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Inhibition of nitric oxide production by quercetin in endotoxin/cytokine-stimulated microglia

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ABSTRACT

Aims: Flavonoids possess several biological and pharmacological activities. Quercetin, a naturally occurring flavonoid, has been shown to down-regulate inflammatory responses and provide neuroprotection. However, the mechanisms underlying the anti-inflammatory properties of quercetin are poorly understood. In the present study, we investigated the modulatory effect of quercetin against neuroinflammation.
Main methods: We herein describe a potential regulatory mechanism by which quercetin suppresses nitric oxide (NO) production by lipopolysaccharide (LPS)/interferon-γ (IFN-γ)-stimulated BV-2 microglial cells. The underlying regulatory cascades were approached by biochemical and pharmacological strategies.
Key findings: Quercetin produced an inhibitory effect on inducible nitric oxide synthase (iNOS) expression and NO production. Biochemical studies revealed that the anti-inflammatory effect of quercetin was accompanied by the down-regulation of extracellular signal-regulated kinase, c-Jun N-terminal kinase, p38, Akt, Src, Janus kinase-1, Tyk2, signal transducer and activator of transcription-1, and NF-κB. In addition, quercetin scavenged free radicals and produced inhibitory effects on serine/threonine and tyrosine phosphatase activities. Intriguingly, the accumulation of lipid rafts, which is the critical step for signaling, was disrupted by quercetin.
Significance: The data indicate that the anti-inflammatory action of quercetin may be attributable to its raft disrupting and anti-oxidant effects. These distinct mechanisms work in synergy to down-regulate iNOS expression and NO production.

Introduction

Microglia are critical effector cells of immune surveillance and host defense in the central nervous system (CNS) under normal conditions. In response to brain injury, microglia become activated by changing morphology from resting ramified to amoeboid phagocytic cells, proliferating, migrating to injured sites, and producing biologically active molecules such as nitric oxide (NO). The overproduction of NO by microglia in the CNS has pro-inflammatory and regulatory effects and is believed to contribute to progressive neuronal damage in neurodegenerative diseases. Activated microglia and excessive production of NO by the high-output NO synthesizing enzyme inducible nitric oxide synthase (iNOS) are observed in various neurological diseases including stroke, Parkinson's disease, and Alzheimer's disease (Simmons and Murphy 1992; Gonzalez-Scarano and Baltuch 1999). Therefore, novel pharmacological agents that can inhibit iNOS expression and NO production by microglia represent a promising therapeutic strategy to control the potentially detrimental pro-inflammatory activity of microglia in NO-mediated neurodegenerative damage.

There is growing interest in dietary therapeutic strategies to combat inflammation-associated damage in the CNS. Many studies have demonstrated the beneficial role of a diet rich in polyphenolic compounds in protecting against neurological diseases. Flavonoids, a group of well-known plant-derived polyphenolic compounds, are widely distributed in plants and have been demonstrated to possess health-promoting effects involving anti-oxidant and anti-inflammatory activities (Mora et al. 1990; Chen et al. 2004a). Quercetin, a typical flavonol-type flavonoid, is ubiquitously present in the diet and exhibits a variety of modulating effects. Quercetin has been reported to exhibit beneficial effects against neurological disorders. Specifically, quercetin reduced neuronal damage and improved neurobehavioral deficit in

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ischemic animals (Cho et al. 2006; Pu et al. 2007). In vitro, quercetin inhibited microglia activation and protected neurons against inflammatory damage (Chen et al. 2005; Bureau et al. 2008). As previously reported, quercetin inhibits NO production at the transcriptional level by macrophages and microglia. The suppression of iNOS expression by quercetin is accompanied by inactivation of NF-κB and signal transducer and activator of transcription-1 (STAT1) and elevated expression of heme oxygenase-1 or interleukin-10 (Chen et al. 2005; Comalada et al. 2006). In light of these observations, it is reasonable to propose that the inhibition of NO production by quercetin could be one of the mechanisms responsible for its anti-inflammatory effects and the suppressive mechanisms of iNOS expression could be multifactorial. As NO derived from microglia is a critical factor in brain damage, we extended this study by examining the effects of quercetin on upstream signaling execution critical to iNOS transcription in murine BV-2 microglial cells and attempted to clarify the underlying molecular basis. We hope the results will highlight the therapeutic potential of quercetin as a novel anti-inflammatory adjuvant in neurodegenerative diseases.

Materials and methods

Cell culture

Murine BV-2 microglia cell line was kindly donated by Dr. Hong JS (Research Triangle Park, NIEH, NIH). BV-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies), 100 U/ml penicillin (Gibco Life Technologies) and 100 μg/ml streptomycin (Gibco Life Technologies), and were maintained in a humidified incubator with 5% CO_2. In all experiments, cells were treated with endotoxin lipopolysaccharide (LPS) (100 ng/ml *Escherichia coli*, serotype 0111:B4, Sigma Chemical Co.)/interferon gamma (IFN-γ) (10 U/ml, Sigma Chemical Co.) in serum-free DMEM. Pharmacological agents were dissolved in DMSO and the final concentration of DMSO added to cells never exceeded 0.1%.

NO determination

NO production by cultured cells was determined by the measurement of total nitrite, a stable oxidation product of NO based on the Griess reaction (Green et al. 1992). Briefly, 50 μl of culture supernatant was reacted with an equal volume of Griess reagent (1 part 0.1% naphthylethylenediamine, 1 part 1% sulfanilamide in 5% H_3PO_4) in 96-well plates for 10 min at room temperature in the dark. The absorbance at 550 nm was determined using a microplate reader (PowerWaveX 340, Bio-Tek Instruments, Inc.). A standard nitrite curve was generated in the same fashion using $NaNO_2$.

Isolation of RNA and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were extracted by the single-step method developed by Chomczynski and Sacchi (1987). cDNA synthesis was carried out at 42 °C for 1 h using 5 μg total RNAs and random primer. DNA fragments of specific genes and internal controls were co-amplified in a tube containing Taq DNA polymerase (Promega, WI, USA) and 0.8 μM of each sense and antisense primer. The PCR reaction was performed with a DNA thermal cycler (Perkin Elmer-Cetus) under the following conditions: 1 cycle of 94 °C for 3 min; 28 cycles of 94 °C for 50 s, 58 °C for 40 s, and 72 °C for 45 s; and then 1 cycle of 72 °C for 5 min. In preliminary experiments, we found that the PCR and product amplification were linear ($r = 0.946–0.977$) under this PCR condition. The amplified DNA fragments were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide (EtBr). The DNA band's intensity was determined by a computer image analysis system (Alpha Innotech Corporation, IS1000). Oligonucleotides (synthesized by Gibco Life Technologies) used in this study were as follows: 5′-ACAACGTGGAGAAAACCCCAGGTG and 5′-ACAGCTCCGGGCATCGAAGACC for inducible nitric oxide synthase (iNOS); 5′-TCCTGTGGCATCCACGAAACT and 5′-GGAGCAATGATCTTGATCTTC for β-actin.

Western blot

Cells were washed twice with phosphate-buffered saline (PBS) and harvested in Laemmli SDS sample buffer. The protein concentration in the supernatant was determined by Bradford assay. Protein extracts were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). Membranes were first incubated with 5% nonfat milk in PBS for 1 h at room temperature to reduce nonspecific binding. Membranes were washed with PBS containing 0.1% Tween-20 (PBST), and then incubated for 1 h at room temperature with the indicated antibodies including iNOS, β-tubulin, caveolin-1, β-actin, phosphorylated and non-phosphorylated forms of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, Akt, signal transducer and activator of transcription-1 (STAT1), Src, Janus kinase-1 (Jak1), and Tyk2 (Santa Cruz Biotechnology). After the membranes were washed again with PBST buffer, a 1:10,000 (v/v) dilution of horseradish peroxidase-labeled IgG was added at room temperature for 1 h. The blots were developed using ECL Western blotting reagents (Amersham Pharmacia Biotech).

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described previously (Chen et al. 2004b). Nuclear extract (5 μg) was used for EMSA. The oligonucleotide of NF-κB was synthesized and labeled with biotin (5′-AGTTGAGGGGACTTTCCCAGGC). The binding reaction mixture included 1 μg of poly (dI-dC), 0.1 μg poly L-lysine, and 100 fmol biotin-labeled DNA probe in 20 μl binding buffer (10 mM HEPES, pH 7.6; 50 mM NaCl; 0.5 mM $MgCl_2$; 0.5 mM EDTA; 1 mM dithiothreitol; and 5% glycerol). The DNA/protein complex was analyzed on 6% native polyacrylamide gels.

Phosphatase assay

Cells were resuspended with PBS, subjected to three rounds of freeze/thaw, and then sonicated for 10 s. Serine/threonine and tyrosine phosphatase activities were measured using a commercially available serine/threonine phosphatase assay kit and tyrosine phosphatase assay kit (Molecular Probes), respectively. The generated fluorescent product was determined by a fluorometer (E_x 358 nm and E_m 452 nm).

Free radical determination

Intracellular oxidative stress was assayed by measuring the intracellular oxidation of dichlorofluorescein, as described previously (Chen and Liao 2002). Cultures were loaded with 10 μM 2′,7′-dichlorofluorescein at 37 °C for 1 h, washed, and then subjected to the treatment. The fluorescence signal of oxidized 2′,7′-dichlorofluorescein was measured using a fluorometer (E_x 485 nm and E_m 510 nm).

Cholera toxin B subunit staining

For cholera toxin B subunit staining, cells were fixed with 3.7% formaldehyde for 10 min. After being washed with PBS, cells were incubated with Alexa Fluor 488-conjugated cholera toxin B subunit