The inhibitory action of quercetin on lipopolysaccharide-induced nitric oxide production in RAW 264.7 macrophage cells

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The effect of quercetin on lipopolysaccharide (LPS)-induced nitric oxide (NO) production was studied. Quercetin pretreatment significantly inhibited NO production in an LPS-stimulated RAW 264.7 murine macrophage cell line. Post-treatment with quercetin partially inhibited NO production. The inhibitory action of quercetin was due to neither the cytotoxic action nor altered LPS binding. The expression of inducible-type NO synthase (iNOS) was markedly down-regulated by quercetin. Quercetin suppressed the release of free nuclear factor (NF)-κB by preventing degradation of IκB-α and IκB-β. Moreover, quercetin blocked the phosphorylation of extracellular signal regulated kinase 1/2 (Erk1/2), p38, and c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) and, further, the activity of tyrosine kinases in LPS-stimulated RAW cells. Quercetin also inhibited interferon (IFN)-γ-induced NO production. Taken together, these results indicate that the inhibitory action of quercetin on NO production in LPS- and/or IFN-γ-stimulated macrophages might be due to abrogation of iNOS protein induction by impairment of a series of intracellular signal pathways.

INTRODUCTION

Nitric oxide (NO) is an important regulatory and effector molecule with various biological functions.1-6 NO is synthesized from L-arginine by constitutively expressed NO synthase and an inducible isoform of NO synthase (iNOS).1,3,7 NO production is markedly augmented in several cell types including macrophages and vascular endothelial cells by lipopolysaccharide (LPS) and pro-inflammatory cytokines.1,5-10 The augmentation of NO synthase (NOS) production is markedly down-regulated by quercetin. Quercetin depresses the release of free nuclear factor (NF)-κB by preventing degradation of IκB-α and IκB-β. Moreover, quercetin blocked the phosphorylation of extracellular signal regulated kinase 1/2 (Erk1/2), p38, and c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) and, further, the activity of tyrosine kinases in LPS-stimulated RAW cells. Quercetin also inhibited interferon (IFN)-γ-induced NO production. Taken together, these results indicate that the inhibitory action of quercetin on NO production in LPS- and/or IFN-γ-stimulated macrophages might be due to abrogation of iNOS protein induction by impairment of a series of intracellular signal pathways.

MATERIALS AND METHODS

Materials

LPS was extracted from Klebsiella O3 as described previously21 and dissolved at a concentration of 1 mg/ml in...
0.01 M phosphate-buffered saline (PBS) at pH 7.2. Quercetin and recombinant mouse interferon (IFN-γ) was obtained from Wako Pure Chemical, Osaka, Japan and Genzyme, Cambridge, MA, USA, respectively.

**Cell culture**

The murine macrophage cell line RAW 264.7, obtained from Riken cell Bank (Tsukuba, Japan), was maintained in RPMI 1640 medium containing 5% fetal bovine serum and antibiotics at 37°C with 5% CO₂ and seeded in 35-mm culture dishes or a 96-well plate for experiments. The cells were pretreated with quercetin (50 µM) for 18 h and subsequently stimulated with LPS (10 µg/ml) and/or IFN-γ (20 ng/ml) for 18 h unless otherwise stated. The experiments were performed in triplicate.

**Determination of nitrite concentration**

NO was measured as its end product, nitrite, using the Griess reagent as described elsewhere. The culture supernatant (100 µl) was mixed with 100 µl of Griess reagent for 10 min, and the absorbance at 570 nm was measured in a microplate reader. The concentration of nitrite in the samples was determined with reference to a sodium nitrite standard curve. Data represent the mean of triplicate determinations ± SD.

**Laser flow cytometric analysis**

RAW cells were pretreated with quercetin (50 µM) for 18 h. Quercetin-pretreated and untreated RAW cells were incubated with 1 µg/ml FITC-conjugated LPS (Sigma, St Louis, MO, USA) or a 1:40 dilution of FITC-conjugated anti-mouse CD14 antibody (PharMingen, San Diego, CA, USA) at 4°C for 1 h. A 1:100 dilution of anti-mouse IFN-γ receptor antibody (PharMingen) was also used, followed by FITC-conjugated second antibody. The cells were then washed and suspended in PBS. The fluorescence intensity was analyzed by a laser flow cytometry (FACS Caliber, Becton Dickinson, San Jose, CA, USA). Anti-Toll-like receptor 4 antibody (HTA125) was provided by Dr. K. Miyake and used at a 1:40 dilution.

**Immunoblotting**

Quercetin-pretreated and untreated RAW cells were seeded in 35-mm plastic dishes (4 x 10⁵ cells/dish) and incubated with LPS for 1 or 8 h. The cells were lysed in lysis buffer (0.5 M Tris-HCl, 4% SDS, 2 mercaptoethanol) and boiled for 5 min at 100°C. Aliquots (20 µg/lane) containing equal amounts of protein were electrophoresed under reducing conditions in a 4–20% gradient polyacrylamide gel and transferred to a polyvinylidene difluoride membrane filter. The membranes were treated with an appropriately diluted rabbit polyclonal antibody against iNOS (Upstate Biotechnology, Lake Placid, NY, USA), IκB-α, IκB-β (Santa Cruz Biotechnology, Santa Cruz, CA, USA), extracellular signal regulated kinase 1/2 (Erk1/2), phospho-Erk1/2, p38, phospho-p38 and c-Jun NH₂-terminal kinase/stress-activated protein kinase (JNK/SAPK), phospho-JNK/SAPK, STAT1, or phospho-STAT1 (Y701; New England Biolabs, Beverly, MA, USA) for 1 h. A monoclonal antibody to phosphotyrosine (py20; Transduction Laboratories, Lexington, KY, USA) was also used. The membranes were further treated with a 1:3000 dilution of horseradish peroxidase-conjugated protein G for 1 h. Immune complexes were detected with an enhanced chemiluminescence substrate (New England Nuclear, Boston, MA, USA) and exposed to Kodak XAR X-ray film.

**Luciferase reporter gene assay for NF-κB activation**

RAW cells (3 x 10⁵/ml) were plated in 35-mm plastic dishes. On the following day, the cells were transfected with 0.5 µg of pNF-κB-Luc plasmid (a luciferase reporter gene driven by five tandem repeats of NF-κB, PathDetect System, Stratagene, La Jolla, CA, USA) and 0.5 µg of pCMV-β-gal plasmid (Gibco-BRL) by the lipofectin method (Gibco-BRL). The transfected cells were pretreated with quercetin for a given time followed by treatment with LPS (10 µg/ml) and/or IFN-γ (20 ng) for 8 h. The cells were lysed using lysis reagent (Promega, Madison, WI, USA) prior to measurement of luciferase activity. The luciferase activity was determined on cell lysates with a luminometer. β-Galactosidase activity was used to normalize transfection efficiencies. All bar diagrams are shown as the mean of duplicate determination ± SD for two experiments in which each transfection was performed in duplicate.

**Statistical analysis**

Experimental results are expressed as the mean ± SD. Statistical significance was determined by Student’s t-test.

**RESULTS**

**Inhibition of LPS-induced NO production by quercetin**

The effect of pretreatment with various concentrations of quercetin on LPS-induced NO production was studied (Fig. 1A). RAW cells were pretreated with quercetin at a concentration range of 1–100 µM for 18 h, followed by
an 18 h incubation with LPS (10 µg/ml), IFN-γ (20 ng/ml), or the combination of LPS and IFN-γ. Quercetin pretreatment reduced LPS-induced NO production in a concentration-dependent manner, and a significant reduction of NO production was observed at 10 µM of quercetin (P < 0.01). Similarly, NO production induced by IFN-γ alone or the combination of LPS and IFN-γ was inhibited by quercetin. Quercetin pretreatment inhibited spontaneous NO production in untreated control cells. In addition, the dye exclusion assay and mitochondrial dehydrogenase activity assay demonstrated no toxic effect of quercetin (50 µM) on the cell viability of RAW (data not shown). Next, the effect of pretreatment time of quercetin on LPS-induced NO production was studied (Fig. 1B). RAW cells were pretreated with quercetin (50 µM) for various periods, followed by an 18 h incubation with LPS and/or IFN-γ. Quercetin pretreatment for any time tested induced significant reduction in LPS-induced NO production (P < 0.01). Pretreatment of RAW cells with quercetin for ≥3 h inhibited IFN-γ-induced NO production (P < 0.01). The inhibition of IFN-γ-induced NO production by quercetin required a longer pretreatment time compared with LPS. To assess whether the addition of quercetin after LPS stimulation affected NO production, cells were post-exposed to quercetin at various times after LPS stimulation (Fig. 1C). Quercetin post-treatment inhibited LPS-induced NO production (P < 0.01), although the inhibition with quercetin post-treatment was much less compared with pretreatment.

The effect of various exposure times of quercetin on LPS-induced NO production was studied (Fig. 2). The cells were exposed to quercetin for 1, 3, 6, or 24 h and then washed to remove quercetin. One of three independent experiments is shown.

Fig. 1. The inhibitory action of quercetin on NO production in LPS-stimulated RAW cells. (A) The effect of pretreatment with various concentrations of quercetin on LPS-induced NO production. RAW cells were pretreated with quercetin at concentrations of 1–100 µM for 18 h. Quercetin-pretreated RAW cells were incubated with LPS (10 µg/ml), IFN-γ (20 ng/ml), and the combination of LPS and IFN-γ. (B) The effect of various pretreatment times of quercetin on LPS-induced NO production. RAW cells were incubated with quercetin (50 µM) for various times, and then were stimulated with LPS and/or IFN-γ. (C) RAW cells were incubated with LPS and/or IFN-γ, and quercetin (Q; 50 µM) was added to the cultures at 10, 60, 180 and 360 min after the stimulation. RAW cells were post-treated with quercetin for 18 h. One of three independent experiments is shown.

Fig. 2. The inhibition of LPS-induced NO production by the exposure to quercetin. RAW cells were treated with quercetin for various times and then washed to remove the quercetin. Pretreated cells were incubated with LPS (10 µg/ml), IFN-γ (20 ng/ml), or the combination of LPS and IFN-γ for 18 h. One of three independent experiments is shown.
The exposure of quercetin for 1 h inhibited LPS-induced NO production but not IFN-γ-induced NO production \((P < 0.01)\). However, IFN-γ-induced NO production was inhibited by the 3 h exposure of quercetin \((P < 0.01)\).

Inhibition of iNOS protein expression by quercetin

The effect of quercetin on the expression of iNOS protein was studied (Fig. 3). Quercetin-pretreated or control untreated RAW cells were stimulated with LPS for 18 h. The expression of iNOS protein was readily detected in LPS-stimulated RAW cells. However, the pretreatment of RAW cells with quercetin completely abrogated the appearance of iNOS proteins in response to LPS. iNOS expression was not detected in quercetin-pretreated RAW cells in response to IFN-γ alone or the combination of LPS and IFN-γ.

No alteration in LPS binding, and the expression of CD14, Toll-like receptor 4 and IFN-γ receptor in quercetin-pretreated RAW cells

To study the inhibitory action of quercetin on LPS-induced NO production and iNOS expression, we compared the binding of FITC-conjugated LPS, the cell surface expression of CD14, Toll-like receptor 4 and IFN-γ receptor between quercetin-pretreated and control untreated RAW cells. Laser flow cytometric analysis demonstrated that there was no significant difference in the binding of FITC-conjugated LPS and the expression of CD14, Toll-like receptor 4 and IFN-γ receptor between those cells (data not shown). Moreover, there was no significant difference in the phagocytic activity (data not shown). This result suggested that quercetin might affect the intracellular signal transduction for NO production in LPS and/or IFN-γ-stimulated RAW cells.

Inhibition of NF-κB activation in LPS-stimulated RAW cells by quercetin

It has been reported that the activation of NF-κB is involved in NO production in LPS-stimulated RAW cells.\(^7,11,12,23-25\)
Therefore, we tested the effect of quercetin on LPS-induced NF-κB activation using a luciferase reporter gene assay (Fig. 4). LPS definitely enhanced the reporter gene activity indicating NF-κB activation in untreated RAW cells. However, quercetin pretreatment blocked LPS-induced enhancement of NF-κB-dependent reporter gene activity. Although IFN-γ slightly enhanced NF-κB activation, quercetin pretreatment inhibited it \((P < 0.01)\). Subsequently, the effect of quercetin on IκB-α and IκB-β expression was examined to clarify the inhibitory action of quercetin on LPS-induced NF-κB activation (Fig. 5). The expression of IκB-α and IκB-β proteins in the exposure of quercetin-pretreated RAW cells to LPS was studied by immunoblotting. LPS caused reduced expression of IκB-α and IκB-β in untreated RAW cells, whereas the IκB-α and IκB-β expression was not changed in quercetin-pretreated cells. IFN-γ treatment also caused the loss of IκB-α and IκB-β, and the loss was inhibited by quercetin pretreatment. Quercetin was suggested to inhibit the release of free NF-κB through the stabilization of IκB-α and IκB-β.

**Inhibition of the phosphorylation of a series of mitogen-activated protein (MAP) kinases including Erk1/2, p38, and JNK/SAPK by quercetin**

LPS is known to activate a series of MAP kinases, such as Erk1/2, p38, and JNK/SAPK, in NO production.\(^{26,27}\) Therefore, the effect of quercetin on the activation of the MAP kinase signal pathways was examined by immunoblotting using anti-phospho-MAP kinase antibodies (Fig. 6). LPS clearly induced phosphorylated forms of Erk1/2, p38, and JNK/SAPK in RAW cells. The combination of LPS and IFN-γ also lead to the phosphorylation of three MAP kinases. Quercetin pretreatment inhibited the phosphorylation of all three MAP kinases in LPS-stimulated RAW cells. In addition, IFN-γ alone did not affect the phosphorylation of those MAP kinases.

IFN-γ is known to activate the JAK-STAT signal pathway and induces NO production.\(^{28-30}\) In order to study the effect of quercetin on the JAK-STAT signal pathway, the phosphorylation of STAT1 was examined by immunoblotting using anti-phospho-STAT1 antibody (Fig. 7). Quercetin pretreatment inhibited the phosphorylation of STAT1 in RAW cells stimulated with IFN-γ alone or IFN-γ and LPS, although IFN-γ significantly induced the phosphorylation of STAT1.

**Inhibition of tyrosine kinases by quercetin**

The effect of quercetin on the activity of tyrosine kinases was studied by immunoblotting using anti-phosphotyrosine antibody. RAW cells were pretreated with or without quercetin and were further stimulated with LPS, IFN-γ, or the combination of LPS and IFN-γ. Anti-phosphotyrosine
antibody strongly reacted with phosphorylated tyrosine in the samples from untreated control RAW cells, while phosphorylated tyrosine was much less in quercetin-pre-treated RAW cells (Fig. 8). This suggested that quercetin might inhibit NO production in LPS and/or IFN-γ-stimulated RAW cells by down-regulating the activity of tyrosine kinases.

**DISCUSSION**

In the present study, we demonstrated that quercetin inhibits NO production in LPS-stimulated RAW murine macrophage cells through down-regulating the expression of iNOS. The inhibition of iNOS expression by quercetin was due to neither altered LPS binding nor cytotoxicity. The inhibition appears to be mediated by the impairment of intracellular signal transduction. Previously, quercetin was reported to inhibit LPS-induced NO production in RAW cells,18,31 peritoneal macrophages,32 and astrocytes.33,34 However, there are few reports on the detailed mechanism of how quercetin inhibits LPS-induced iNOS expression in RAW cells. In the present study, we clearly demonstrated that quercetin impairs a series of signal pathways. It inhibited the activation of NF-κB and MAP kinases including Erk1/2, p38, and JNK/SAPK in LPS-stimulated RAW cells. Moreover, quercetin inhibited the activation of JAK/STAT as well as the phosphorylation of tyrosine kinases. Since these signal pathways are involved in iNOS expression, quercetin may inhibit LPS-induced NO production in RAW cells through the impairment of several signal pathways in iNOS expression.

The inhibition of NF-κB activation by quercetin is important for the suppression of LPS-induced iNOS expression since NF-κB activation plays a critical role in the expression of iNOS in LPS-stimulated macrophages. The stabilization of IκB-α and IκB-β with quercetin was suggested to inhibit NF-κB activation through preventing the liberation of free NF-κB. On the other hand, the wine polyphenolic quercetin did not inhibit LPS-induced NF-κB activation although it decreased NO production in RAW cells.18 This discrepancy cannot be explained at present. In addition, flavonoids are reported to inhibit LPS-induced NF-κB activation.18,35–37 In the present study, NF-κB was activated slightly by IFN-γ-stimulated RAW cells. This might be dependent on autocrine mechanisms involving tumor necrosis factor-α (TNF-α) and interleukin-1. Otherwise, IFN-γ might trigger the activation of NF-κB directly through an unknown mechanism. There was no marked difference in the NF-κB reporter gene activity between IFN-γ alone and the combination of IFN-γ and LPS. This indicated that the IFN-γ signal might play a dominant role in the treatment with combined IFN-γ and...
LPS. It corresponded to the finding that there was no marked difference in NO production and iNOS expression between IFN-γ alone and combined IFN-γ and LPS.

Quercetin inhibits the signal pathways in IFN-γ-induced NO production. Based on the inhibitory action of quercetin on tyrosine kinases in the present study, quercetin may inhibit the activity of JAK. In fact, the present study demonstrated that quercetin prevents the phosphorylation of STAT1. Interestingly, the inhibitory action of quercetin on IFN-γ-induced NO production was less marked compared with that on LPS-induced NO production. It might be dependent on the difference of the signal transduction between LPS and IFN-γ. The JAK/STAT pathway might be less sensitive to the inhibitory action of quercetin.

LPS activates a series of MAP kinases and induces NO production in RAW cells through the expression of iNOS protein.26–29 We have reported that a series of MAP kinases, Erk1/2, p38, and JAK/SAPK, play an important role in LPS-induced NO production in RAW cells.29 It is reasonable that the down-regulation of MAP kinases by quercetin causes attenuated LPS-induced NO production. For the first time, we have demonstrated that quercetin inhibits the signaling of all MAP kinases in LPS-induced NO production.

Recently we reported that a series of flavonoids protect mice from LPS-induced lethality.15 Quercetin pretreatment results in marked reduction of TNF-α production and free radicals and reduces the mortality in lethal endotoxic shock in vivo.16 Based on the present study, quercetin pretreatment may abolish a number of signal pathways and abrogate the production of noxious mediators. It is likely that the down-regulation of LPS signal transduction by quercetin is closely associated with its protective action on lethal endotoxic shock. Quercetin could be used as a new potent therapeutic agent for prevention and control of sepsis and septic shock due to an excess of NO production.

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REFERENCES


