JPP Journal of Pharmacy and Pharmacology

JPP 2002, 54: 383–389

Received June 11, 2001
Accepted October 30, 2001
ISSN 0022-3573

1-*O*-Hexyl-2,3,5-trimethylhydroquinone inhibits IκB phosphorylation and degradation-linked inducible nitric oxide synthase expression: beyond antioxidant function

Wei Liu*, Masataka Itoigawa, Tokutaro Miki, Hiroshi Nishikawa, Satoru Sugiyama and Naohisa Ishikawa

Abstract

Inducible nitric oxide (NO) production in macrophages plays an important role in atherosclerosis, the protective effects of vitamin E and its derivatives perhaps being partly mediated by alteration in this parameter. We have investigated the influence of a novel synthesized vitamin E derivative, 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ), on NO production in the RAW 264.7 mouse macrophage cell line. HTHQ dose-dependently inhibited lipopolysaccharide (LPS)-induced NO production through reducing LPS-triggered inducible nitric oxide synthase (iNOS) expression. The phosphorylation and subsequent degradation of IκB caused by LPS in RAW 264.7 cells was markedly blocked. The free radical scavenging activity of HTHQ was only 2-fold that of vitamin E, whereas its inhibition of NO production was found to be nearly 500-fold stronger. Our results indicated that HTHQ suppressed NO production in macrophages by blocking IκB degradation and thus inhibiting iNOS expression. The inhibitory activity of HTHQ on NO production did not parallel its free radical scavenging activity, implying a possible involvement of additional functions.

Department of Pharmacology, Aichi Medical University School of Medicine, Nagakute, Aichi 480-1195, Japan

Wei Liu, Masataka Itoigawa, Satoru Sugiyama, Naohisa Ishikawa

Nippon Hypox Laboratories, Inc., 9420 Nanbu, Nanbu-cho, Minamikoma-gun, Yamanashi 409-2212, Japan

Tokutaro Miki, Hiroshi Nishikawa

Correspondence: N. Ishikawa, Department of Pharmacology, Aichi Medical University School of Medicine, Nagakute, Aichi 480-1195, Japan. E-mail: nao@aichi-med-u.ac.jp

Present address: *W. Liu, Cell Biology and Metabolism Branch, The National Institute of Child Health & Human Development, NIH, Bethesda, MD 20892-5430, USA.

Introduction

Vitamin E, a well-known natural lipophilic chain-breaking antioxidant (Tappel 1980), and certain derivatives have been used widely to protect against experimental atherogenesis. Activities such as inhibition of oxidized low-density lipoprotein (ox-LDL) uptake (Iuliano et al 2000; Ricciarelli et al 2000), prevention of monocyte adhesion (Islam et al 1998) and decrease of endothelial apoptosis (Li et al 1999) have been considered to contribute to the anti-atherogenic properties of vitamin E. Recently it was reported that vitamin E and its derivatives exerted potent inhibitory effects on nitric oxide (NO) induction in macrophages (Hassoun et al 1995) and vascular smooth muscle cells (Hattori et al 1995), suggesting a possible involvement of NO suppression in the mechanism of vitamin E-mediated protection.

NO, a potent messenger molecule produced by nitric oxide synthase (NOS), mediates a wide range of biological processes involved in neurotransmission, antimicrobial defense, and vascular homeostasis. Inducible NOS (iNOS), expressed mainly in activated macrophages, is transcriptionally controlled following general or local inflammatory responses. Its production is considered to be important during atherosclerosis, because iNOS has been detected in human atherosclerotic

plaques, co-localizing with 3-nitrotyrosine in macrophages (Buttery et al 1996). A main regulator of iNOS gene expression is the transcription factor nuclear factor kappaB (NF-κB), whose activation requires ubiquitin-dependent degradation of the corresponding inhibitory protein IκB, that retains inactive NF-κB in the cytosol. Accumulating evidence suggests a redox regulation of NF-κB (Piette et al 1997; Hirota et al 1999), and several vitamin E derivatives have been reported to inhibit the activation of NF-κB through their antioxidative actions (Suzuki & Packer 1993).

In this study, we have examined the effects of 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ), a novel synthesized vitamin E derivative with a shorter side-chain which lacks the pyrane-ring, on the production of NO and expression of iNOS in RAW 264.7 cells. The results suggested that HTHQ suppressed iNOS expression in macrophages through inhibition of IκB phosphorylation and degradation, involving a mechanism not entirely dependent on free radical scavenging activity.

Materials and Methods

Cell culture

RAW 264.7 mouse macrophage cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated foetal bovine serum, 100 U mL^{-1} penicillin, and 100 $\mu g\ mL^{-1}$ streptomycin. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. The cells were washed twice with phosphate-buffered saline (PBS) before use, and the culture medium was replaced by RPMI-1640 with no serum. Maintaining cells in serum-free medium for 24 h had no affect on cell viability.

Reagents

HTHQ was synthesized in Nippon Hypox Lab. Inc. (Yamanashi, Japan) and vitamin E was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Lipopolysaccharide (LPS; *Escherichia coli*, Serotype 0127:B5) was obtained from Sigma (St Louis, MO). HTHQ and vitamin E were dissolved in ethanol and added to the culture medium. Control experiments demonstrated that treatment with the same concentrations of ethanol alone had no effect on LPS-induced iNOS expression and IκB phosphorylation and degradation. Rabbit polyclonal anti-iNOS antibodies, goat anti-rabbit IgG, and rabbit anti-actin antibodies were purchased from Transduction Laboratories (Lexington, KY), Tago (Burlingame, CA), and Sigma (St Louis, MO), respectively. Rabbit polyclonal anti-phospho-IκBα(Ser32) and anti-IκBα antibodies were purchased from New England Biolabs (Beverly, MA).

Free radical scavenging capacity of HTHQ and vitamin E

The free radical scavenging capacities of HTHQ and vitamin E were measured with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as described by Blois (1958). Briefly, 30 μL HTHQ or vitamin E, dissolved in ethanol, were mixed with 3 mL 0.1 mM DPPH solution. After incubation at room temperature for 30 min, absorbance at 520 nm was measured with a spectrophotometer (JASCO, Japan). Percentage radical scavenging of HTHQ or vitamin E was calculated when the absorbance obtained in the absence of agents was 100%. Concerning the effects of HTHQ and vitamin E on free radical scavenging capacity, the EC50 values were estimated by probit-transformation technique.

Determination of NO synthesis

The amount of NO formed was estimated from the accumulation of stable nitrite metabolites in the medium using an NO_2/NO_3 assay kit (2,3-diaminonaphthalene Kit, Dojindo, Kumamoto, Japan). Briefly, RAW 264.7 cells were treated at 37°C for 24 h, the culture medium was centrifuged at 200 *g* for 15 min, and then the supernatant was sampled. To an 80-μL sample was added 10 μL 2,3-diaminonaphthalene followed by 15-min incubation, and addition of 10 μL 2.8 M NaOH. Fluorescence was then measured with a fluorescence spectrophotometer (Hitachi, Japan) using an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The amount of nitrite was calculated from a sodium nitrite standard curve. To measure the effects of HTHQ and vitamin E on NO production, the EC50 values were estimated by probit-transformation technique.

Immunoblotting

Western blot analysis was performed essentially as described by Liu et al (1999). Briefly, RAW 264.7 cells were stimulated with 100 $ng\ mL^{-1}$ LPS for various periods up to 2 h. HTHQ or vitamin E was pretreated

for appropriate periods, before adding the LPS solution. LPS-treated or untreated cells were lysed in sample buffer (×2; 62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol and 10% glycerol) and the proteins were separated by electrophoresis in 8% (for iNOS detection) or 12.5% (for IκB and actin detection) SDS polyacrylamide gels. Proteins were then transferred to polyvinylidene difluoride membranes. After blocking with 5% milk, the membranes were reacted with the specific antibody i.e. anti-iNOS antibody (diluted 1:10 000), anti-phospho-IκB antibody (diluted 1:1 000), anti-IκB antibody (diluted 1:1 000) or anti-actin antibody (diluted 1:100). Thereafter the membranes were treated with horseradish peroxidase-conjugated goat anti-rabbit IgG, which was visualized using the Western Blot Chemiluminescence Reagent (DuPont NEN, Boston, MA).

Statistical analysis

Free radical scavenging capacities and inhibitory actions on LPS-induced NO production were shown as mean ± s.d. of negative log EC50 (M) value obtained for HTHQ or vitamin E. Significance of the difference between HTHQ and vitamin E was first evaluated by two-way (drug and concentration) analysis of variance and performed with a statistical analysis system (SAS Institute Inc., Tokyo). When the values were found to be statistically significant, the difference between two means was tested using Scheffe's method. A statistically significant difference was considered at $P < 0.05$.

Results

Free radical scavenging capacity of HTHQ

As a novel synthesized vitamin E derivative, HTHQ lacked the pyrane-ring and had a shorter side-chain compared with vitamin E (Figure 1). In the DPPH test (Figure 2) HTHQ and vitamin E exhibited scavenging activity from a concentration of 3 μM. Incubation with 10 μM HTHQ for 30 min resulted in 56% scavenging, whereas the capacity of vitamin E at the same concentration was 24%. The mean values of negative log EC50 (M) for free radical scavenging effects of HTHQ and vitamin E were -5.02 ± 0.06 and -4.75 ± 0.09 (n = 4, for each), respectively. These results indicated the relative free radical scavenging activity of HTHQ to be slightly stronger than that of vitamin E, but only by two-times and this was not significantly different.

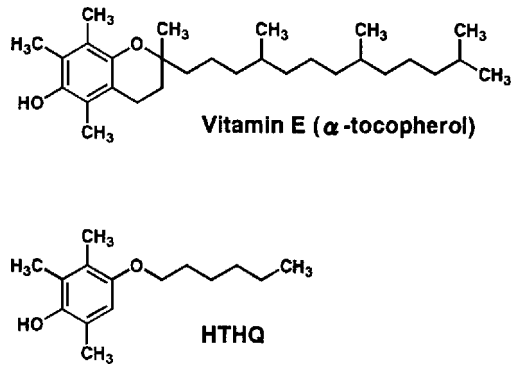

Figure 1 Chemical structures of 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ) and vitamin E.

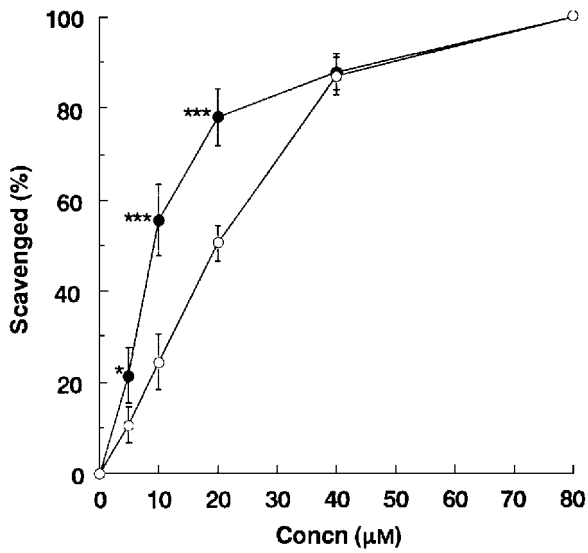

Figure 2 Free radical scavenging capacity of 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ). Various concentrations of HTHQ (●) and vitamin E (○) dissolved in ethanol were mixed with 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution. After 30-min incubation at room temperature, absorbance at 520 nm was measured with a spectrophotometer. Percentage radical scavenging was calculated and the results are presented as means ± s.d. of triplicate measurements. $^{*}P < 0.005$, $^{***}P < 0.001$, compared with the value obtained with the same concentration of vitamin E.

HTHQ inhibits LPS-induced NO production

The effects of HTHQ on induction of NO synthesis in the macrophage cell line RAW 264.7 were assessed fluorometrically, by estimating the amount of nitrite accumulated in the culture medium using an NO_2/NO_3 assay kit with high sensitivity. As shown in Figure 3, stimulation of RAW 264.7 cells with 100 ng mL^{-1} LPS

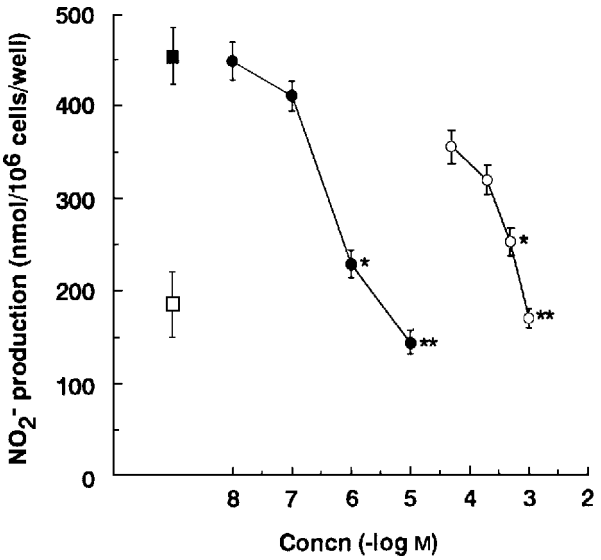

Figure 3 Effects of 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ) on LPS-induced NO production. RAW 264.7 cells were treated for 24 h with 100 ng mL^{-1} lipopolysaccharide (LPS) with or without 1-h pretreatment of the indicated concentrations of HTHQ (●) or vitamin E (○). Nitrite in the culture medium was determined as described in the Methods section. NO_2^- production levels measured without drugs are shown with open and closed squares, for LPS-untreated and -treated groups, respectively. $^*P < 0.05$, $^{**}P < 0.01$, compared with the value obtained in the LPS-treated group without drugs.

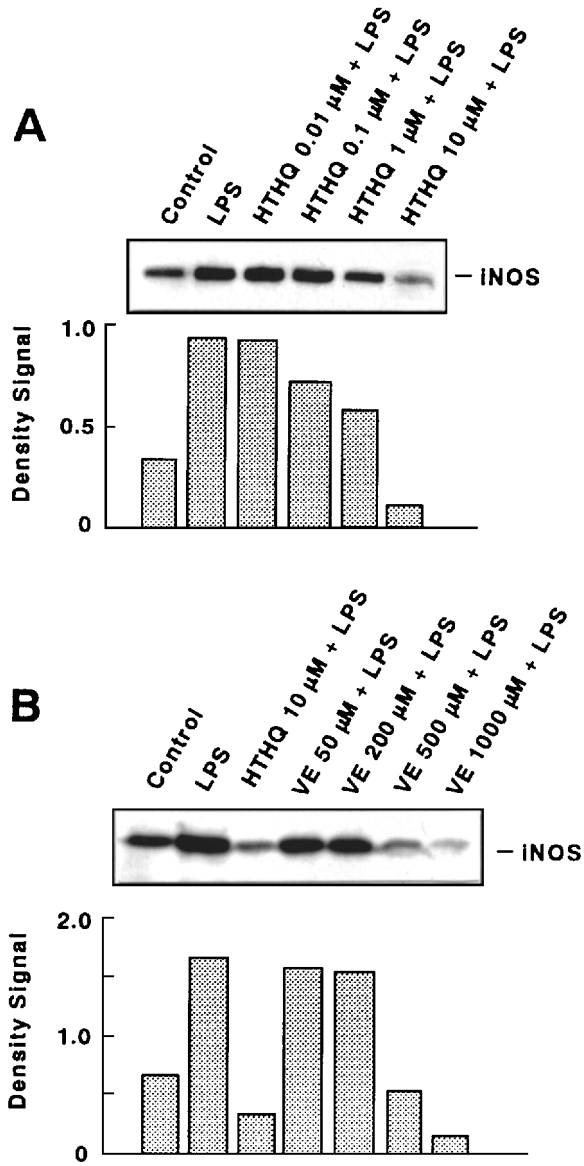

Figure 4 Effects of 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ) on LPS-induced expression of iNOS protein. A and B; RAW 264.7 cells were treated for 24 h with 100 ng mL^{-1} lipopolysaccharide (LPS) with or without 1-h pretreatment of the indicated concentrations of HTHQ or vitamin E(VE). The iNOS protein level was determined by Western blotting using a specific anti-iNOS antibody. Upper panels show a representative picture from three experiments. The lower panels show the density signal for the bands measured by densitometry.

for 24 h led to a clear production of nitrite. Pretreatment of the cells with HTHQ or vitamin E concentration-dependently reduced the LPS-induced NO production. The mean values of negative log EC50 (M) of HTHQ and vitamin E in inhibiting LPS-induced NO-production were -5.97 ± 0.49 and -3.28 ± 0.45 (n = 4, for each), respectively. The relative NO-inhibitory capacity of HTHQ was approximately 500-times greater than that of vitamin E, being significantly different ($P < 0.01$).

HTHQ inhibition of iNOS expression

The effects of HTHQ and vitamin E on iNOS protein expression were assessed in RAW 264.7 cells cultured in serum-free medium and treated with 100 ng mL^{-1} LPS with or without HTHQ or vitamin E pretreatment at the same concentrations used with NO induction. As shown in Figure 4, the results of Western blotting using a specific anti-iNOS antibody indicated that HTHQ and vitamin E attenuated LPS-induced iNOS expression. The inhibitory effects of HTHQ were much stronger, corresponding well with the results shown in Figure 3.

HTHQ blocks phosphorylation and proteolysis of IκB

To investigate the HTHQ-induced inhibition of iNOS expression, we studied the impact on NF-κB activation by examining the levels of IκB phosphorylation and

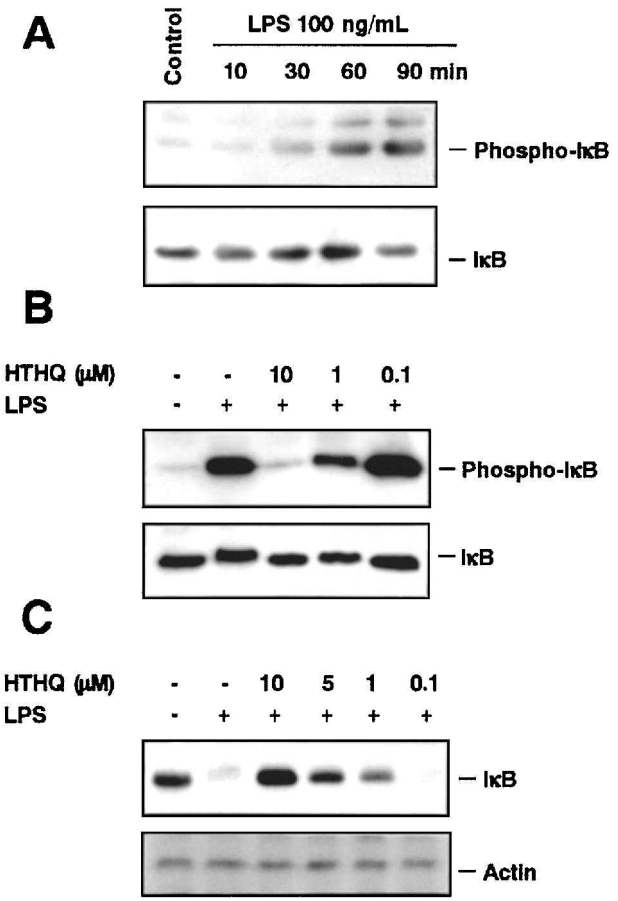

Figure 5 Effects of 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ) on LPS-induced phosphorylation and degradation of IκB. A. RAW 264.7 cells were treated with 100 ng mL^{-1} lipopolysaccharide (LPS) for the indicated time up to 90 min, then IκB phosphorylation or IκB protein levels were determined by Western blotting using specific anti-phospho-IκB or anti-IκB antibodies. B. RAW 264.7 cells were pretreated with the indicated amounts of HTHQ for 30 min, and then were stimulated with 100 ng mL^{-1} LPS for a further 1 h. Levels of phospho-IκB and IκB protein were determined by Western blotting using specific anti-phospho-IκB and anti-IκB antibodies, respectively. C. RAW 264.7 cells were pretreated with the indicated amounts of HTHQ for 30 min, and then were stimulated with 100 ng mL^{-1} LPS for a further 2 h. Levels of IκB and actin proteins were assessed by Western blotting with anti-IκB and polyclonal anti-actin antibodies, respectively.

proteolysis. As shown in Figure 5A, stimulation of RAW 264.7 cells with LPS for less than 90 min led to a time-dependent promotion of IκB phosphorylation as assessed using a phospho-specific anti-IκBα antibody that detected IκB only when phosphorylated at serine 32. Pretreatment of the cells with HTHQ markedly reduced the LPS-induced IκB phosphorylation (Figure 5B). In HTHQ-pretreated cells, LPS-triggered IκB proteolysis was also partially prevented (Figure 5C). These results demonstrated that HTHQ might inhibit iNOS expression by preventing the phosphorylation and subsequent degradation of IκB.

Discussion

We have demonstrated that HTHQ, a novel synthesized vitamin E derivative, exerted significant inhibitory actions on iNOS expression and NO production in macrophages, apparently linked to reduced phosphorylation and degradation of IκB. The lack of correlation between the inhibitory activity on iNOS expression and free radical scavenging activity suggested an involvement of HTHQ actions beyond antioxidation in NO suppression.

Accumulating evidence has indicated that NO is an important regulator in the control of vascular homeostasis. Although the precise actions in atherosclerosis remain to be determined, it is likely that the effects of NO vary greatly depending on its local concentration. Reportedly, high-output NO production with increased iNOS expression in inflammatory cytokine-treated macrophages plays a role in causing endothelial damage (Palmer et al 1992), death of macrophages (Terenzi et al 1995), and other types of tissue injury associated with the pathogenesis of atherosclerosis (Buttery et al 1996). On the other hand, regulated low amounts of NO synthesized constitutively by the endothelial NOS (eNOS) in endothelial cells exert anti-atherogenic effects by inhibiting leucocyte adhesion (Bath et al 1991), platelet aggregation (Radomski & Moncada 1993) and smooth muscle proliferation (Dhaunsi et al 1997). In this context it is of interest that vitamin E was reported to preserve endothelium-derived NO bioactivity (Anderson et al 1994; Keaney et al 1994; Lee & Csallany 1994; Li et al 1999). In this study, we have shown that HTHQ, a novel vitamin E derivative, markedly affected macrophage-derived NO production, providing supporting evidence for an earlier report (Hattori et al 1995) and demonstrating far greater attenuation than vitamin E. These results suggested that the effects on iNOS-mediated NO production might be involved in the reported anti-atherogenic mechanisms with this class of compound.

Results from many studies have suggested redox-dependent regulation of NF-κB activity (Hirota et al 1999; Oka et al 2000). As well known antioxidants, vitamin E and many of its derivatives have been reported to inhibit NF-κB activation (Suzuki et al 1995; Li et al 2000). However, it is likely that the effects of

vitamin E and its derivatives vary, depending on the cell type and the agonists (Liu et al 1995; Nakamura et al 1998). HTHQ, as a new synthesized vitamin E derivative, has been reported to be a potent anti-lipid-peroxidative compound (Nihro et al 1994; Hino et al 1998). The results obtained in this study demonstrated that HTHQ effectively prevented phosphorylation and degradation of IκB, resulting in the inhibition of NF-κB activation, when stimulated with LPS. In addition, this inhibitory effect of HTHQ was much stronger than that of vitamin E and appeared linked to its influence on iNOS expression and subsequent NO production. Considering the potent inhibitory effects of HTHQ on NF-κB, we propose that this novel synthesized derivative may exert actions such as suppression of adhesion molecule expression, and prevention of ox-LDL-induced apoptosis in endothelial cells.

Interestingly, we found that the inhibitory activity of HTHQ on NO production did not parallel its antioxidative potential, the discrepancy being many-fold compared with vitamin E. In fact, although the antioxidant functions of vitamin E are no doubt crucial, there is a growing body of evidence that vitamin E derivatives may exert non-antioxidant effects on various aspects of cell metabolism (Tasinato et al 1995; Grau & Ortiz 1998; Teupser et al 1999). Asmis & Jelk (2000) demonstrated that vitamin E supplementation did not protect macrophages or foam cells from ox-LDL-mediated cell lysis. Furthermore, Suzuki & Packer (1993) reported that in Jurkat T cells, the short chain homologue, 2,2,5,7,8-pentamethyl-6-hydroxychromane, but not α-tocopherol itself, inhibited tumour necrosis factor-induced NF-κB activation. Our results for HTHQ, a homologue of vitamin E with a shorter side chain lacking the pyrane-ring, provided consistent evidence in RAW 264.7 cells, suggesting that the chromanol portion of the vitamin E molecule was important for inhibition of NF-κB activation through action on IκB. Prevention of the phosphorylation and degradation of IκB by HTHQ suggested the possibility that vitamin E was a direct effector of signalling molecules modifying gene expression.

In conclusion, HTHQ dose-dependently inhibited LPS-induced NO production while decreasing iNOS expression in RAW 264.7 cells. Preventing IκB phosphorylation and subsequent IκB degradation, HTHQ negatively regulated the activity of NFκB, a critical transcription factor for iNOS. While it showed only a 2-fold higher antioxidative activity than vitamin E, the inhibitory effects were nearly 500-fold, suggesting involvement of actions beyond simple antioxidant effects.

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