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Inhibition of the expression of inducible nitric oxide synthase and cyclooxygenase-2 in macrophages by 7HQ derivatives: involvement of IκB-α stabilization

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Abstract

Nitric oxide is an important biological mediator associated with multiple pathophysiological phenomena, such as platelet aggregation, vasodilation, septic shock, and autoimmune diseases. Prostaglandins, derived from cyclooxygenases, play prominent roles in homeostasis and inflammation. In this study, we characterized the effects of 7HQ derivatives (7-[(4-methylene-5-oxo-2-*R*-2-tetrahydrofuranyl) methoxy]-3,4-dihydrocarbostyril, where *R* is methyl, phenyl, *p*-fluorophenyl and *p*-phenylphenyl; 7HQ-1,-2,-3 and-4, respectively) in murine RAW 264.7 cells, a macrophage-like cell line. Lipopolysaccharide, the active component of endotoxin, significantly induced the expression of inducible nitric oxide synthase and cyclooxygenase-2, leading to the accumulation of nitrite and prostaglandin E_2, respectively. These actions of lipopolysaccharide were inhibited by 7HQ derivatives; additionally, the inhibition of the expression, rather than the activity, of inducible nitric oxide synthase correlated well with that of nitric oxide formation. Western blotting and electrophoretic mobility shift assay results demonstrated that the 7HQ derivatives could effectively inhibit IκB-α degradation and nuclear factor κB (NF-κB) translocation. At higher concentrations, 7HQ derivatives also inhibited cyclooxygenase-2 enzyme activity. These results suggest that 7HQ derivatives exhibit inhibitory effects on lipopolysaccharide-induced nitric oxide production and expression of inducible nitric oxide synthase and cyclooxygenase-2 through inhibition of IκB-α degradation and NF-κB activation.

Keywords: 7HQ derivative; Nitric oxide (NO) synthase, inducible; Cyclooxygenase-2; Macrophage

1. Introduction

Nitric oxide is a critical mediator of various physiological and pathological processes including vasodilation, neurotransmission and inflammation (Schmidt and Walter, 1994). Nitric oxide is generated by nitric oxide synthase. Unlike the constitutive form, inducible nitric oxide synthase has been implicated in inflammation and other immunological responses (MacMicking et al., 1997). Bacterial endotoxin (e.g. lipopolysaccharide), a component of Gram-negative bacteria, is responsible for triggering the development of septicemia (Mayeux, 1997). It provokes fever, shock, and other manifestations of infection (Beutler and Kruys, 1995). Macrophages produce reactive oxygen intermediates and release various cytokines in response to lipopolysaccharide. These mediators include tumor necrosis factor α, interleukin 1 and interleukin 6, nitric oxide, and arachidonic acid metabolites (Ding et al., 1988; Decker, 1998). Furthermore, the interaction of proinflammatory cytokines with lipopolysaccharide has a synergistic effect, contributing to induction of inducible nitric oxide synthase (Wolkow, 1998).

In the innate immune response, the host immediately secretes cytokines and other mediators to defend it from microbial pathogens. It has been noted that the phylogenetically conserved cellular signaling mechanisms in innate immunity that provide an immediate reaction to utilize the nuclear factor κB (NF-κB) system at the heart of this first-line defense (Hatada et al., 2000). In addition to its well-known role in innate immunity, NF-κB has important

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functions in adaptive immunity (Robert and Kupper, 1999). In response to proinflammatory stimuli, the activation of NF-κB liberates NF-κB heterodimers from cytoplasmic IκB (inhibitor of NF-κB) proteins and results in the passage of NF-κB into the nucleus, where it initiates gene transcription upon binding to its specific sequences on the regulatory segments of genes (Baeuerle, 1998).

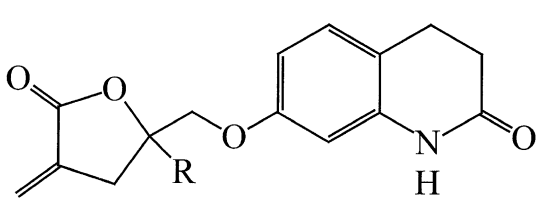
Prostaglandins mediate many physiologic functions to maintain homeostasis. There are two isozymes, cyclooxygenase-1 and cyclooxygenase-2, catalyzing prostaglandins synthesis. Cyclooxygenase-2 is an inducible enzyme and is expressed in response to growth factors or other physiologic and inflammatory stimuli (Simon, 1999). Following stimulation with lipopolysaccharide, macrophages express the cyclooxygenase-2 message (Herschman, 1996). Moreover, cyclooxygenase-2-related metabolites, such as prostaglandin E_2 and prostaglandin I_2, are involved in the induction of inflammation and pain (Bley et al., 1998).

To develop new compounds that are inhibitors of either inducible nitric oxide synthase or cyclooxygenase-2 may require a better understanding of inflammatory states. In the present study, we investigated the effects of 7HQ derivatives on the expression of inducible nitric oxide synthase and cyclooxygenase-2 in RAW 264.7 macrophages stimulated by lipopolysaccharide.

2. Materials and methods

2.1. Materials

7HQ derivatives (7HQ-1, -2, -3 and -4) (Fig. 1) were chemically synthesized by Dr. C.C. Tzeng (School of Chemistry, Kaohsiung Medical University, Kaohsiung, Taiwan) (Tzeng et al., 2000). Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, streptomycin and amphotericin B were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, USA). [α-^{32}P] ATP (3000 Ci/mmol) and the prostaglandin E_2 enzyme immunoassay system were purchased from Amersham International (Buckinghamshire, UK). Rabbit polyclonal antibodies specific for inducible nitric oxide synthase, IκB-α, cyclooxygenase-2 and alkaline phosphatase-coupled anti-rabbit antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipopolysaccharide (from *Escherichia coli* serotype 055: B5), *N*-(1-naphthyl)-ethylenediamine, sulfanilamide, arachidonic acid, 3-[4,5-dimethylthiazol -2-yl]-2,5-diphenyltetrazolium bromide, nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, phenylmethane sulphonyl fluoride, β-mercaptoethanol, dithiothreitol, and dimethylsulfoxide were purchased from Sigma (St. Louis, MO, USA). All materials for SDS-PAGE were obtained from Bio-Rad Laboratories (Hercules, CA, USA).



7HQ-1 R=CH_3
7HQ-2 R=C_6H_5
7HQ-3 R=*p*-C_6H_4-F
7HQ-4 R=*p*-C_6H_4-C_6H_5

Fig. 1. Chemical structures of 7HQ derivatives.

2.2. Cell culture

RAW 264.7 cells, a murine macrophage cell line, were obtained from the ATCC (American Type Culture Collection). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Cells were incubated at 37°C under 5% CO_2 in cell culture flasks. 7HQ derivatives were dissolved in dimethylsulfoxide and further diluted in culture medium. The final dimethylsulfoxide concentration in the cell supernatant was less than 0.1%, which did not interfere with the testing systems.

2.3. Nitrite assay

Cells were grown in 24-well plates and then incubated with or without lipopolysaccharide in the absence or presence of different 7HQ derivatives in various concentrations for 24 h. The nitrite accumulation in the supernatant was assessed by Griess reaction (Green et al., 1982). Each 100 μl of culture supernatant was mixed with an equal volume of Griess reagent (0.1% *N*-(1-naphthyl)-ethylenediamine, 1% sulfanilamide in 5% phophoric acid) and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in an automated microplate reader, and a series of known concentrations of sodium nitrite was used as a standard. In other experiments to measure inducible nitric oxide synthase activity, RAW 264.7 macrophages were stimulated with lipopolysaccharide for 10 h, washed and incubated in fresh medium for a further 14 h in the absence or presence of 7HQ derivatives.

2.4. Western blotting

Cells were plated at a density of 5×10^5 cells/ml in 60-mm tissue culture dishes overnight to allow macrophage adherence. Confluent macrophages were incubated with or without lipopolysaccharide in the absence or presence of different 7HQ derivatives in various concentrations for the desired times. Cells were washed twice with ice-cold phosphate-buffered saline and lysed in a buffer containing 50 mM Tris (pH = 7.4), 150 mM NaCl, 1% Triton X-100,

1 mM EDTA, 1 mM EGTA and 1 mM phenylmethane sulphonyl fluoride. Proteins from lysates were separated on polyacrylamide gels (10% for inducible nitric oxide synthase, 15% for IκB-α and 7.5% for cyclooxygenase-2) and transferred onto polyvinylidene difluoride membranes. After blockade with 5% non-fat skimmed milk for 1 h, the membranes were incubated with polyclonal rabbit antibodies and then with anti-rabbit IgG conjugated to alkaline phosphatase. Finally, the membranes were developed in alkaline phosphatase buffer with 330 μg/ml nitro blue tetrazolium and 167.5 μg/ml 5-bromo-4-chloro-3-indoyl phosphate. Signal intensities were quantified by densitometric analysis.

2.5. Electrophoretic mobility shift assay

After treatment, the cells were suspended in buffer A containing 10 mM HEPES (pH = 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol and 0.2 mM phenylmethane sulphonyl fluoride for 10 min on ice and centrifuged at 2000 rpm for 3 min. The pellet was resuspended in buffer C containing 20 mM HEPES (pH = 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.2 mM phenylmethane sulphonyl fluoride, vortexed vigorously, and allowed to stand on ice for 20 min. The supernatants with soluble nuclear proteins were collected by centrifugation at 14,000 rpm for 5 min and stored at −70°C. For the NF-κB–DNA binding assay, each nuclear extract was mixed with the labeled double-strand NF-κB oligonucleotide, 5′-ATCGAGTTGAGGGGACTTTCCCAGGC-3′ (underline indicates κB consensus sequence) for 30 min. The binding reaction mixtures included 0.25 μg of poly (dI-dC) and 20,000 dpm ^{32}P-labeled DNA probes in binding buffer consisting of 10 mM Tris (pH = 7.5), 1 mM EDTA, 4% Ficoll, 1 mM dithiothreitol, and 75 mM KCl. The DNA/protein complex was analyzed on 4.5% native polyacrylamide gels.

2.6. Prostaglandin E_2 determination

The production of prostaglandin E_2, one of the mediators released after activation of cyclooxygenase, was used as a marker of cyclooxygenase activity. The induction assay was slightly modified according to that described by Salvemini et al. (1993). Briefly, RAW 264.7 cells were pretreated with lipopolysaccharide (1 μg/ml) for 18 h and then equilibrated in fresh Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 2.1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 2 mM EDTA, and 1 mg/ml glucose). The cells were thereafter incubated in the absence or presence of 7HQ derivatives for another 1 h, and 30 μM arachidonic acid was added for 10 min. All incubations were terminated by adding 4 mM EDTA and 30 μM indomethacin. The supernatants were assayed for prostaglandin E_2 by enzyme immunoassay system. All determinations were performed in duplicate.

2.7. Cytotoxicity assay

Cell viability was determined by the enzymatic reduction of the yellow dye, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), to a blue formazan product in intact cells. Macrophages (10^4/0.1 ml) were treated with different concentrations of 7HQ derivatives for 24 h. MTT dye (10 μl of 5 mg/ml) was added later and then the cells were incubated for 1 h at 37°C. After removal of the supernatant, the cells were lysed and absorbance was detected at 550 nm.

Plasma membrane integrity was determined by measuring lactate dehydrogenase, a stable cytosolic enzyme that is released upon cell lysis. After treatment with different 7HQ derivatives for 24 h, the supernatants were collected for the lactate dehydrogenase releasing assay.

2.8. Statistics

Results are expressed as means ± S.E.M. A one-way analysis of variance was performed for multiple comparisons, and if there was significant variation between treatment groups, the mean values were compared with the respective control using Student's *t*-test. P values less than 0.05 were considered significant.

3. Results

3.1. 7HQ derivatives inhibit nitrite production in lipopolysaccharide-treated macrophages

In order to investigate whether 7HQ derivatives altered nitric oxide synthesis by lipopolysaccharide-treated RAW 264.7 macrophages, we determined the nitrite content of the cell culture supernatant. As shown in Fig. 2, co-incubation of lipopolysaccharide-treated cells with various test compounds resulted in a concentration-dependent reduction in nitrite accumulation. 7HQ-1, -2, -3, and -4 inhibited NO generation with IC_{50} values of 2.7 ± 0.79, 0.44 ± 0.07, 9.16 ± 1.89 and 3.71 ± 1.56 μM, respectively. Among them, 7HQ-2 was the most potent inhibitor. The cytotoxic effects of the 7HQ derivatives were also examined in the lactate dehydrogenase releasing assay (5.8 ± 0.6%, 4.4 ± 1.2%, 2.4 ± 1.1% and 8.7 ± 1.5% for 7HQ derivatives, respectively; $P > 0.05$ as compared with control value of 5.3 ± 1.0%, $n = 4$) and the MTT uptake assay (3.2 ± 1.7%, 7.0 ± 4.4%, 4.2 ± 3.4% and 3.8 ± 2.7% cytotoxicity for 7HQ derivatives, respectively; $P > 0.05$ as compared with control value of 0 ± 0%, $n = 4$). The results showed that all 7HQ derivatives had little influence in these two assays even at high concentrations (data not show).

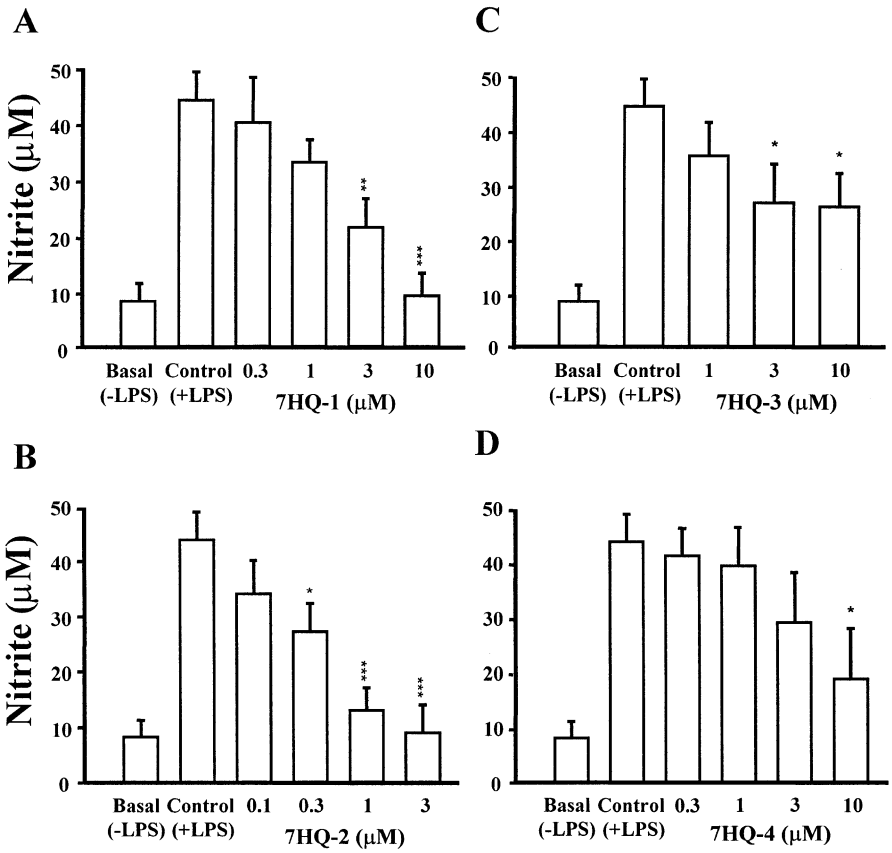


Fig. 2. Effects of 7HQ-1 (A), 7HQ-2 (B), 7HQ-3 (C) and 7HQ-4 (D) on nitrite accumulation in RAW 264.7 macrophages. Cells were pretreated with 7HQ derivatives for 30 min and then incubated with lipopolysaccharide (1 μg/ml) for 24 h. The nitric oxide production was measured by the Griess reaction. Data are expressed as means ± S.E.M. of seven determinations. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ compared with the respective control.

Exposure of the macrophages to lipopolysaccharide led to a time-dependent increase in nitrite production and inducible nitric oxide synthase expression (Chen and Wang, 1999). In search of an explanation for the decrease in nitrite production elicited by the 7HQ derivatives, the activity of inducible nitric oxide synthase was measured. After induction with lipopolysaccharide (10 h), the later addition of the 7HQ compounds did not significantly alter the activity of inducible nitric oxide synthase (data not shown).

3.2. 7HQ derivatives decrease the expression of inducible nitric oxide synthase in lipopolysaccharide-treated macrophages

We determined whether these compounds affect the expression of inducible nitric oxide synthase. According to the Western blotting analyses, the 7HQ derivatives attenuated the expression of inducible nitric oxide synthase in a concentration-dependent manner (Fig. 3). This influence on protein expression correlated with the parallel reduction in nitrite accumulation. It suggests that the inhibition of the expression of this enzyme plays a crucial role in 7HQ-derivative signaling.

3.3. 7HQ derivatives suppress the activation of NF-κB in lipopolysaccharide-treated macrophages

Activation of NF-κB has been shown to be involved in the induction of nitric oxide synthase in macrophages (Xie et al., 1994). Thus, to investigate whether 7HQ derivatives selectively inhibited the activation of NF-κB, NF-κB binding activity was analyzed by gel mobility shift assay. As shown in Fig. 4, stimulation of the cells with lipopolysaccharide caused a profound increase in the nuclear translocation of NF-κB; the corresponding band was decreased in cells pretreated with the various 7HQ derivatives. The result reveals that the inhibition of NF-κB nuclear translocation may be the key event for the inhibitory effect of 7HQ derivatives.

3.4. 7HQ derivatives stabilize the degradation of IκB-α in lipopolysaccharide-treated macrophages

The nuclear translocation of NF-κB is preceded by the phosphorylation and proteolytic degradation of the inhibitory subunit IκB-α (Stancovski and Baltimore, 1997). To verify whether the effect of the 7HQ derivatives on NF-κB activation is related to IκB-α degradation, the

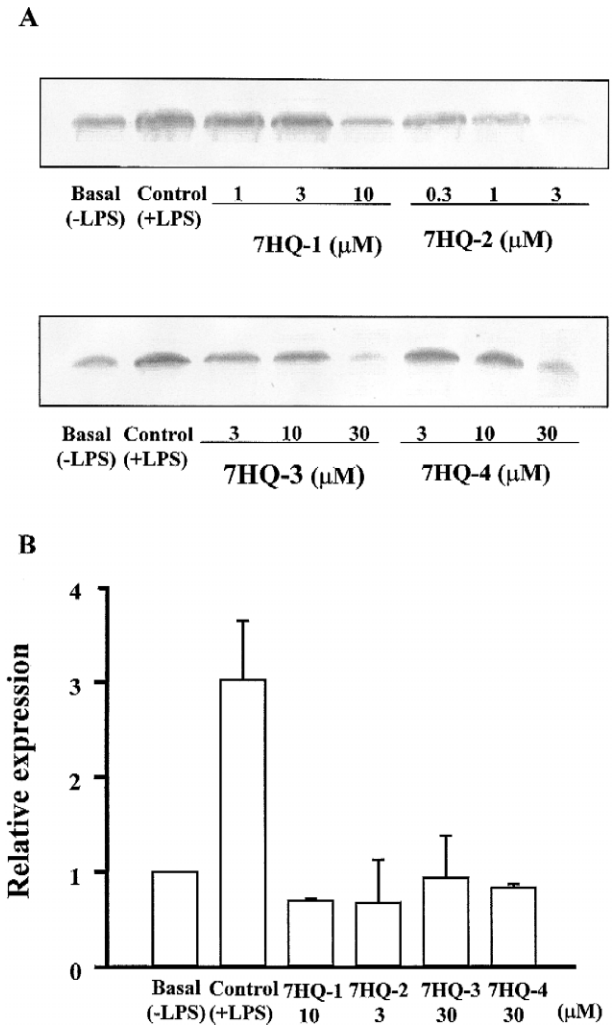


Fig. 3. Effects of 7HQ derivatives on lipopolysaccharide-induced inducible nitric oxide synthase expression in RAW 264.7 macrophages. Cells were pretreated with 7HQ derivatives for 30 min and then incubated with lipopolysaccharide (1 μg/ml) for 24 h to induce protein expression. After the above treatment, cells were washed and harvested as described in Section 2. Inducible nitric oxide synthase expression was analyzed by Western blotting (A) and then quantitatively assessed using a densitometer (B). Data of relative expression are expressed as means ± S.E.M. of three determinations.

protein level of IκB-α was examined by Western blotting analysis. In cells stimulated with lipopolysaccharide, the levels of the IκB-α protein gradually diminished and almost disappeared after stimulation for 30 min (Fig. 5, upper lanes). However, treatment of cells with the 7HQ derivatives prior to lipopolysaccharide stimulation abated the decrease in IκB-α expression and restored it to nearly the control level (Fig. 5, lower lanes).

3.5. 7HQ derivatives attenuate cyclooxygenase-2 expression and enzyme activity in lipopolysaccharide-treated macrophages

There is evidence suggesting that a marked production of proinflammatory prostaglandins occurs at the site of

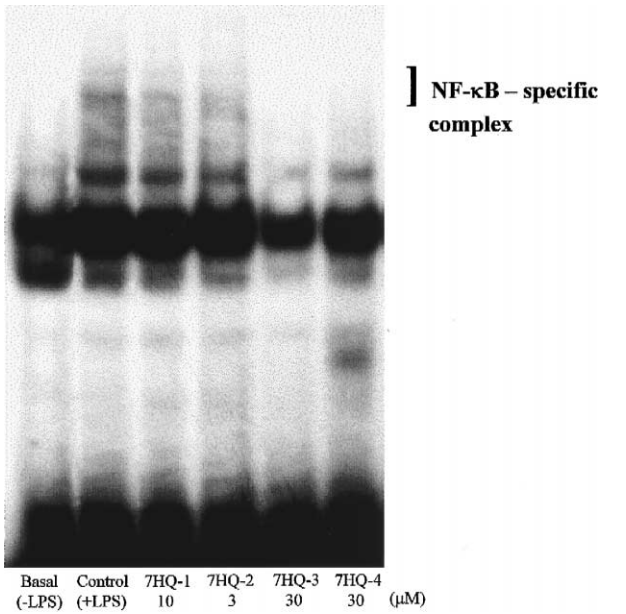


Fig. 4. Effects of 7HQ derivatives on lipopolysaccharide-induced NF-κB activation in RAW 264.7 macrophages. Cells were pretreated with 7HQ derivatives for 30 min, and then lipopolysaccharide was added to the cells for another 45 min. Cells were harvested and nuclear extracts were prepared for the determination of NF-κB as described in Section 2. The detection of NF-κB was performed by electrophoretic mobility shift assay.

injury, and cyclooxygenase-2 is reported to be central to the inflammatory process (Dubois et al., 1998). Furthermore, it is suggested that the maximum expression of cyclooxygenase-2 protein is reached after stimulation of RAW 264.7 cells with lipopolysaccharide for 8–12 h (Hwang et al., 1997). We aspired to learn about the influence of 7HQ derivatives on the expression and activity of cyclooxygenase-2. The results demonstrated that all of the 7HQ derivatives incubated with lipopolysaccharide for 8 h significantly inhibited cyclooxygenase-2 expression (Fig. 6). After the induction of this enzyme, the addition of the series of compounds did not significantly alter prostaglandin E_2 production. At the concentrations we used in the present experiment, these compounds had little effect

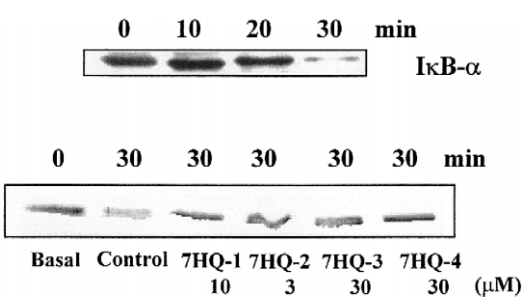


Fig. 5. Effects of 7HQ derivatives on lipopolysaccharide-induced IκB-α degradation in RAW 264.7 macrophages. Cells were pretreated with (lower lanes) or without (upper lanes) 7HQ derivatives for 30 min, and then lipopolysaccharide was added to the cells for the indicated times. After the above treatment, cells were washed and harvested as described in Section 2. IκB-α expression was analyzed by Western blotting.

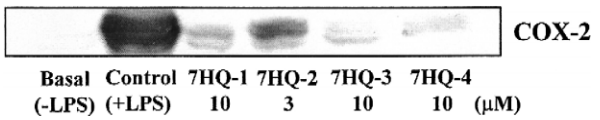


Fig. 6. Effects of 7HQ derivatives on lipopolysaccharide-induced cyclooxygenase-2 expression in RAW 264.7 macrophages. Cells were pretreated with 7HQ derivatives for 30 min and then incubated with lipopolysaccharide (1 μg/ml) for 8 h to induce cyclooxygenase-2 expression. After the above treatment, cells were washed and harvested as described in Section 2. Cyclooxygenase-2 expression was analyzed by Western blotting.

on the activity of the enzyme except at higher concentrations. The relative enzyme activity in the presence of the 7HQ derivatives is presented as a percentage of prostaglandin E_2 formation compared with that in the lipopolysaccharide-treated cells (control, 100%). The values are 7HQ-1 (10 μM), 86.7 ± 2.37%; 7HQ-2 (3 μM), 72.3 ± 15.8%; 7HQ-3 (30 μM), 75.9 ± 3.7%; 7HQ-4 (10 μM), 70.5 ± 6.1%, respectively ($P < 0.01$, $n = 3$).

4. Discussion

This study demonstrates that four structures with a dihydrocarbostyril nucleus suppress NO production in RAW 264.7 cells at a transcriptional level by preventing the activation of NF-κB. In addition to the above effects, the 7HQ derivatives had an inhibitory influence on lipopolysaccharide-induced cyclooxygenase-2 activity. Of the four compounds, 7HQ-2 was the most powerful inhibitory agent. These results suggest that the phenyl group substitution of 7HQ-2 may be beneficial for its inhibitory action. Furthermore, the observed inhibitory effects were not due to cytotoxicity, since cell viability was not altered.

The inhibitory effect on nitric oxide production of the 7HQ derivatives is unlikely to be due to direct modification of the activity of inducible nitric oxide synthase, because the 7HQ derivatives did not significantly inhibit nitrite accumulation when inducible nitric oxide synthase had already been expressed. We considered that it mainly caused a decrease in the expression of inducible nitric oxide synthase protein.

The expression of the gene for inducible nitric oxide synthase in murine macrophages is regulated by transcriptional activation (Lorsbach et al., 1993; Ohata et al., 1998). A κB binding site for NF-κB in the promoter region of inducible nitric oxide synthase has been characterized (Lowenstein et al., 1993), and NF-κB is the necessary transcriptional factor responsible for the induction of inducible nitric oxide synthase gene expression by lipopolysaccharide (Xie et al., 1994). Inappropriate regulation of NF-κB has been associated with chronic inflammatory disease and perpetuates the local inflammatory response (Barnes and Karin, 1997). In unstimulated cells, NF-κB is bound to IκB, keeping it in the cytosol. Stimulation of cells leads to phosphorylation of IκB-α at serine 32 and 36 via IκB kinase (IKK). This phosphorylation, in turn, induces ubiquitination of the molecule, which targets it for degradation by proteasomes (Israël, 1997). We show here clearly that 7HQ derivatives have potent inhibitory effects on NF-κB activation at various concentrations by stabilizing the IκB-α protein and preventing its degradation. Moreover, oxidative stress has been implied to involve NF-κB activation (Piette et al., 1997), and antioxidants diminish or completely eliminate NF-κB activation in many types of cells (Allen and Tresini, 2000). However, unless they are shown to be directly and specifically involved in IKK activation, reactive oxidative intermediates are unlikely to have a general signaling role in NF-κB activation (Li and Karin, 1999). We also examined the free radical-scavenging activity of 7HQ derivatives using the stable radical 1,1-diphenyl-2-picrylhydrazyl. 7HQ derivatives did not act as antioxidants even at the highest effective inhibitory concentrations on nitric oxide production (data not shown). These results demonstrate that the inhibitory action of 7HQ derivatives is not related to free radical-scavenging activity.

The products of proinflammatory genes such as cyclooxygenase initiate many events associated with sepsis. There is evidence suggesting that nitric oxide could regulate the cyclooxygenase enzymes (Salvemini, 1997). Moreover, under inflammatory situations, nitric oxide probably acts as a precursor of peroxynitrite, a potent modulator of prostaglandin endoperoxide synthase, to activate cyclooxygenase (Landino et al., 1996). These reports suggest that both nitric oxide and cyclooxygenase activity play important parts in the pathology of inflammation and sepsis. In order to investigate the effects of 7HQ derivatives on cyclooxygenase-2, we examined the expression of cyclooxygenase-2 protein. As demonstrated in Fig. 6, the results showed that the expression of cyclooxygenase-2 protein was inhibited by 7HQ derivatives. Besides enzyme expression, our results revealed that the 7HQ derivatives exhibited modest inhibitory effects against cyclooxygenase-2 activity at higher concentrations. Because the murine cyclooxygenase-2 gene contains a NF-κB site, it is likely that the inhibitory effects of 7HQ derivatives on IκB degradation as well as NF-κB nuclear translocation may account for their action on cyclooxygenase-2.

In summary, we suggest that 7HQ derivatives exert an inhibitory action on the expression of inducible nitric oxide synthase and cyclooxygenase-2 via the inhibition of IκB degradation and NF-κB activation. The combination of inhibition of inducible enzyme formation and inhibition of enzyme activity may make 7HQ derivatives potential compounds for anti-inflammatory therapy.

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