Foresti R: Heme oxygenase-1 as a target for drug discovery. Antioxid Redox Signal *20*: 1810-1826, 2014.
20 Fujisawa S, Ishihara M and Kadoma Y: Kinetics of the radical scavenging activity of β-carotene-related compounds. SAR QSAR Environ Res *15*: 33-41, 2004.
21 Murakami Y, Kawata A, Seki Y, Koh T, Yuhara K, Maruyama T, Machino M, Ito S, Kadoma Y and Fujisawa S: Comparative inhibitory effects of magnolol, honokiol, eugenol and *bis*-eugenol on cyclo-oxygenase-2 expression and nuclear factor-kappa B activation in RAW264.7 macrophage-like cells stimulated with fimbriae of *Porphyromonas gingivalis*. In Vivo *26*: 941-950. 2012.
22 Kadoma Y, Ishihara M, Okada N and Fujisawa S: Free radical interaction between vitamin E (alpha-, beta-, gamma- and delta-tocopherol), ascorbate and flavonoids. In Vivo *20*: 823-827, 2006.
23 Kadoma Y and Fujisawa S: Radical-scavenging activity of dietary phytophenols in combination with co-antioxidants using the induction period method. Molecules *16*: 10457-10470. 2011.
24 Nishino A, Yasui H and Maoka T: Reaction and Scavenging mechanism of β-carotene and zeaxanthin with reactive oxygen species. J Oleo Sci *66*: 77-84, 2017.
25 Trevithick-Sutton CC, Foote CS, Collins M and Trevithick JR: The retinal carotenoids zeaxanthin and lutein scavenge superoxide and hydroxyl radicals: a chemiluminescence and ESR study. Mol Vis *12*: 1127-1135, 2006.
27 Fiedor J, Fiedor L, Haessner R and Scheer H: Cyclic endoperoxides of β-carotene, potential pro-oxidants, as products of chemical quenching of singlet oxygen. Biochim Biophys Acta *1709*: 1-4, 2005.
27 Ozhogina OA and Kasaikina OT: β-Carotene as an interceptor of free radicals. Free Radic Biol Med *19*: 575-581, 1995.
28 Shimidzu N, Goto M and Miki W: Carotenoids as singlet oxygen quenchers in marine organisms. Fish Sci *62*: 134-137, 1996.
29 Rodriguez EB and Rodriguez-Amaya DB: Lycopene epoxides and apo-lycopenals formed by chemical reactions and autoxidation in model systems and processed foods. J Food Sci *74*: C674-682, 2009.
30 Satoh K, Ida Y, Sakagami H, Tanaka T and Fujisawa S: Effect of antioxidants on radical intensity and cytotoxic activity of eugenol. Anticancer Res *18*: 1549-1552, 1998.
31 Chang HT, Cheng H, Han RM, Wang P, Zhang JP and Skibsted LH: Regeneration of β-carotene from radical cation by eugenol, isoeugenol, and clove oil in the Marcus theory inverted region for electron transfer. J Agric Food Chem *65*: 908-912, 2017.
32 Liebler DC and McClure TD: Antioxidant reactions of β-carotene: identification of carotenoid-radical adducts. Chem Res Toxicol *9*: 8-11 1996.
33 Morgan MJ and Liu ZG: Crosstalk of reactive oxygen species and NF-κB signaling. Cell Res *21*: 103-115, 2011.
34 Zhang J, Wang X, Vikash V, Ye Q, Wu D, Liu Y and Dong W: ROS and ROS-Mediated Cellular Signaling. Oxid Med Cell Longev *2016*: 4350965, 2016.
35 Salvemini D, Kim SF and Mollace V: Reciprocal regulation of the nitric oxide and cyclooxygenase pathway in pathophysiology: relevance and clinical implications. Am J Physiol Regul Integr Comp Physiol *304*: R473-487, 2013.
36 Mohamed R, Pineda M and Aguilar M: Antioxidant capacity of extracts from wild and crop plants of the Mediterranean region. J Food Sci *72*: S59-63, 2007.
37 Choi SK, Park YS, Choi DK and Chang HI: Effects of astaxanthin on the production of NO and the expression of COX2 and iNOS in LPS-stimulated BV2 microglial cells. J Microbiol Biotechnol *18*: 1990-1996, 2008.
38 Jung WJ and Sung MK: Effects of major dietary antioxidants on inflammatory markers of RAW 264.7 macrophages. Biofactors *21*: 113-117, 2004.
39 Murakami Y, Kawata A, Ito S, Katayama T and Fujisawa S: Radical-scavenging and anti-inflammatory activity of quercetin and related compounds and their combinations against RAW264.7 cells stimulated with *Porphyromonas gingivalis* fimbriae. relationships between anti-inflammatory activity and quantum chemical parameters. In Vivo *29*: 701-710. 2015.
40 Linnewiel K, Ernst H, Caris-Veyrat C, Ben-Dor A, Kampf A, Salman H, Danilenko M, Levy J and Sharoni Y. Structure–activity relationship of carotenoid derivatives in activation of the electrophile/antioxidant response element transcription system. Free Radic Biol Med *47*: 659-667, 2009.
41 Ben-Dor A, Steiner M, Gheber L, Danilenko M, Dubi N, Linnewiel K, Zick A, Sharoni Y and Levy J: Carotenoids activate the antioxidant response element transcription system. Mol Cancer Ther *4*: 177-186, 2005.
42 Gammone MA, Riccioni G and D'Orazio N: Carotenoids: Potential allies of cardiovascular health? Food Nutr Res *59*: 26762, 2015.
43 Gloria NF, Soares N, Brand C, Oliveira FL, Borojevic R and Teodoro AJ: Lycopene and β-carotene induce cell-cycle arrest and apoptosis in human breast cancer cell lines. Anticancer Res *34*: 1377-1386, 2014.
44 Allred AL and Rochow EG: A scale of electronegativity based on electrostatic force. J Inorg Nucl Chem *5*: 264-268, 1958.
45 Negishi E: Organoboron and organoaluminum compounds as unique nucleophiles in organic synthesis. in New applications of organometallics reagents in organic synthesis, Seyferth D, Ed., Elsevier, Amsterdam, pp. 93-125, 1976.
46 Sun Medical Co. Ltd. Material Safety Datasheet, http://www.sunmedical.co.jp/english/product/resincement/superbond-c&b/msds/pdf/m_spbond-c&b_01.pdf. 29th June 2017.
47 Takagi M, Hisata K, Baba M and Masuhara E: Toxicity of tri-*n*-butyl borane-initiated MMA-TBB resin system. Acute toxicity. Jpn J Oral Maxillofac Sur *22*: 49533-49549, 1973.
48 Hall IH, Burnham BS, Chen SY, Sood A, Spielvogel BF and Morse KW: The anti-inflammatory activity of boron derivatives in rodents. Met Based Drugs *2*: 1-12, 1995.
49 Ameen HNM, Hussain SA, Ahmed ZA and Aziz TA: Anti-inflammatory effects of boron alone or as adjuvant with dexamethasone in animal models of chronic and granulomatous inflammation. Int J Basic Clin Pharmacol *4*: 701-707, 2015.

50 Fujisawa S, Imai Y and Masuhara E: Studies on dental self-curing resin (XI). Charactarization of the various complexes of tri-*n*-butylborane as an initiator. Rept Inst Eng Tokyo Med Dent Univ *3*: 64-71, 1969.

51 Son Y, Lee JH, Cheong YK, Chung HT and Pae HO: Antidiabetic potential of the heme oxygenase-1 inducer curcumin analogues. Biomed Res Int *2013*: 918039, 2013.

52 Shen Y, Ward NC, Hodgson JM, Puddey IB, Wang Y, Zhang D, Maghzal GJ, Stocker R and Croft KD: Dietary quercetin attenuates oxidant-induced endothelial dysfunction and atherosclerosis in apolipoprotein E knockout mice fed a high-fat diet: a critical role for heme oxygenase-1. Free Radic Biol Med *65*: 908-915, 2013.

53 Murakami Y, Ishii H, Takada N, Tanaka S, Machino M, Ito S and Fujisawa S: Comparative anti-inflammatory activities of curcumin and tetrahydrocurcumin based on the phenolic O-H bond dissociation enthalpy, ionization potential and quantum chemical descriptor. Anticancer Res *28*: 699-707, 2008.

Received November 21, 2017
Revised December 16, 2017
Accepted December 20, 2017