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Inhibition of iNOS gene expression by quercetin is mediated by the inhibition of IκB kinase, nuclear factor-kappa B and STAT1, and depends on heme oxygenase-1 induction in mouse BV-2 microglia

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Abstract

In the present study, experiments were performed to explore the action of quercetin, the most widely distributed flavonoids, and its major metabolite, quercetin-3′-sulfate, on lipopolysaccharide (LPS)- and interferon-γ (IFN-γ)-induced nitric oxide (NO) production in BV-2 microglia. Quercetin could suppress LPS- and IFN-γ-induced NO production and inducible nitric oxide synthase (iNOS) gene transcription, while quercetin-3′-sulfate had no effect. LPS-induced IκB kinase (IKK), nuclear factor-κB (NF-κB) and activating protein-1 (AP-1) activation, and IFN-γ-induced NF-κB, signal transducer and activator of transcription-1 (STAT1) and interferon regulatory factor-1 (IRF-1) activation were reduced by quercetin. Moreover quercetin was able to induce heme oxygenase-1 expression. To address the involvement of heme oxygenase-1 induction in iNOS inhibition, heme oxygenase-1 antisense oligodeoxynucleotide was used. Quercetin-mediated inhibition of NO production and iNOS protein expression were partially reversed by heme oxygenase-1 antisense oligodeoxynucleotide, but was mimicked by hemin, a heme oxygenase-1 inducer. The involvement of signal pathways in quercetin-induced heme oxygenase-1 gene expression was associated with tyrosine kinase and mitogen-activated protein kinases activation. All these results suggest quercetin should provide therapeutic benefits for suppression of inflammatory-related neuronal injury in neurodegenerative diseases.

Keywords: Quercetin; Inducible nitric oxide synthase; Nuclear factor-κB; Signal transducer and activator of transcription-1; Heme oxygenase-1; Microglia

1. Introduction

Microglial cells are thought to be functionally equivalent to peripheral macrophages in the central nervous system (CNS). Microglia becomes activated in brain injury and various neurodegenerative diseases such as stroke, trauma, Alzheimer's disease, multiple sclerosis, and human immunodeficiency virus-associated dementia (Chao et al., 1992; Dickson et al., 1993). Activated microglia is thought to be involved in neuronal inflammation by over-producing various bioactive molecules such as nitric oxide (NO) (Nakashima et al., 1995). Since significant sustained amount of NO can be produced by inducible nitric oxide synthase (iNOS) whose gene expression is mostly regulated at the transcriptional level, agent aimed at inhibition of iNOS expression is thus a potential therapeutic strategy to reduce neuronal injury in neurodegenerative diseases. Transcriptional factors nuclear factor-κB (NF-κB) and activating

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protein-1 (AP-1) are involved in the gene transcription by bacterial endotoxin (lipopolysaccharide, LPS), while signal transducer and activator of transcription-1 (STAT1) and interferon regulatory factor-1 (IRF-1) are activated by interferon-γ (IFN-γ) (Xie et al., 1993; Kamijo et al., 1994; Martin et al., 1994).

Heme oxygenase-1 is a redox-sensitive inducible protein that provides efficient cytoprotection against oxidative stress (Morse and Choi, 2002). A substantial body of evidence demonstrates an imbalance in the redox state is known to promote activation of the heme oxygenase-1 system in different cell types. Owing to the important biological function in cytoprotection, this enzyme can be regarded as a potential therapeutic target in a variety of oxidant- and inflammatory-mediated diseases (Motterlini et al., 2000; Scapagnini et al., 2002). One of multiple mechanisms responsible for the antiinflammation and cell protection is ascribed to the downregulation of iNOS (Lee et al., 2003; Sumi and Ignarro, 2004; Yang et al., 2004).

Flavonoids are widely distributed in the plant kingdom and have been identified to exhibit a wide variety of clinically relevant properties such as anti-tumor, anti-platelet, anti-ischemic, and anti-inflammatory activities (Avila et al., 1994; Gerritsen et al., 1995). Antioxidant and free radical scavenging activities might contribute to their biological activities (Hodnick et al., 1990; Mora et al., 1990). Quercetin is a typical flavonol-type flavonoid ubiquitously present in the diet and has been shown to exhibit broad-modulating effects (Formica and Regelson, 1995; Lamson and Brignall, 2000). As previously reported, quercetin could reduce LPS-induced NO production at the transcriptional level from macrophages (Kim et al., 1999; Chen et al., 2001; Mu et al., 2001; Raso et al., 2001; Shen et al., 2002; Blonska et al., 2004; Jung and Sung, 2004). In vivo animal study further confirmed the inhibitory activity of quercetin pretreatment on LPS-induced NO production (Shen et al., 2002). It is thus suggested that inhibition of iNOS expression by quercetin may be one of the mechanisms responsible for its anti-inflammatory effects. Further study reported that the inhibition of NF-κB activation by quercetin is associated with the suppression of iNOS transcription in macrophages (Mu et al., 2001).

Dietary flavonoid glycosides are hydrolyzed in the intestine, absorbed as aglycones and metabolized to methylated, glucurono-sulfated derivatives (Manach et al., 1995). In human trials, the bioavailability of orally administered quercetin is only 24%. The absorbed quercetin in the plasma is present in its major metabolite, quercetin-3′-sulfate, with higher amount than its unaltered form (Hollman et al., 1995; Morand et al., 1998). Although it is suggested that repeated dietary intake of quercetin can lead to accumulation in plasma and prevent oxidative stress-related chronic diseases, whether its sulfate form of metabolite also accounts for the protective function is an unanswered question.

Recent studies have demonstrated important neuroprotective actions of dietary flavonoids found in fruits, vegetables, and plant-derived drinks (Youdim et al., 2002). In order to understand the mechanisms underlying flavonoid-mediated neuroprotection, it is important to establish whether they are able to cross the blood–brain-barrier and enter the CNS. Evidence from the study using in vitro models mimicking aspects of the blood–brain-barrier presented that certain flavonoids and their physiologically relevant metabolites can cross the brain endothelium (Youdim et al., 2003). They also reported that flavonoids are able to travel the blood–brain-barrier in vivo and the permeability of certain flavonoids is influenced by their lipophilicity and interactions with efflux transports (Youdim et al., 2004). Therefore in the present study we investigate the effects and mechanisms of quercetin and quercetin-3′-sulfate on iNOS and heme oxygenase-1 induction in BV-2 microglia. These results would highlight the therapeutic potential of quercetin as a novel anti-inflammatory drug in neurodegenerative diseases.

2. Materials and methods

2.1. Materials

Oligonucleotides specific for NF-κB, AP-1, and STATs binding were synthesized on a DNA synthesizer (PS250; Cruachem Ltd., Glasgow, UK), using the cyanoethyl phosphoroamidate method, and purified by gel filtration. The sequences of the double-stranded oligonucleotides used to detect the DNA-binding activities of NF-κB, AP-1, and STAT are as follows: NF-κB, 5′-GATCAGTTGAGG-GGACTTTCCCAGGC-3′; AP-1, 5′GATCCGCTTGATG-ACTCAGCCGGAA-3′; and STAT, 5′-ATCGTTCATTT-CCCGTAAATCCCTA-3′. Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). [α-^{32}P] dATP (3000 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were obtained from NEN (Boston, MA, USA). The enhanced chemiluminescence detection agents were purchased from Amersham Biosciences (Piscataway, NJ, USA). Rabbit polyclonal anti-iNOS and anti-β-tubulin antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). Rabbit polyclonal antibodies specific for p65 NF-κB, p50 NF-κB, IKKα, IKKβ, c-*fos*, c-Jun, STAT-1, heme oxygenase-1, protein A/G agarose beads, horseradish peroxidase-coupled anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody specific for Tyr 701-phosphorylated active STAT1 was purchased from New England Biolabs (Beverly, MA, USA). All materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Hercules, CA, USA). Murine IFN-γ was purchased from R & D (Minneapolis, MN, USA). Phenol-extracted LPS (L8274) from *Escherichia coli*, and other chemicals (*N*-acetyl-L-cysteine, glutathione, vitamin C, 1,1-diphenyl-2-picryl-hydrazyl, *N*(omega)-nitro-L-arginine methyl ester (L-NAME), hemin) were obtained from

Sigma-Aldrich (St. Louis, MO, USA). Quercetin and quercetin-3′-sulphate were synthesized as we previously described (Hou et al., 2003). PD98059, KT5720, KT5823, PP2, herbimycin A, SB203580 and U0126 were purchased from Calbiochem (Sn Diego, CA, USA). Y27632 and SP600125 were purchased from Tocris Cookson (Avonmouth, UK). The iNOS reporter plasmid was constructed and kindly provided by Dr. C.K. Glass (Department of Medicine, University of California, San Diego, Ca, USA) (Ricote et al., 1998). The iNOS promoter-luciferase reporter plasmid containing binding sites for AP-1 and NF-κB, which are required for response to LPS, and STAT1, which is required for response to IFN-γ. The κB-Luc plasmid was constructed under the control of three NF-κB binding sites. The luciferase reporter construct of the human IRF-1 gene promoter (−1.3 kb pairs) containing one GAS element was kindly provided by Dr. Yoshihiro Ohmori (Cleveland Clinic Foundation, Cleveland, OH, USA).

2.2. Cell culture

BV-2 microglia were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in an atmosphere of 5% CO_2. Cells were seeded into 24-well plates for nitrite measurement, reporter assay, immunoblots and RT-PCR, or into 10-cm dishes for electrophoretic mobility shift assay (EMSA) and IKK kinase assay.

2.3. Nitrite assay

Measurement of nitrite production as an assay of NO release was performed. Accumulation of nitrite in the medium was determined by colorimetric assay with Griess reagent. The cells were treated with LPS, IFN-γ and/or the indicated agents for 24 h. Aliquots of conditioned media were mixed with an equal volume of Griess reagent [1% sulfanilamide and 0.1% *N*-(1-naphthyl)-ethylenediamine in 5% phosphoric acid]. Nitrite concentrations were determined by comparison with the OD_{550} using standard solutions of sodium nitrite prepared in cell culture medium. Each experiment was performed in duplicate and repeated at least three times.

2.4. Immunoblotting analysis

After agent treatment, the medium was aspirated. Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), and 100 μl of whole-cell lysis buffer (20 mM Tris–HCl, pH 7.5, 125 mM NaCl, 1% Triton X-100, 1 mM $MgCl_2$, 25 mM β-glycerophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) was then added to each well. After cell harvest, cell lysates were centrifuged. Equal amounts of the soluble protein were electrophoresed on a SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. Nonspecific binding was blocked with Tris-buffered-saline with Tween (TBST) (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.02% Tween 20) containing 5% nonfat milk for 1 h at room temperature. After immunoblotting with the first specific antibodies, membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. After three washes with TBST, the protein bands were detected with enhanced chemiluminescence detection reagent. To make sure equal amounts of sample protein were applied for electrophoresis and immunoblotting, β-actin was used as an internal control.

2.5. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The expression of iNOS and heme oxygenase-1 mRNA was determined by RT-PCR analysis. After drug treatment, cells were homogenized with 1 ml of RNAzol B reagent (Invitrogen), and total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction. RT was performed using StrataScript RT-PCR kit (Stratagene, La Jolla, CA, USA), and 10 μg of total RNA was reverse transcribed to cDNA following the manufacturer's recommended procedures. RT-generated cDNA encoding iNOS, heme oxygenase-1 and β-actin genes were amplified using PCR. The oligonucleotide primers used correspond to the mouse macrophages iNOS (sense: 5′-TCA TTG TAC TCT GAG GGC TGA CAC A-3′ and anti-sense: 5′-GCC TTC AAC ACC AAG GTT GTC TGC A-3′), heme oxygenase-1 (sense: 5′-GAG AAT GCT GAG TTC ATG-3′ and antisense: 5′-ATG TTG AGC AGG AAG GC-3′), and mouse β-actin (sense: 5′-GAC TAC CTC ATG AAG ATC CT-3′ and anti-sense: 5′-CCA CAT CTG CTG GAA GGT GG-3′). PCR was performed in a final volume of 50 μl containing *Taq*DNA polymerase buffer, all four dNTPs, oligonucleotide primers, *Taq*DNA polymerase, and RT products. After an initial denaturing for 2 min at 94 °C, 30 cycles of amplification (iNOS: 94 °C for 1 min, 52 °C for 45 s, and 72 °C for 90 s; heme oxygenase-1:94 °C for 45 s, 55 °C for 30 s, and 72 °C for 30 s) were performed followed by a 10-min extension at 72 °C. PCR products were analyzed on 1% agarose gel. The mRNA of β-actin served as an internal control for sample loading and mRNA integrity.

2.6. Immunoprecipitation and kinase assay

To determine the effect of quercetin on IKK activity, after stimulation, anti-IKKα and anti-IKKβ (1 μg each) with protein A/G agarose beads were added to the prepared cell extracts as mentioned above. Immunoprecipitation proceeded at 4 °C overnight. The precipitated beads were washed three times with 1 ml of ice-cold cell lysis buffer and twice with kinase buffer (25 mM HEPES, pH 7.4, 20 mM

$MgCl_2$, 0.1 mM Na_3VO_4, and 2 mM dithiothreitol). The immune-complex kinase assay was then performed at 30 °C for 30 min in 20 μl of kinase reaction buffer containing 1 μg of GST-IκBα, 25 μM ATP, and 3 μCi of [γ-^{32}P]ATP. The reaction was terminated with 5X Laemmli sample buffer, and the products were resolved by 12% SDS-PAGE gel electrophoresis. The phosphorylated IκBα was visualized by autoradiography. In some experiments, IKK immunoprecipitate was subjected to SDS-PAGE and immunoblotting to verify that equal amounts of kinase were undergoing kinase reaction.

2.7. Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSA)

Nuclear extracts from stimulated or non-stimulated cells were prepared by cell lysis followed by nuclear lysis. Cells were suspended in 30 μl of buffer containing 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride; vortexed vigorously for 15 s; allowed to stand at 4 °C for 10 min; and centrifuged at 2000 rpm for 2 min. The pelleted nuclei were resuspended in buffer 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 phenylmethylsulfonyl fluoride for 20 min on ice, and then the lysates were centrifuged at 15,000 rpm for 2 min. The supernatants containing the solubilized nuclear proteins were stored at −70 °C until used for EMSA. Binding tests for NF-κB, AP-1, and STAT-1 were performed. Briefly, binding reaction mixtures (15 μl) contained 0.25 μg of poly(dI-dC) (Amersham Biosciences) and 20,000 dpm of [α-^{32}P]-labeled DNA probe in binding buffer consisting of 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 4% Ficoll, 1 mM dithiothreitol, and 75 mM KCl; the binding reaction was started by the addition of cell extracts and continued for 30 min. Samples were analyzed on native 5% polyacrylamide gels. For supershift experiments, 5 μg of p65, p50, c-*fos*, c-Jun, or STAT-1 antibody was mixed with the nuclear extract proteins.

2.8. Free radical scavenging capacity of quercetin and quercetin-3′-sulfate

The free radical-scavenging capacity of quercetin and quercetin-3′-sulfate was measured with 1,1-diphenyl-2-picryl-hydrazyl (DPPH) as we described previously (Hsu et al., 2002). The DPPH radical has a deep, violet color as a result of its unpaired electron, and radical scavenging can be followed spectrophotometrically by absorbance loss at 517 nm when the pale, yellow, non-radical form is produced. A 5 μl aliquot of quercetin (1–10 μM) or quercetin-3′-sulfate (10 μM) was mixed with 995 μl 100 μM DPPH solution (in ethanol) in a cuvette. Following 30-min incubation in the dark, the change of absorbance at 517 nm was measured on a spectrophotometer (Model U-3200, Hitachi Instruments, San Jose, CA). The DPPH test was also done with vitamin C as positive control.

2.9. Transfection and reporter gene assay

Using electroporation (280 V, 1070 μF, 30 ms time constant), cells (2×10^7 cells/cuvette) were cotransfected with 1 μg β-galactosidase expression vector (pCR3lacZ; Pharmacia, Sweden) and 1 μg iNOS-Luc, 1 μg pGL2-ELAM-Luc (κB-Luc), 1 μg AP-1-Luc or 1 μg IRF-1 promoter plasmid. After electroporation, cells were cultured in 24-well plate at 2×10^6 cells/well. After 24-h incubation, cells were incubated with the indicated concentrations of agents. After another 24-h incubation, the media were removed, and the cells were washed once with cold PBS. To prepare lysates, 100 μl of reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 30 s. Aliquots of cell lysates (5 μl) containing equal amounts of protein (10–20 μg) were placed into the wells of an opaque, black 96-well microplate. An equal volume of luciferase substrate (Promega) was added to all samples, and the luminescence was measured in a microplate luminometer (Packard, Meriden, CT). Luciferase activity values were normalized to transfection efficiency monitored by β-galactosidase expression, and was presented as the percentage of luciferase activity measured with LPS or IFN-γ stimulation alone and in the absence of quercetin.

2.10. Oligodeoxynucleotide synthesis and transfection procedures

The sequences of heme oxygenase-1 oligodeoxynucleotide used for transfection studies were as following: antisense: 5′-TGC TGT CGG GCT GTG GAC GCT CCA T-3′, sense: 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′. The oligodeoxynucleotide was obtained from Gene Tools (Philomath, USA). The oligodeoxynucleotide was purified by reverse-phase high-performance liquid chromatography and lyophilized after synthesis by the manufacturer. Before use oligodeoxynucleotide was resolved in sterile water. The special delivery formation consists of two components. One component comprises a pre-paired duplex of Morpholino oligo and partially complementary DNA oligo. The other component is a weakly basic delivery reagent, ethoxylated polyethylenimine (EPEI). BV-2 cells (2×10^5 cells/well, 24-well) were cultured in appropriate medium to adhere overnight. Before transfection, Morpholino/oligodeoxynucleotide component (2 μl, 0.5 mM stock) was mixed with EPEI (3 μl, 200 μM stock) and incubated for 20 min at room temperature for triplicate samples. After the complexes were formed (20 min incubation) they were then diluted with the serum free medium (in 900 μl final volume). The solution was added to the cells according to the manufacturer's instructions. Five hours later removed the solution and replaced with fresh culture medium.

2.11. Statistical evaluation

Values are expressed as the mean ± S.E.M. of at least three experiments. Analysis of variance was used to assess the statistical significance of the differences, with a P value of <0.05 being considered statistically significant.

3. Results

3.1. Quercetin attenuates LPS- and IFN-γ-induced NO formation, iNOS protein and mRNA levels

Measuring nitrite as the index of NO production by the Griess method, we found that in BV-2 microglia, LPS (0.1 μg/ml) and IFN-γ (10 ng/ml) treatment for 24 h resulted in a large amount of NO release. The increased NO release was accompanied by the induction of iNOS protein. Co-addition of quercetin inhibited the formation of NO in a concentration-dependent manner, with IC_{50} values of 3 μM and 5 μM for LPS and IFN-γ stimulation, respectively (Fig. 1A). However, treatment of quercetin-3′-sulfate (10 μM) didn't have the similar effect. Immunoblot analysis revealed the consistent reduction of iNOS protein expression in the presence of quercetin (Fig. 1B). To elucidate the effect of quercetin on iNOS gene expression, the steady-state level of mRNA following drug treatment was measured by RT-PCR. LPS (0.2 μg/ml) incubation has been shown to induce iNOS mRNA at least after 3 h (Cho et al., 2001). As illustrated in Fig. 1C, quercetin, at the dose of 10 μM, significantly decreased LPS- and IFN-γ-induced iNOS mRNA level. The inhibitory effect of quercetin on iNOS gene transcription was confirmed by iNOS reporter assay. The result showed that quercetin at 10 μM inhibited LPS- and IFN-γ-induced iNOS luciferase activity (Fig. 1D). In order to clarify if the inhibitory effects of quercetin on LPS- and IFN-γ-induced iNOS protein expression owing to its cytotoxicity, we used the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay as an index for mitochondria function and found that incubation with quercetin and quercetin-3′-sulfate at 30 μM for 40 h induced no significant cell toxicity (data not shown). When cells were analyzed by flow cytometry with propidium iodide staining, none of quercetin and its sulfate at 30 μM affected the cell cycle progress (data not shown).

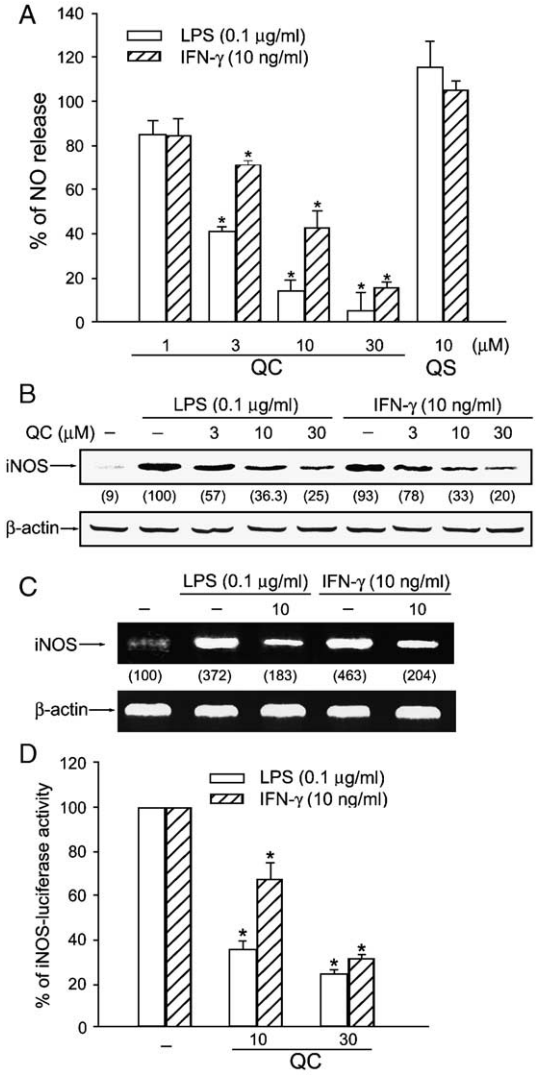

Fig. 1. Quercetin (QC), but not quercetin-3′-sulfate (QS), inhibits LPS- and IFN-γ-induced NO production and iNOS expression in BV-2 microglia. Cells were treated with QC or QS at the concentrations indicated for 30 min, followed by the stimulation with LPS (0.1 μg/ml) or IFN-γ (10 ng/ml) for 24 h. After incubation, the stable NO metabolite nitrite present in the medium was analyzed by the Griess method (A), and cell lysates were subjected to SDS-PAGE for iNOS measurement (B). The β-actin protein level was considered as an internal control. In some experiments, total mRNA from cells treated with QC plus LPS or IFN-γ for 12 h was isolated, and RT-PCR for iNOS steady state mRNA was performed. Internal control of β-actin mRNA was also determined (C). In (D), cells were transfected with iNOS-Luc for 24 h. Then cells were incubated with LPS, IFN-γ and/or QC. Twenty-four hours later, luciferase activity in the cell lysates was determined, and normalized by lacZ transfection efficiency, and expressed as a percentage of the control response without stimulus treatment. The data in (A) and (D) are the mean ± S.E.M. from at least three independent experiments, each performed in duplicate. *$P<0.05$ indicates the statistical significance as compared with control of LPS or IFN-γ treatment alone. The changes of iNOS mRNA and protein levels indicated as percentages of the control response (data shown in parentheses) are representative of three independent experiments with similar results.

3.2. Quercetin inhibits LPS-induced IKK, NF-κB and AP-1 activation

It is well known that NF-κB, AP-1, and STAT1 are important transcription factors for the induction of iNOS gene by LPS and/or IFN-γ (Xie et al., 1993). Because LPS-induced iNOS expression was found to involve the IKK/NF-κB pathway (Huang et al., 2003), the effects of quercetin on the LPS-induced IKK activity was examined. Results shown in Fig. 2A indicated that IKK activity as assessed from the phosphorylation of IκBα in vitro was attenuated by quercetin in a dose-dependent manner. In accordance with IKK inhibition, quercetin inhibited LPS-elicited the DNA binding activity of NF-κB (p65/p50 heterodimer) in a dose-

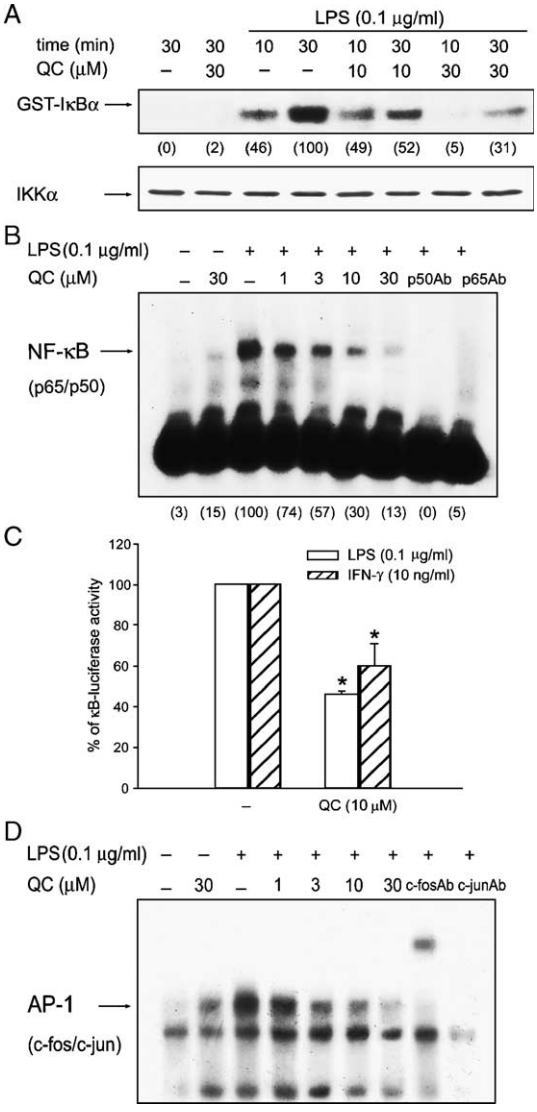

Fig. 2. Quercetin (QC) inhibits IKK, NF-κB and AP-1 activation. (A) BV-2 cells were pretreated with QC at the concentrations indicated for 30 min, and LPS (0.1 μg/ml) was then treated for 10 or 30 min. Using specific IKKα, IKKβ antibody and protein A/G beads, IKK from equal protein amounts of cell lysates was immunoprecipitated, followed by kinase activity assay and immunobloting. GST-IκBα was used as IKK substrate, and its auto-radioactivity reflecting phosphorylation state indicated the enzymatic activity of IKK. The equal density of IKKα indicated the same kinase level performed in enzyme assay. The results are representative of three different experiments. (B, D) Nuclear extracts from cell lysates were assayed for binding activity with specific oligonucleotides containing respective binding sequences for NF-κB (B) or AP-1 (D). Cells were treated with 0.1 μg/ml LPS and/or QC for different concentrations (1–30 μM). After 1 h incubation, equivalent amounts of nuclear extracts were analyzed by EMSA. Specific antibodies for p65, p50, c-*fos* and c-Jun were included in the binding solution as indicated. (C) Cells were transfected with κB-Luc for 24 h. Then cells were incubated with LPS, IFN-γ and/or QC. Twenty-four hours later, luciferase activity in the cell lysates was determined, and normalized by lacZ transfection efficiency, and expressed as a percentage of the control response without stimulus treatment. The data in (C) are the mean±S.E.M. from at least three independent experiments, each performed in duplicate. *$P<0.05$ indicates the statistical significance as compared with control of LPS and IFN-γ treatment alone.

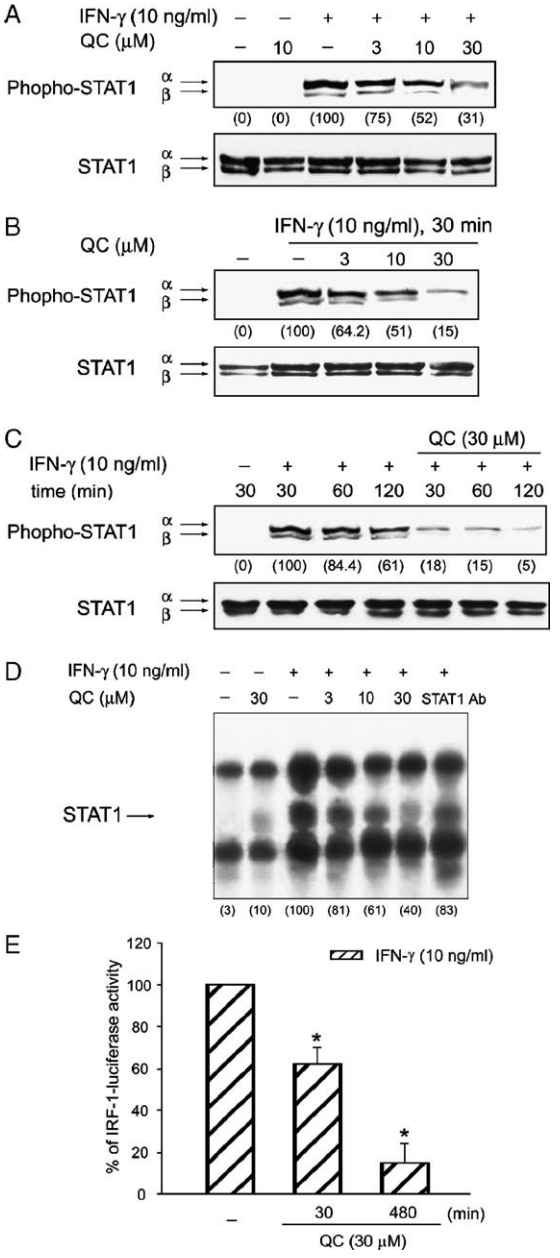

Fig. 3. Quercetin (QC) inhibits STAT1 phosphorylation, STAT1-specific DNA-protein complex formation and IRF-1-Luc activity. BV-2 microglia were pretreated with QC at the concentrations indicated for 30 min before the stimulation with IFN-γ (10 ng/ml) for different periods. Equivalent protein amounts of total cell lysates (A) and nuclear extracts (B, C) were subjected to SDS-PAGE, followed by immunoblotting and detection with antibody specific to phospho-STAT1. The STAT1α/β level was considered as an internal control. (D) In some experiments, after 1 h stimulation with IFN-γ, equivalent amounts of nuclear extracts were assayed for binding activity with specific oligonucleotides containing respective binding sequences for STAT1. (E) Cells were transfected with IRF-1-Luc for 24 h. Then cells were incubated with IFN-γ and/or QC. Twenty-four hours later, luciferase activity in the cell lysates was determined, and normalized by lacZ transfection efficiency, and expressed as a percentage of the control response without stimulus treatment. The data in (E) are the mean±S.E.M. from at least three independent experiments, each performed in duplicate. *$P<0.05$ indicates the statistical significance as compared with control of IFN-γ treatment alone.

dependent manner (Fig. 2B). The inhibitory effect of quercetin on NF-κB activation was further confirmed by NF-κB reporter assay. The result showed that quercetin at 10 μM inhibited LPS-induced NF-κB luciferase activity (Fig. 2C). Moreover EMSA indicated that the LPS-induced DNA binding activity of the increased formation of c-*fos* and c-Jun heterodimer in AP-1 complex was suppressed by quercetin treatment (Fig. 2D).

3.3. Quercetin inhibits IFN-γ-induced STAT1 and NF-kB activity

Since Tyr701 phosphorylation of STAT1 following IFN-γ stimulation is associated with iNOS expression (Lowenstein et al., 1993; Chen et al., 2002), we examined the effect of quercetin on STAT1 phosphorylation. As shown in Fig. 3A, upon treatment of BV-2 microglia with IFN-γ, a dramatic STAT1 phosphorylation at Tyr701 was observed. Quercetin individually did not affect STAT1 phosphorylation, but concentration-dependently attenuated the response of IFN-γ. Consistent with this finding, nuclear level of the phosphorylated STAT1 after IFN-γ exposure increased with less extent in quercetin treated cells (Fig. 3B and C). Fig. 3D further showed that IFN-γ-elicited STAT1 activation, as assessed by EMSA, was inhibited by quercetin. This effect of quercetin displays the concentration-dependency within 3–30 μM. With use of the reporter construct containing the STAT1 binding element in IRF-1 promoter, the inhibitory effect of quercetin on STAT1 activation was further evidenced (Fig. 3E). As NF-κB activity is essential for iNOS gene transcription, we no doubt examined this event in IFN-γ-treated cells. Fig. 2C showed that, similar to LPS stimulation, IFN-γ-elicited NF-κB reporter activity was attenuated by quercetin.

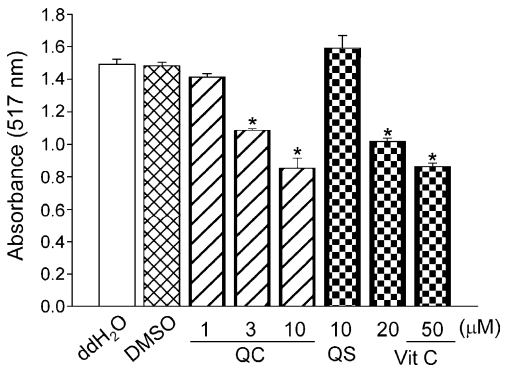

Fig. 4. Effects of quercetin (QC) and quercetin-3′-sulfate (QS) on DPPH scavenging activities. DPPH (100 μM) was treated with vehicle (DMSO), QC (1–10 μM), QS (10 μM), or vitamin C (20 or 50 μM) at room temperature for 30 min, and then the absorbance at 517 nm was measured. *$P<0.05$ indicates the significant antioxidative effect.

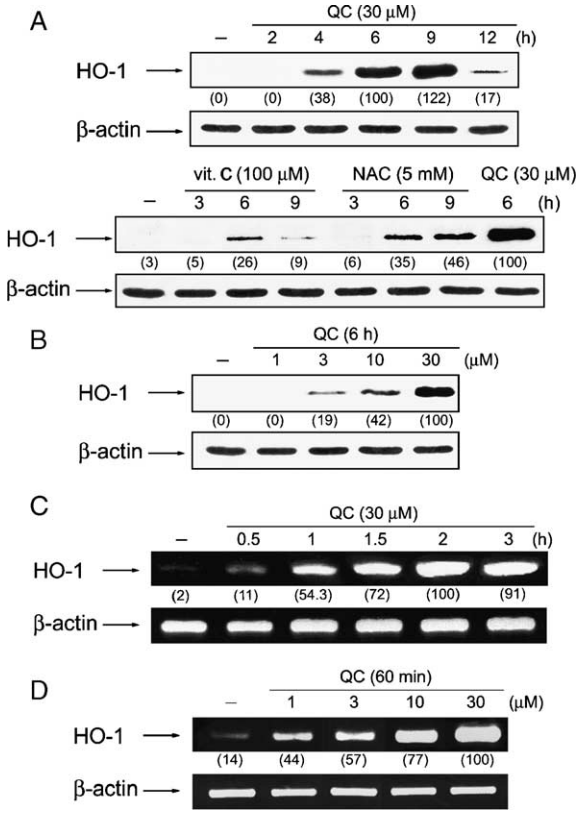

Fig. 5. Effects of antioxidants on heme oxygenase-1 (HO-1) induction. Cells were treated with indicated concentrations of quercetin (QC), *N*-acetyl-L-cysteine (NAC) (5 mM), or vitamin C (100 μM) for different intervals. The cell lysates were prepared and the expression levels of heme oxygenase-1 were examined by immunoblot analysis (A, B). In some experiments, total mRNA of cells treated with QC at concentrations and periods as indicated was isolated, and RT-PCR for heme oxygenase-1 mRNA was performed. Internal control of β-actin mRNA was also determined (C, D). The results are representative of three separate experiments.

3.4. Antioxidant-linked heme oxygenase-1 induction contributes to quercetin inhibition of iNOS expression

As previous studies demonstrated quercetin possesses antioxidant activity, we next attempt to elucidate the antioxidant property of quercetin and its conjugated derivative using the in vitro DPPH assay. Compared with vitamin C at 20 and 50 μM, quercetin elicited comparable antioxidant activity at 3 and 10 μM, while antioxidant activity of quercetin-3′-sulfate was not significant (Fig. 4). According to the abilities of some antioxidants [e.g. pyrrolidine dithiocarbamate (PDTC) and curcumin] to induce heme oxygenase-1 accompanied by the anti-inflammatory effects (Hartsfield et al., 1998; Motterlini et al., 2000), including the inhibition of iNOS/NO response (Koppal et al., 2000; Lin et al., 2003; Otterbein et al., 2003), we verify this event in the case of quercetin. At basal state a weak immunoreactivity of heme oxygenase-1 protein was detected in BV-2 cells, while quercetin treatment markedly induced it. This action displayed the dose- (Fig. 5B) and time-dependency, occurring after 4 h exposure and

peaking within 6–9 h (Fig. 5A). Meanwhile vitamin C (100 μM) and *N*-acetyl-L-cysteine (5 mM) also induced heme oxygenase-1 expression in a time-dependent manner. Since heme oxygenase-1 is an inducible gene product, we determined whether the effect of quercetin results from increased mRNA level. Using RT-PCR analysis, we found that heme oxygenase-1 mRNA level was time- and concentration-dependently increased by quercetin (Fig. 5C and D). The incubation period as short as 30 min was sufficient to induce heme oxygenase-1 mRNA, which was further increased and maintained at least for 3 h.

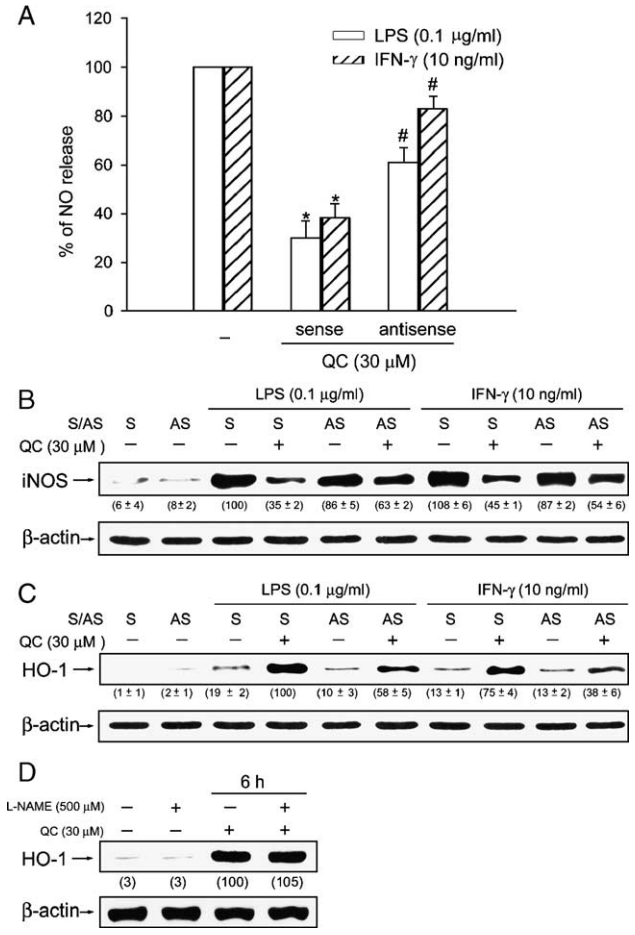

Fig. 6. Heme oxygenase-1 (HO-1) induction involves in the inhibition of iNOS/NO expression by quercetin (QC). Cells pretreated the heme oxygenase-1 antisense or sense (1 μM) for 12 h were incubated with LPS (0.1 μg/ml), IFN-γ (10 ng/ml) and/or QC (30 μM). Twenty-four hours later, NO production (A), iNOS (B) and heme oxygenase-1 (C) expression were determined. (D) BV-2 microglia were pretreated with L-NAME (500 μM) for 30 min and then stimulated with QC (30 μM) for 6 h. Then heme oxygenase-1 expression was determined. The β-actin level was considered as an internal control (B–D). The data in (A) are the mean±S.E.M. from at least three independent experiments, each performed in duplicate. $^{*}P<0.05$ indicates the statistical significance as compared with control of LPS- or IFN-γ treatment alone. $^{\#}P<0.05$ indicates the statistical significance as compared with QC inhibition in the presence of heme oxygenase-1 sense oligonucleotides. Results are representative of three independent experiments with similar results.

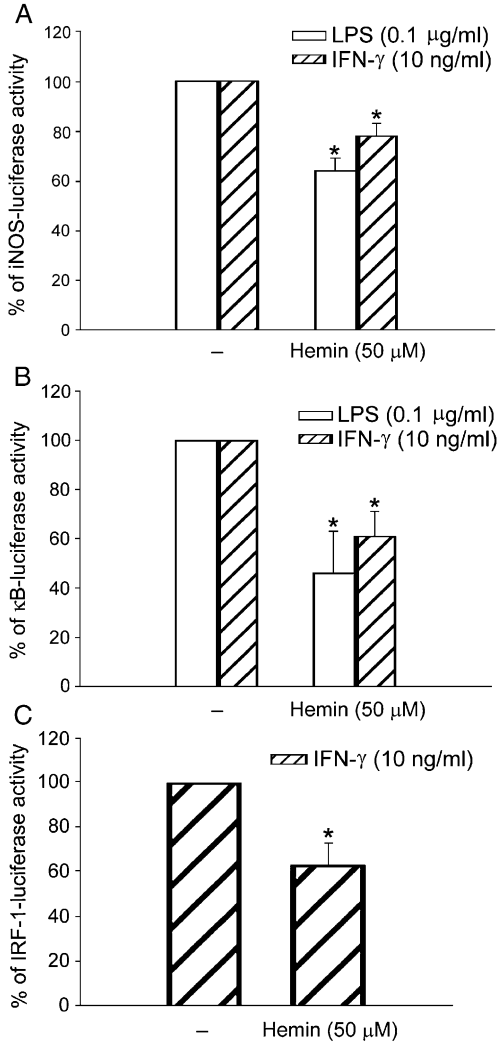

Fig. 7. Hemin inhibits LPS- and IFN-γ-induced iNOS, NF-κB and IRF-1 gene expressions. BV-2 microglia cells were transfected with iNOS-Luc (A), κB-Luc (B), and IRF-1-Luc (C) for 24 h. Then cells were incubated with LPS, IFN-γ and/or hemin. Twenty-four hours later, luciferase activity in the cell lysates was determined, and normalized by lacZ transfection efficiency, and expressed as a percentage of the control response without stimulus treatment. All data are the mean±S.E.M. from at least three independent experiments, each performed in duplicate. $^{*}P<0.05$ indicates the statistical significance as compared with control response of LPS or IFN-γ treatment alone.

To understand whether heme oxygenase-1 upregulation by quercetin acts as a feedback regulator in iNOS expression, we used antisense strategy. As shown in Fig. 6A and B, quercetin-induced inhibition of LPS- and IFN-γ-mediated NO production and iNOS expression were attenuated in cells treated with heme oxygenase-1 antisense oligodeoxynucleotide. Under this condition, quercetin-induced marked heme oxygenase-1 protein expression was accordingly diminished by 40–50% (Fig. 6C), suggesting heme oxygenase-1 induction as a negative feedback regulator for iNOS expression. At basal state, LPS and IFN-γ alone can only induce a slight increase of heme oxygenase-1 protein. Otherwise, when cells were treated with NOS inhibitor, L-

NAME (500 μM), we found that the effect of quercetin on HO-1 induction was not affected (Fig. 6D).

3.5. The heme oxygenase-1 inducer, hemin, inhibits LPS- and IFN-γ-induced iNOS, NF-κB and IRF-1 gene expressions

Results described above suggested that heme oxygenase-1 upregulation is involved in the inhibition of LPS- and IFN-γ-induced NO production by quercetin. To further confirm this issue, a well-known heme oxygenase-1 inducer hemin was used in the study. We pretreated the BV-2 cells with hemin (50 μM) for 6 h to induce heme oxygenase-1 protein expression. In the presence of LPS (0.1 μg/ml) or IFN-γ (10 ng/ml), hemin exhibited a significant inhibition of iNOS reporter activity (Fig. 7A), NF-κB reporter activity (Fig. 7B) and IFN-γ-induced IRF-1 reporter activity (Fig. 7C). These data suggest that the inhibitory action of hemin paralleled with quercetin on LPS- and IFN-γ-induced iNOS gene expression and activated transcription factors.

3.6. Signaling pathways of quercetin-induced heme oxygenase-1 expression

Numbers of pathways have been implicated in transmitting the extracellular signals to the nuclei for heme oxygenase-1 gene expression. To investigate the signal transduction pathway(s) involved in regulating heme oxygenase-1 expression in response to quercetin, we examined the effects of various pharmacological inhibitors of signaling intermediates on heme oxygenase-1 protein levels. As shown in Fig. 8, we found that treatment of cells with the tyrosine kinase inhibitors (PP2 and herbimycin A), p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580), c-Jun NH_2-terminal kinase (JNK) inhibitor (SP600125), and mitogen-activated protein (MAP)/ERK kinase (MEK) inhibitors (PD98059 and U0126) reduced quercetin-induced heme oxygenase-1 induction. KT5823 (a protein kinase G inhibitor), KT5720 (a protein kinase A inhibitor), Y27632 (a Rho kinase inhibitor), and GF109203X (a protein kinase C inhibitor) did not have any effects. The action of these inhibitors alone was also shown in Fig. 8. We found that most pharmacological inhibitors do not affect heme oxygenase-1 induction, however, Y27632, U0126 and PD98059 induce a slight increase. These results suggest the participation of tyrosine kinase, possibly the Src members, and MAPK family in quercetin-induced heme oxygenase-1 expression in BV-2 cells.

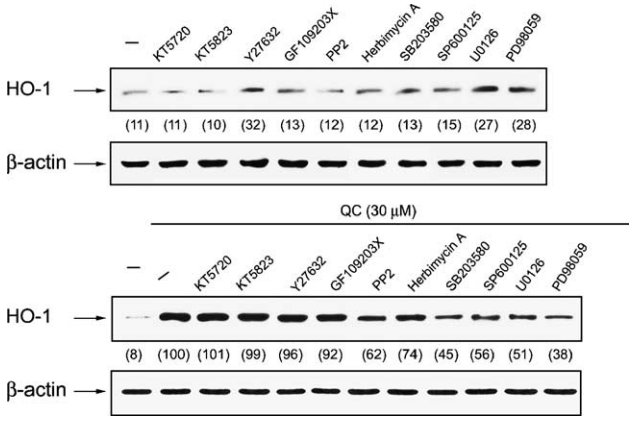

Fig. 8. Effects of pharmacological manipulations on quercetin-induced heme oxygenase-1 induction. BV-2 microglia were pretreated with KT5720 (1 μM), KT5823 (1 μM), Y27632 (30 μM), GF109203X (3 μM), PP2 (1 μM), herbimycin A (3 μM), SB203580 (10 μM), SP600125 (10 μM), U0126 (1 μM) or PD98059 (30 μM) for 30 min, and then stimulated with or without QC (30 μM) for 6 h. Cell lysates were harvested for immunoblotting with heme oxygenase-1 antibody. The β-actin protein level was considered as an internal control. The trace shown is a representative of three separate experiments.

4. Discussion

Several studies have demonstrated that polyphenolic flavonoids derived from herbs possess several biological activities, including free radical scavenging, anti-inflammatory and anti-cancer activities. Since microglia-associated NO production plays a crucial role in inflammation and neurodegenerative diseases, prevention of iNOS expression represents an important therapeutic goal. Although there are several publications analyzing the effects of quercetin, the most common flavonoid glycoside consumed in the diet daily, on murine iNOS expression in macrophages and glial cells (Chen et al., 2001, 2004b; Blonska et al., 2004; Jung and Sung, 2004), currently only one report showed the inhibitory effect of flavonoid baicalein on iNOS expression in microglia (Chen et al., 2004a). To address whether quercetin possesses such action in microglia, we elucidated in this study the ability and molecular mechanisms of quercetin in inhibition of LPS- and IFN-γ-induced NO production and iNOS gene expression in BV-2 microglia. To our knowledge, this is the first time that the inhibitory effect of quercetin on STAT1 activity has been reported.

The murine iNOS promoter contains numerous consensus sequences for transcription factors, NF-κB, AP-1 and STAT1, and among them NF-κB plays an essential role (Xie et al., 1993). Previous studies have indicated these factors are regulated by redox changes, especially for NF-κB where antioxidants have long been considered as inhibitors of NF-κB (Meyer et al., 1994; Hecker et al., 1996; Zingarelli et al., 2003). Our present study using in vitro DPPH assay confirmed quercetin as an antioxidant whose potency is approximately 5-fold greater than vitamin C. Accordingly inhibitory effects of quercetin obtained from EMSA and promoter activity assays point to a redox-sensitive step in the activation of NF-κB, AP-1 and STAT1. Inhibition of LPS- and IFN-γ-induced iNOS gene expression by quercetin was well correlated with inhibition of these transcription factors. In contrast quercetin-3'-sulfate had no antioxidant and NO inhibitory activities. Furthermore we also found two common antioxidants, *N*-acetyl-L-cysteine and PDTC, could exert similar suppressive effect on LPS- and IFN-γ-

induced NO production as quercetin did in BV-2 cells (data not shown). We provided new evidence from in vitro model indicating that antioxidative activity of quercetin contributes to the suppression of signaling pathways leading to NF-κB, AP-1 and STAT1 activation, which are involved in regulating the key intracellular processes causing inflammatory consequences. These anti-inflammatory mechanisms also influence NO production and affect extent of iNOS gene expression in BV-2 cells. Accumulating evidence has indicated heme oxygenase-1 functions as a "therapeutic funnel". Since heme oxygenase-1 has been shown to have anti-inflammatory, anti-apoptotic, and anti-proliferative effects, investigation on its salutary effects in diseases and interest in searching compounds of heme oxygenase-1 inducible activity are now active and rapidly evolving (Morse and Choi, 2002). In parallel to the inhibition of iNOS/NO response at the same concentration range, we found quercetin can induce increases in heme oxygenase-1 mRNA and protein expression in microglial cells. This effect is also mimicked by some antioxidants, such as vitamin C, *N*-acetyl-L-cysteine in BV-2 cells (our present findings), curcumin and caffeic acid phenethyl ester in vascular endothelial cells and astrocytes (Motterlini et al., 2000; Scapagnini et al., 2002). Quercetin pretreatment significantly inhibited NO production in LPS- and IFN-γ stimulated BV-2 cells. Data from antisense approach further suggest the involvement of heme oxygenase-1 expression by quercetin in its downregulation action on iNOS and NO. We also found that hemin attenuated LPS- and IFN-γ-induced iNOS, NF-κB and IRF-1 reporter activities accompanied with heme oxygenase-1 protein induction (Fig. 7). This notion is consistent to previous findings proposing that heme oxygenase-1 may serve to negatively modulate iNOS-dependent NO production via NF-κB interruption (Lee et al., 2003; Sumi and Ignarro, 2004; Yang et al., 2004). Notably the effects of quercetin on iNOS inhibition and heme oxygenase-1 induction in BV-2 microglia occur at concentrations close to those present in the human diet (Manach et al., 1995).

Previous papers indicated that activation of intracellular signaling pathway was important for heme oxygenase-1 induction. In general, heme oxygenase-1 gene expression is induced by stimuli that activate MAPKs (Immenschuh and Ramadori, 2000; Zhang et al., 2002). Three major subgroups of the MAPK family identified to date include extracellular signal-regulated kinase 1/2 (ERK1/2), JNK and p38 MAPK. Depending on the stimuli specificity, contradictory results on the regulatory role of different MAPK pathways for heme oxygenase-1 gene expression were observed (Ludwig et al., 1998; Oguro et al., 1998; Alam et al., 2000; Ning et al., 2002; Zhang et al., 2002; Chen et al., in press). In the case of quercetin, our present results indicated the signal transduction involving the MAPK family and tyrosine kinase.

There is growing interest in dietary therapeutic strategies to combat oxidative stress-induced damage to the CNS associated with specific pathophysiological processes. Recently, several reports described the cytoprotective and neuroprotective effects of flavonoids and their relevant conjugates and metabolites (Schroeter et al., 2001; Spencer et al., 2001). Evidence from these studies suggests that flavonoid neuroprotection may result from their hydrogen-donating antioxidants or interactions with different signaling cascades, and the general bioavailability and particular presence in the brain in vivo appear to play an important role in the expression of neuroprotective capacity of flavonoids. Among the few investigations addressed this issue, the current study provided strong evidence that structurally different flavonoids are able to enter different regions of the brain, albeit to differing extents (Youdim et al., 2004). Surprisingly, Dajas et al. (2003) increased the possibility of quercetin crossing the blood–brain-barrier by mixing it with lecithin, generating a liposomal preparation. This preparation is a recognized way of transporting molecules in the body, increasing the time of interaction of a given molecules with its target. Therefore, quercetin and other flavonoids are likely to exert protective effects at glia and vessels in the brain.

In conclusion, our results showed the anti-inflammatory activity of quercetin in inhibiting iNOS gene transcription induced by LPS and IFN-γ in microglial cells. This action is resulting from the suppression of NF-κB, AP-1 and STAT1, and involves the induction of heme oxygenase-1 as well. The results of these experiments contribute to an integrated mechanism extending previous hypotheses, and highlight the therapeutic potential of quercetin as a novel anti-inflammatory and anti-neurodegenerative drug. It is warranted to determine the efficacy of this application for clinical use.

Acknowledgements

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