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Mechanisms of suppression of nitric oxide production by 3-*O*-methylquercetin in RAW 264.7 cells

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Abstract

Rhamnus nakaharai Hayata (Rhamnaceae) is used as a folk medicine in Taiwan for treating constipation, inflammation, tumors, and asthma. 3-*O*-Methylquercetin (3-MQ), a main constituent of the plant, has been reported to have potential for use in the treatment of asthma. The mechanisms of anti-inflammation of 3-MQ are still unclear. Nitric oxide (NO) production induced by lipopolysaccharide (LPS) through iNOS expression in RAW 264.7 cells, a mouse macrophage cell line, may reflect the degree of inflammation and may provide a measure for assessing the effect of drugs on the inflammatory process. Therefore, we were interested in investigating the mechanisms of suppression of NO production by 3-MQ in RAW 264.7 cells. 3-MQ (1–10 μM) concentration-dependently inhibited LPS (100 ng/mL)-induced NO production in RAW 264.7 cells. The IC_{50} value was calculated to be 4.23 μM. 3-MQ (1–10 μM) significantly and concentration-dependently inhibited LPS (100 ng/mL)-induced iNOS protein and mRNA expressions in cells. The IC_{50} values were calculated to be 4.36 and 6.53 μM, respectively. There was no significant difference among these three IC_{50} values of 3-MQ. In conclusion, 3-MQ may exert its anti-inflammatory effect through the inhibition of iNOS DNA transcription.
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Keywords: 3-*O*-Methylquercetin; *Rhamnus nakaharai*; Rhamnaceae; iNOS protein expression; iNOS mRNA expression; Anti-inflammation

1. Introduction

Nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthase (NOS) in various animal cells and tissues. At least three NOSs have been identified, including endothelial NOS (eNOS), neural NOS (nNOS), and inducible NOS (iNOS). The small amount of NO produced by constitutive NOS (cNOS), including eNOS and nNOS, is an important regulator of physical homeostasis, whereas the large amount of NO produced by iNOS has been closely correlated with the pathophysiology of a variety of diseases and inflammation. After exposure to inducers, such as lipopolysaccharide (LPS) from Gram-negative bacteria, lipoteichoic acid (LTA) from Gram-positive bacteria, and/or cytokines, iNOS can quantitatively be induced in various cells, such as macrophages, smooth muscle cells, and hepatocytes, to trigger cytotoxicity, tissue damage, inflammation, sepsis, and stroke (Marletta, 1993). Therefore, NO production induced by LPS or LTA through iNOS induction may reflect the degree of inflammation and may provide a measure for assessing the effect of drugs on the inflammatory process.

3-*O*-Methylquercetin (3-MQ) was originally characterized to have antiviral activity from total extracts of different *Euohorbia* species and from the alcoholic extracts of the fruit of *Veronia amygdalina* Del. (Compositae) (Van Hoof et al., 1984). It has been reported that poliovirus RNA synthesis (Castrillo et al., 1986) and replication (Castrillo and Carrasco, 1987) are

Abbreviations: 3-MQ, 3-*O*-methylquercetin; LSP, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SNP, sodium nitroprusside; AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; 7-NINA, 7-nitroindazole monosodium salt; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate buffer solution; NF-κB, nuclear-factor κB; IkB, inhibitory subunit of NF-κB; IKK, IκB kinase; ATP, adenosine triphosphate; TNF-α, tumor necrosis factor-α

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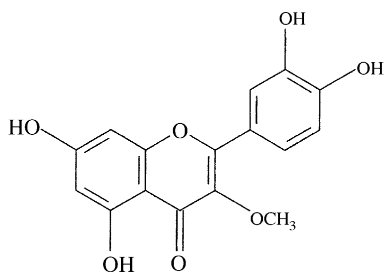

Fig. 1. Chemical structure of 3-*O*-methylquercetin (3-MQ, mol. wt. 316).

selectively and potently blocked by 3-MQ. 3-MQ is also a main constituent of *Rhamnus nakaharai* Hayata (Rhamnaceae) which is used as a folk medicine in Taiwan for treating constipation, inflammation, tumors, and asthma (Chiu and Chang, 1998). 3-MQ has been reported to more selectively inhibit PDE3 than PDE4 in our laboratory (Ko et al., 2003). Recently, we reported that 3-MQ has the potential for use in the treatment of asthma, with both anti-inflammatory and bronchodilating effects, at a dose which does not affect blood pressure (Ko et al., 2004). At the doses used (3–30 μmol/kg, i.p.), 3-MQ significantly suppressed total inflammatory cells, macrophages, neutrophils, and eosinophils, and significantly attenuated secretion of tumor necrosis factor (TNF)-α (Ko et al., 2004). In addition, 3-MQ (30 μmol/kg, i.p.) significantly attenuated secretions of interleukine (IL)-4 and IL-5 (Ko et al., 2004). However, the mechanisms of the anti-inflammatory effect of 3-MQ are still unclear. Therefore, we were interested in investigating the mechanisms of suppression of NO production by 3-MQ in RAW 264.7 cells, a mouse macrophage cell line.

2. Materials and methods

2.1. Reagents and drugs

3-MQ (Fig. 1) was isolated from *Rhamnus nakaharai* Hayata (Lin et al., 1995), and identified by spectral methods, including UV, IR, MS, and NMR spectroscopic techniques. The purity of 3-MQ was 99%. Fresh stem bark of the plant was collected at Ali, Wu-Tai Shian, Ping-Tung Hsien, Taiwan in July of 1990 and identified by Professor Chung-Nan Lin, School of Pharmacy, Kaoshiung Medical University, Kaoshiung, Taiwan. Voucher specimens (9001) are deposited in the herbarium of the School of Pharmacy, Kaoshiung Medical University. Ammonium persulfate, bovine serum albumin, β-mercaptoethanol, dimethylsulfoxide, lipopolysaccharide (*Escherichia coli*, serotype 0127:B8), sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochoride, and sodium nitroprusside dihydrate were purchased from Sigma Chemical, USA. Sodium nitroprusside (SNP), sodium nitrite, sodium chloride, and sodium bicarbanate were purchased from Merck, Germany. Fetal bovine serum, L-glutamine, penicilline/streptomycin, Dulbecco's modified Eagle's medium (DMEM), sodium pyruvate, and non-essential amino acid were purchased from Invitrogen, USA. Acrylamide, bromophenol blue, protein assay dye reagent concentrate, glycine, sodium dodecylsulfate (SDS), tris(hydroxymethyl)-aminomethane, and *N*,*N*,*N*,*N*,-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad, USA. Rabbit anti-mouse iNOS antibody was purchased from Santa Cruz Biotechnology, USA. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody was purchased from Chemicon International, USA. Glyceraldehyde-3-phosphate dehydrogenase mouse monoclonal antibody (GAPDH) was purchased from Biogenesis, USA. HRP-conjugated goat anti-mouse IgG antibody was purchased from Transduction Lab, USA. iNOS was purchased from Cayman, USA. 2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT) and 7-nitroindazole monosodium salt (7-NINA) were purchased from Tocris, UK.

2.2. Cell culture

RAW 264.7 cells, purchased from American Type Culture Collection, USA, were cultured in DMEM, supplemented with 5% fetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 2 mM L-glutamine, and 1 mM non-essential amino acids in a 10-cm plate at a density of 1×10^6 cells/mL for 24 h, at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Treatment of macrophages with LPS

RAW 264.7 cells were incubated in 12-well plates (15×10^4 cells/well) for 24 h. After incubation, macrophages were incubated with or without 100 ng/mL of LPS to stimulate NO production for another 24 h. When macrophages were incubated with LPS, vehicle (dimethyl sulfoxide:ethyl alcohol, 1:1) or various concentrations (0.3–10 μM) of 3-MQ were added at the same time. The suppressive effect of 3-MQ on NO production in the medium was assessed using the Griess reagent. However, macrophages (225×10^4 cells/plate) were incubated in 10-cm plates for 24 h, and vehicle or various concentrations (1–10 μM) of 3-MQ were added 30 min prior to LPS-addition and incubated for another 24 h, when the suppressive effect of 3-MQ on iNOS gene expression was assessed.

2.4. Nitrite measurement

Nitrite was measured by adding 100 μL of the Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μL of medium for 5 min. The optical density at 550 nm (OD_{550}) was measured with a microplate reader. Concentrations were calculated by comparison with the OD_{550} of a standard solution of sodium nitrite prepared in culture medium.

2.5. Cell viability

To evaluate whether the suppressive effect of 3-MQ on NO production is related to cell viability, RAW 264.7 cells treated with and without LPS were washed with 500 μL of phosphate buffer solution (PBS) and resuspended with 1 mL of culture medium. Cell viability was assessed by the trypan blue dye exclusion method. Shortly after mixing an equal volume of the cell suspension and trypan blue dye, the number of live cells was

counted using a hemacytometer. Live cells treated with LPS in the absence of 3-MQ were set as 100%. Percentages of relative survival in the presence of vehicle or various concentrations of 3-MQ were compared against each other.

2.6. Western blotting

To assess the suppressive effect of 3-MQ on iNOS gene expression, RAW 264.7 cells treated with or without LPS were washed, harvested, and sonicated. The total protein in the lysates was assayed according to the method described by Bradford (1976). Lysates (40 μg protein/lane) were separated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and transferred onto an immobilon polyvinylidenedifluoride membrane (Millipore, USA). Membranes were blocked at 4 °C for 30 min in TBS-T (11 mM Tris–base, 155 mM NaCl, 0.1% Tween 20, pH 7.4) containing 5% non-fat milk. After washing with TBS-T for 10 min three times, membranes were probed with primary antibodies, such as rabbit anti-mouse iNOS antibody and GAPDH mouse monoclonal antibody for 2 h at room temperature. After washing with TBS-T three times, the blots were incubated with secondary antibodies, such as HRP-conjugated goat anti-rabbit IgG antibody and HRP-conjugated goat anti-mouse IgG antibody for 1 h at room temperature. Finally, the blots were incubated with an enhanced chemiluminescent substrate (Pierce, USA) in a dark room for 5 min. After exposure to X-ray film, the density of the band was quantitatively measured using a densitometer (GelDoc 2000, Bio-Rad, USA).

2.7. RT-PCR

Total cellular RNA from RAW 264.7 cells was extracted using RNeasy mini kits (Qiagen, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was performed by reverse transcription (RT) of 1 μg of each total RNA using the SuperScript One-Step RT-PCR System (Gibco BRL/Invitrogen, USA). PCR primers for mouse iNOS and GAPDH were synthesized according to the following oligonucleotide sequences (Promega, USA). iNOS: forward primer 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′, and reverse primer 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′; and GAPDH: forward primer 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, and reverse primer 5′-CATGTAGGCCATGAGGTCCACCAC-3′. After pre-denaturation at 95 °C for 5 min, cDNAs of iNOS and GAPDH, respectively, were amplified with the SuperScript One-Step RT-PCR System (Gibco BRL/Invitrogen, USA) for 30 cycles of 30 s at 95 °C, 30 s at 54 °C, and 45 s at 72 °C followed by an extension at 72 °C for 5 min. Equal amounts of PCR products were electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining. The intensity of the band was quantitatively measured using a densitometer (GelDoc 2000, Bio-Rad, USA).

2.8. Determination of iNOS activity

The specific iNOS activity was determined by conversion of [^{3}H]-arginine to [^{3}H]-citrulline as described by Kobuchi et al. (1997) with minor modifications. Briefly, the reaction mixture (100 μL) contained 1 mM NADPH, 10 μM FAD, 1 mM dithiothreitol, 100 μM BH_4, 10 μM L-arginine supplemented with L-[^{3}H]-arginine (0.5 μCi/mL reaction medium), and 2 units of iNOS in 50 mM HEPES (pH 7.5). After incubation for 1 h at 37 °C, the reaction was terminated by addition of 200 μL of cold-stop buffer (50 mM morpholinoethanesulfonic acid, pH 5.5, and 5 mM EDTA). The reaction mixture was then applied to a 0.5-mL column of Dowex 50W-X8 cation exchange resin (Na^+ form, Sigma, USA), which was pre-equilibrated with stop buffer, and eluted with 1 mL of stop buffer. Radiolabeled citrulline in the combined eluate was measured using a liquid scintillation counter.

2.9. NO production from nitroprusside

To understand the inhibitory effects of 3-MQ on NO production, SNP was freshly prepared at 25 mM in PBS, and 1 mL of it was added into each six-well plate in the absence or presence of various concentrations (3–30 μM) of 3-MQ or its vehicle. NO production was determined each hour using the Griess reagent, as mentioned above. To understand the scavenging effects of 3-MQ on preformed NO, SNP was freshly prepared in PBS at 5 mM and equilibrated for 3 h. One milliliter of SNP was added into each six-well plate in the absence or presence of various concentrations (3–30 μM) of 3-MQ or its vehicle, then the nitrite concentration was determined each hour using the Griess reagent, as mentioned above.

2.10. Statistical analysis

All values are given as the mean ± S.E.M. Statistical differences among values were calculated by one-way analysis of variance (ANOVA), and then evaluated by Dunnett's test. The difference between two values, however, was determined using Student's *t*-test. Differences were considered statistically significant if the *P*-value was less than 0.05.

3. Results

3-MQ (0.3–10 μM) concentration-dependently inhibited NO production in LPS-activated RAW 264.7 cells with an IC_{50} value of 4.23 μM (Fig. 2A; Table 1). AMT, a selective iNOS inhibitor, concentration-dependently inhibited NO production with an IC_{50} value of 0.02 μM (Fig. 2B; Table 1). However, 7-NINA, a selective nNOS inhibitor, inhibited NO production with an IC_{50} value of 65.47 μM (Fig. 2C; Table 1). The inhibitory effect of 3-MQ on NO production was not due to cell death because 3-MQ (1–10 μM) did not affect cell survival (Fig. 3). 3-MQ (1–10 μM) did not inhibit iNOS activity (data not shown). 3-MQ (3–30 μM) did not inhibit NO production from SNP, and neither did it scavenge preformed NO from SNP (Fig. 4). However, 3-MQ (1–10 μM) concentration-dependently and significantly inhibited iNOS protein expression in LPS-activated RAW 264.7 cells (Fig. 5). The IC_{50} value of 3-MQ against iNOS protein expression was 4.36 μM (Table 1). Furthermore, from the

Table 1
IC_{50} (μM) values of 3-MQ, AMT, and 7-NINA on LPS-activated NO production, and iNOS protein and iNOS mRNA expressions in RAW 264.7 cells

Test compounds	NO production	iNOS protein expression	iNOS mRNA expression
3-MQ	4.23 ± 0.48 (3)	4.36 ± 1.00 (3)	6.53 ± 3.47 (3)
AMT[a]	0.02 ± 0.00 (3)	ND	ND
7-NINA[b]	65.47 ± 3.34 (2)	ND	ND

All values are expressed as the mean ± S.E.M. (n), and n is the number of experiments. ND: not determined. There was no significant difference among these three values of 3-MQ using one-way ANOVA.

[a] A selective iNOS inhibitor, used as a positive control.

[b] A selective nNOS inhibitor, used as a negative control.

RT-PCR analysis, 3-MQ (1–10 μM) concentration-dependently and significantly inhibited mRNA expression of iNOS in LPS-activated RAW 264.7 cells (Fig. 6). The IC_{50} value of 3-MQ against mRNA expression of iNOS was 6.53 μM (Table 1). There was no significant difference among these three IC_{50} values of 3-MQ (Table 1).

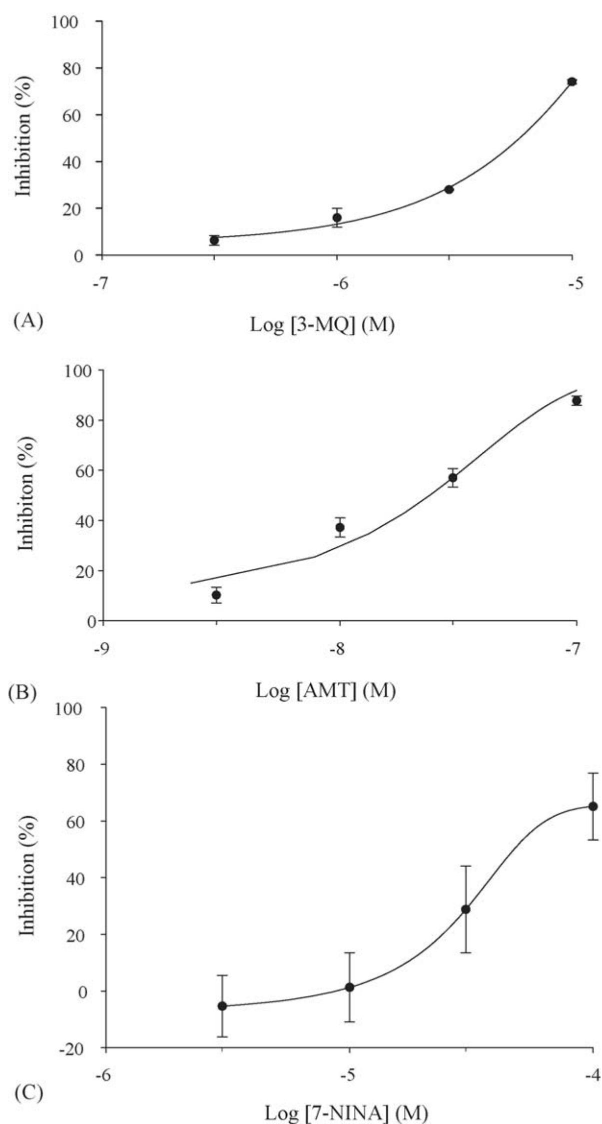

Fig. 2. Log concentration–inhibition curves of 3-MQ (A), AMT (B), and 7-NINA (C) on NO production in LPS-activated RAW 264.7 cells.

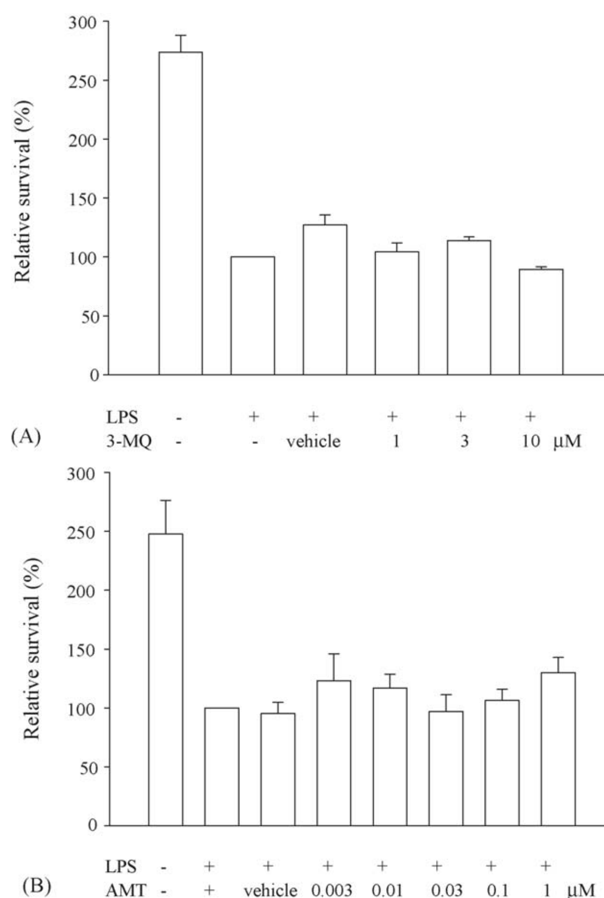

Fig. 3. Relative survival of 3-MQ (A) and AMT (B), a selective iNOS inhibitor, in LPS-activated RAW 264.7 cells.

4. Discussion

Results sometimes are dependent on methods used. For example, the IC_{50} value of quercetin against NO production in LPS-activated RAW 264.7 cells was reported to be 17 μM (Chen et al., 2001), 36 μM (Mastuda et al., 2002), and 107 μM (Kim et al., 1999) by different laboratories. The difference may have been caused from the concentration of LPS and the well size of the culture plate used. If the well diameter is too small, cells may colonize around the well on the bottom due to surface tension of the medium. Thus, a higher concentration of quercetin is required to inhibit NO production. To avoid the

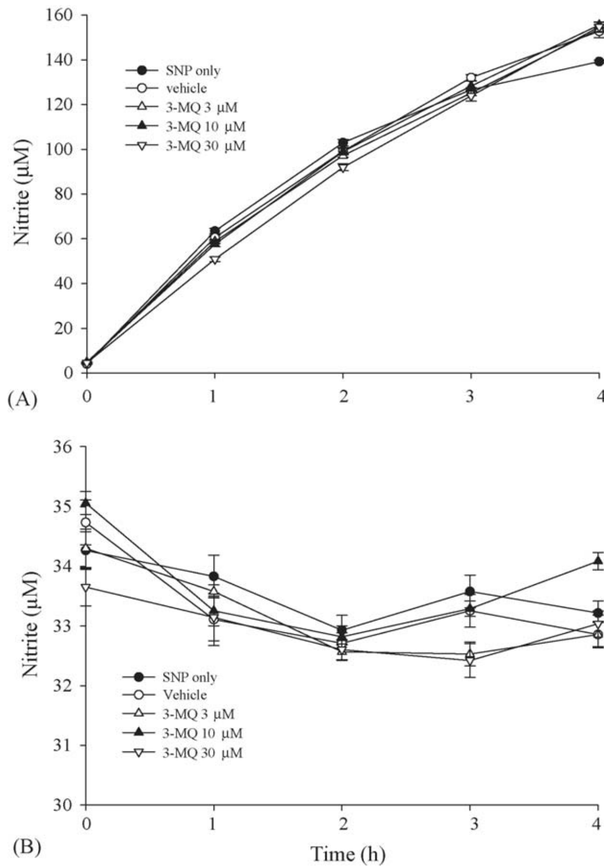

Fig. 4. Inhibitory effects of 3-MQ on NO production (A), and its scavenging effect on preformed NO (B) from SNP.

effect of surface tension of the medium, we used 12-well plates to culture macrophages. Of course, the higher the concentration of LPS which is used, the higher concentration of quercetin which is needed to inhibit NO production. In our present experiment, we used 100 ng/mL of LPS to activate macrophages. At this concentration of LPS, the survival of the macrophages was about one-third of that without LPS (Fig. 3). However, the concentration of LPS used was the least among the three laboratories mentioned above (Chen et al., 2001; Kim et al., 1999; Mastuda et al., 2002). In our experimental conditions, the IC_{50} value of quercetin against NO production was 2.17 μM (unpublished data).

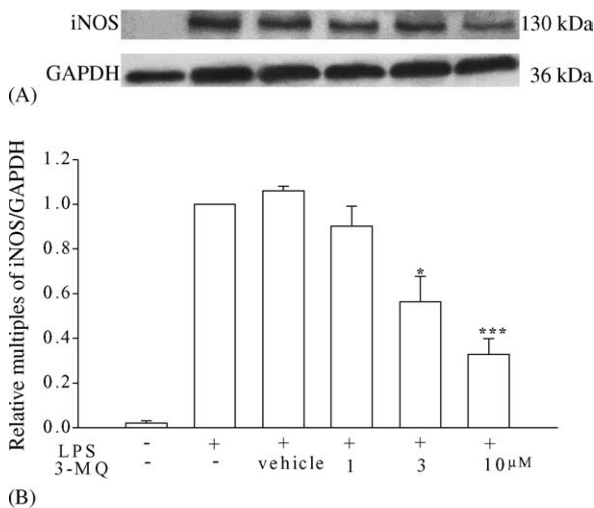

Fig. 5. Inhibition of iNOS protein expression in LPS-activated RAW 264.7 cells by 3-MQ. Macrophages were treated with LPS (100 ng/mL) in various coexisting concentrations of 3-MQ for 24 h. At the end of the incubation, the culture medium was collected for iNOS and GAPDH protein analysis (A). Band intensities were quantified using a densitometer (B). This experiment was independently repeated three times. $^{*}P < 0.05$, $^{***}P < 0.001$ when compared with control (vehicle).

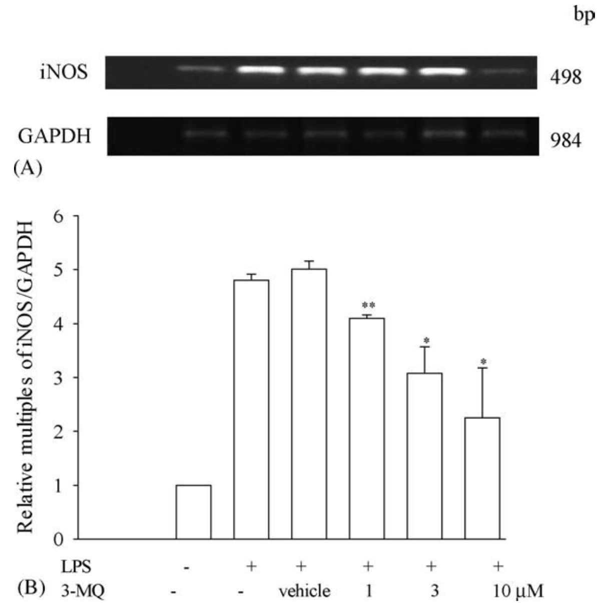

Fig. 6. Inhibition of iNOS mRNA expression in LPS-activated RAW 264.7 cells by 3-MQ. Macrophages were treated with LPS (100 ng/mL) in various coexisting concentrations of 3-MQ for 20 h. At the end of the incubation, the culture medium was collected for iNOS and GAPDH RT-PCR analysis (A). Band intensities were quantified using a densitometer (B). This experiment was independently repeated three times. $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with the control (vehicle).

Various concentrations (1–10 μM) of 3-MQ did not significantly affect the survival of macrophages when compared with the vehicle (Fig. 3). In our results, 3-MQ concentration-dependently inhibited NO production of LPS-activated macrophgaes with an IC_{50} value of 4.23 μM (Table 1). The inhibitory effect was not due to cell survival (Fig. 3) or to extracellular effects, such as direct inhibition or/and scavenging of NO (Fig. 4). It also did not seem to be directly due to inhibit on of iNOS activity. From Western blots and RT-PCR analyses, the IC_{50} values of 3-MQ against iNOS protein and mRNA expressions were similar to that against NO production in LPS-activated macrophages (Table 1). These results suggest that 3-MQ (1–10 μM) may inhibit iNOS DNA transcription through which it exerts its anti-inflammatory effects. 3-MQ has been reported to significantly inhibit thromboxane formation by cyclo-oxygenase inhibition in rabbit platelets, although the activity is less potent than that of indomethacin (Laekeman et al., 1986). Also, 3-MQ has been reported to have potent anti-platelet effects on arachidonic acid- and collagen-induced platelet aggregation (Lin et al., 1995). In addition, 3-MQ at micromolar concentrations has been reported to have xanthine oxidase inhibiting and superoxide scavenging activities (Cimanga et al., 2001).

Therefore, at least in part, the above results may explain and support the use of *Bridelia ferruginea* Benth. (Euphorbiaceae) stem bark, a traditional African medicine used against various diseases, for the treatment of rheumatic pains (Cimanga et al., 2001).

3-MQ and its derivatives, including 3,3′-dimethylquercetin, 3,7′-dimethylquercetin, and 3,7,3′-trimethylquercetin, isolated from *Euphorbia grantii* Oliv. (Euphorbiaceae) stems, an African medicinal plant, have been reported to exhibit remarkable activities against picornaviruses and vesicular stomatitis virus (Van Hoof et al., 1984). 3-MQ has been also reported to selectively inhibit poliovirus replication (Castrillo et al., 1986). The inhibitory effect of 3-MQ is reversible, since cells treated with this compound at the beginning of infection will start to synthesize viral RNA and protein after the compound is removed. The addition of 3-MQ at the beginning of poliovirus infection prevents the appearance of viral proteins, although the shut-off of host translation still takes place (Castrillo et al., 1986). Addition of 3-MQ late in the infection does not inhibit viral protein synthesis, whereas viral RNA synthesis is drastically inhibited, suggesting that RNA synthesis is the target of 3-MQ action (Castrillo et al., 1986). 3-MQ has no effect on RNA synthesis in uninfected cells. However, this compound blocks the synthesis of poliovirus single-stranded RNA and its replicative intermediates (Castrillo and Carrasco, 1987).

In our recent report, 3-MQ at 3 μmol/kg (i.p.) significantly attenuated TNF-α release in bronchoalveolar lavage fluid of sensitized mice (Ko et al., 2004). NF-κB has been reported to be the most important transcription factor in the regulation of iNOS gene expression (Taylor et al., 1998). In resting cells, NF-κB is sequestered in the cytoplasm through its association with inhibitory proteins termed IκB. When cells are stimulated by TNF-α or IL-1, IκB proteins (IκBα and IκBβ) are phosphorylated at serine residues in the N-terminal region (Traenckner et al., 1995) and degraded. After degradation of IκB, NF-κB proteins are released and translocated to the nucleus where they up regulate the transcription of target genes (Baldwin, 1996). IκB proteins are phosphorylated by IκB kinases (IKKα and IKKβ). IKKβ, but not IKKα, is the target for proinflammatory stimuli (Hu et al., 1999). On the other hand, IKKα is essential for development of the skin and skeleton during embryogenesis (Hu et al., 1999). Several tyrosine kinase inhibitors, such as genistein, quercetin, and staurosporine, are able to inhibit NF-κB activation (Natarajan et al., 1998). Quercetin and staurosporine, but not daidzein, an inactive analogue of genistein in tyrosine kinase inhibition, inhibit IKKα and IKKβ, the two key regulated serine kinases in the NF-kB pathway (Peet and Li, 1999). Staurosporine competitively inhibits these two kinases when ATP is used as a substrate. However, quercetin serves as a mixed type inhibitor toward ATP. The binding site of quercetin likely overlaps with both the ATP and IκBα binding pockets (Peet and Li, 1999). Quercetin has been reported to be a non-specific inhibitor of protein kinases (Groundwater et al., 1996) and suppresses TNF-α-induced NF-κB activation (Natarajan et al., 1998). Therefore, 3-MQ, an analogue of quercetin, may non-specifically inhibit protein kinases, such as tyrosine kinase and serine kinases (specially IKKβ) in exerting its anti-inflammatory effects. The signal transductional mechanism of the compound needs to be further investigated.

5. Conclusions

3-MQ (1–10 μM) concentration-dependently inhibited LPS (100 ng/mL)-induced NO production in RAW 264.7 cells. The IC_{50} value was calculated to be 4.23 μM. 3-MQ (1–10 μM) significantly and concentration-dependently inhibited LPS (100 ng/mL)-induced iNOS protein and mRNA expressions in cells. The IC_{50} values were calculated to be 4.36 and 6.53 μM, respectively. There was no significant difference among these three IC_{50} values of 3-MQ. In conclusion, 3-MQ may exert its anti-inflammatory effect through the inhibition of iNOS DNA transcription.

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