Inhibitory Effects of a Rice Hull Constituent on Tumor Necrosis Factor α, Prostaglandin E2, and Cyclooxygenase-2 Production in Lipopolysaccharide-Activated Mouse Macrophages

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ABSTRACT: Isovitexin, isolated from rice hull of *Oryza sativa,* has been characterized as a potent antioxidant. Its antioxidant activity, determined on the basis of inhibition of lipid peroxidation by the Fenton reaction, was comparable with that of α-tocopherol, a well-established antioxidant. Isovitexin was able to reduce the amount of hydrogen peroxide production induced by lipopolysaccharide (LPS) in mouse macrophage RAW264.7 cells. In this study, we assessed its effects on the production of tumor necrosis factor α (TNF-α), prostaglandin E2 (PGE_2), and the expression of cyclooxygenase-2 (COX-2) in LPS-activated RAW 264.7 macrophages. Isovitexin inhibited the release of TNF-α, a proinflammatory cytokine, upon LPS activation with a 50% inhibitory concentration (IC_{50}) of 78.6 μM. Isovitexin markedly reduced LPS-stimulated PGE_2 production in a concentration-dependent manner, with an IC_{50} of 80.0 μM. The expression of COX-2 was also inhibited by isovitexin treatment. Our results suggest that suppression of ROS-mediated COX-2 expression by isovitexin is beneficial in reducing inflammation and carcinogenesis.

KEYWORDS: antioxidant; inflammation; isovitexin; COX-2; PGE_2; tumor necrosis factor

INTRODUCTION

Oxidative stress has been implicated in a variety of pathological processes, including aging, cancer, diabetes mellitus, atherosclerosis, neurological degeneration, and arthritis. Increased uptake of antioxidants may prevent organ injury associated

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with excessive generation of reactive oxygen species (ROS).[1,2] ROS have been shown to initiate a wide range of toxic oxidative reactions.[3] The effects of oxidants on signaling pathways are often characterized as resulting from heightened oxidative stress. Many antioxidants also exhibit anti-inflammatory effects. Theses observations provide a significant molecular basis for understanding the mode of actions of selected dietary ingredients in preventing diseases associated with heightened oxidative stress.

Tumor necrosis factor α (TNF-α), a proinflammatory cytokine, and prostaglandin E2 (PGE_2) are key mediators in inflammatory reaction. TNF-α generated in inflammation may induce tissue damage.[4] Prostaglandins, especially PGE_2, are involved in stimulating cell proliferation, tumor growth, and suppressing the immune response to malignant cells. Overproduction of prostaglandins from upregulation of cyclooxygenase-2 (COX-2) in cells had been associated with malignant growth.[5] COX-2 is rapidly induced by tumor promoters, growth factors, cytokines, and mitogens in various cell types.[6] Many cell types associated with inflammation, such as macrophages and endothelial cells and fibroblasts, may be induced to overexpress COX-2.[7] Treatment with TPA (12-*O*-tetradecanoyl-phobol-13-acetate) in mice led to edema and papilloma formation by enhancing COX-2 expression. Specific COX-2 inhibitors could counteract these biological events. Suppression of COX-2 induction and or its activity may be an effective approach for the prevention of carcinogenesis in several organs. Selected inhibitors of COX-2 may also have a therapeutic role in certain cancers.

Rice is an important dietary staple in Asia, where the incidence of breast and colon cancer is markedly lower than that in the western world.[8] It has been reported that rice constituents counteract chemical-induced mutagenicity, tumor promotion, and carcinogenicity.[9] Constituents from rice bran have been found to be beneficial for cancer prevention by epidemiological survey. Rice bran contains several classes of chemopreventive agents (e.g., flavonoids and their glycosides, tocotrienols, and γ-oryzanol). Isovitexin and related flavonoids are constituents of the rice hull of *Oryza sativa* and have been shown to exhibit potent antioxidant activity,[10] including inhibition of xanthine oxidase, protection of DNA from oxidative damage, and prevention of heavy-metal–induced cell injury.[11] Here, we further studied the antioxidative properties of isovitexin by examining its effects on LPS-induced PGE_2 and TNF-α production and COX-2 expression in the murine macrophage-like cell line RAW264.7.

MATERIALS AND METHODS

Materials

Isovitexin, a glycosylflavonoid, was isolated from rice hull as described previously.[12] The final product showed a major peak on capillary chromatography (P/ACE 5000; 75 μm × 37 cm [Beckman, Fullerton, CA]; borax buffer, 20 mM, pH = 10.0; UV absorbance at 214 nm; 25°C; UV absorbance at 214 nm, 25°C, 10K voltage; injection pressure, 80 psi; migration time: 6.64 min.). LPS (*Escherichia coli* O127:B8) and chemicals were purchased from Sigma (St. Louis, MO) unless specified.

Cell Culture

The mouse monocyte–macrophage cell line RAW 264.7 (ATCC TIB-71) was cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco). Cells were plated in 24-well plates or petri dishes before activation by LPS (100 ng/mL). Isovitexin dissolved in dimethyl sulfoxide (DMSO) with the final DMSO concentration of less than 0.2% (vol/vol) was administered with LPS. Control samples contained the same concentration of DMSO.

Lipid Peroxidation

Ethyllinoleate was oxidized by the Fenton reaction (Fe^{2+}/H_2O_2). In selected experiments, equal concentration (30 μM) of apigenin, isovitexin, and α-tocopherol were added to 0.5 mL of an aqueous solution containing 1.5 mg/L ethyl linoleate, 0.25 mM Trizma–HCl/0.75 mM KCl buffer (pH 7.4), 0.2% *N*-lauroyl sarcosine, 1 μM ferrous chloride, and 0.5 μM hydrogen peroxide in a 2-mL microtube. The mixture was incubated for 16 h at 37°C. The quantity of oxidation was measured by the thiobarbituric acid (TBA) assay.[11] The antioxidative activity of the samples was calculated according to the following formula: antioxidative activity (%) = 1 – (absorbance of sample/absorbance of control) × 100. The level of TBA-reactive substance (TBARS) by autooxidation of ethyllinoleate was calculated as the amount of malondialdehyde.

Determination of TNF-α Release

Murine macrophages were seeded in 24-well plates at a density of 5.0×10^5 cells/well the day before the experiment. Cells were treated with or without isovitexin (10–100 μM) and/or LPS (100 ng/mL) in 500 μL of medium containing 10% fetal bovine serum for 1 h at 37°C. TNF-α levels in the media were then determined by a quantitative sandwich enzyme-linked immunosorbent assay using the commercially available mouse TNF-α immunoassay kit (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. All experiments were done in triplicate.[13]

PGE_2 Assay

Cells were plated at a density of 1.0×10^6 cells/mL in 24-well culture plate and stimulated with LPS (50 ng/mL) in the presence or absence of various concentrations of isovitexin for 18 h. The culture medium of control and treated cells was collected and centrifuged. The level of PGE_2 released into culture medium was determined using an enzyme immunoassay according to the manufacturer's instructions (Amersham, Buckinghamshire, UK).[4]

Western Blotting

The cellular protein fraction was prepared using lysis buffer containing 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris–HCl (pH 7.9), 100 μM β-glycerophosphate, 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Proteins (50 μg) were separated on so-

dium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA). The membrane was incubated with an anti–COX-2 monoclonal antibody (Oncogene Science, Cambridge, UK) containing 1% bovine serum albumin and 0.2% NaN_3 overnight at 4°C. After incubation with horseradish peroxidase–conjugated anti–mouse immunoglobulin G antibody (Oncogene Science, Cambridge, UK), the immunoreactive bands were visualized with enhanced chemiluminescent reagents (ECL; Amersham, Buckinghamshire, UK). Relative protein content in each band was assessed using a densitometer (Alpha Innotech IS-1000; Digital Imaging System, San Leandro, CA).

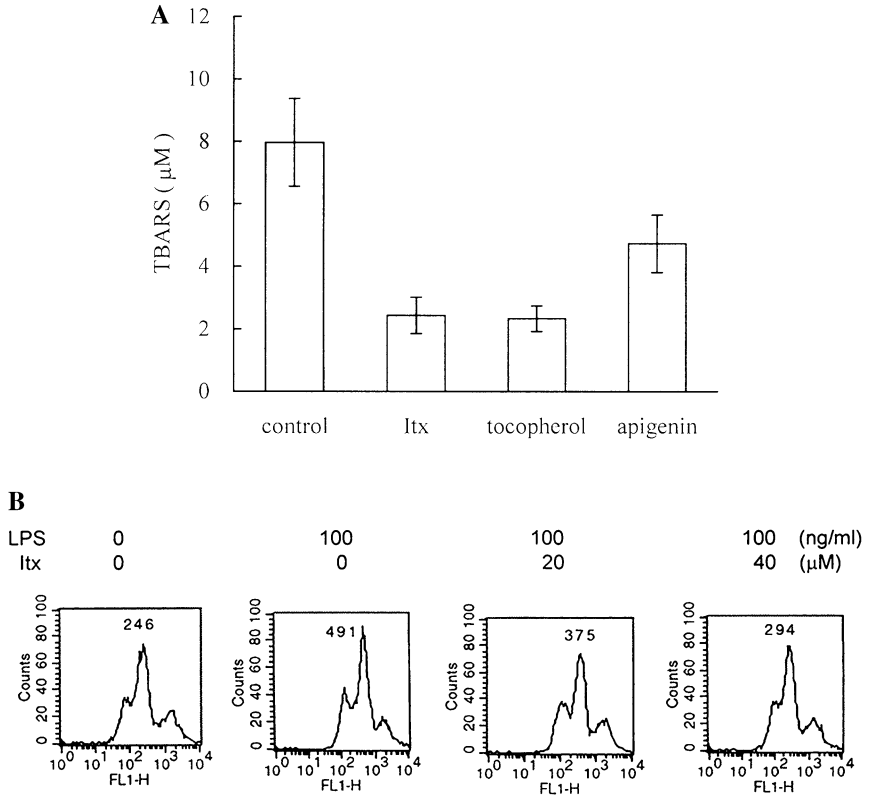

FIGURE 1. Antioxidative activity of isovitexin. (**A**) Isovitexin (Itx) inhibited the Fenton reaction–induced lipid peroxidation. Isovitexin (Itx), α–tocopherol, and apigenin (30 μM) were compared on the basis of their antioxidative activities. Quantity of oxidation was measured using the TBA assay. Antioxidative activity of the samples was measured according to levels of TBA-reactive substance (TBARS). (**B**) Isovitexin suppressed LPS-induced production of hydrogen peroxide. RAW 264.7 cells were treated with LPS (100 ng/mL) alone or LPS and isovitexin (20 and 40 μM) for 30 min. Cells were stained with DCFH-DA and subjected to flow cytometry. Peaks of FL1-H fluorescence intensity are indicated for each treatment.

Flow Cytometric Detection of Hydrogen Peroxide

RAW 264.7 cells were suspended in phenol red–free medium at a density of 10^5 cells/mL. Cells were stained with 100 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA) in the dark for 30 min and then analyzed using a FACScan (Becton Dickinson, San Jose, CA). Oxidation of green DCH fluorescence by hydrogen peroxide in living cells was detected using the FL1-H wavelength band. The fluorescence signals of 10,000 cells were processed using a logarithmic amplifier as described previously.[14]

RESULTS AND DISCUSSION

Antioxidative Activity of Isovitexin

Many antioxidants exert anti-inflammatory effects, and many polyphenolic compounds have been reported to be potent ROS scavengers. The antioxidative activity of isovitexin was evaluated by the TBA method. The inhibitory effect on ethyllinoleate oxidation by isovitexin (69.5% inhibition, TBARS value = 2.43 μM) was comparable to that of α-tocopherol (70.8% inhibition, TBARS value = 2.33 μM) and higher than that of apigenin, an aglycon flavonoid, (40.7% inhibition, TBARS value = 4.73 μM) (FIG. 1A). The results suggest that isovitexin is a potent antioxidant. Isovitexin also lowered the LPS-induced increase in the cellular content of hydrogen peroxide in RAW 264.7 macrophage cells in a dose-dependent manner (FIG. 1B).

Isovitexin Inhibition of TNF-α Production

To determine the effect of isovitexin on LPS-activated TNF-α production, we stimulated the cells with LPS in the presence or absence of isovitexin at concentra-

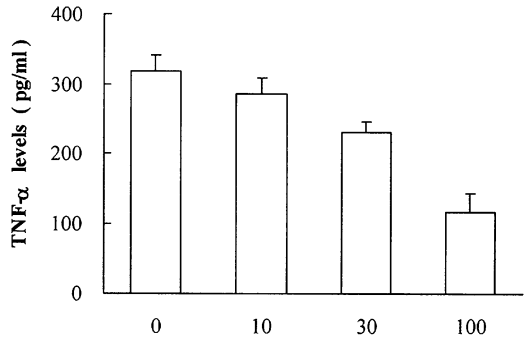

FIGURE 2. Inhibition of TNF-α production by isovitexin. RAW 264.7 cells were treated with LPS (100 ng/mL) in the presence or absence of isovitexin (Itx) (0, 10, 30, and 100 μM, respectively) for 1 h. Amount of TNF-α released into culture medium was determined. Data are presented as means ± standard error of the mean (n = 3).

tions ranging from 10 to 100 μM for 60 min. The production of TNF-α by the unstimulated RAW 264.7 cells was 20 pg/mL ($n = 3$). Incubation of these cells with LPS (100 ng/mL) for 1 h caused a substantial increase in TNF-α production (318.9 pg/mL; $n = 3$). When RAW 264.7 cells were stimulated with LPS in the presence of isovitexin (10–100 μM), a concentration-dependent inhibition of TNF-α production with a 50% inhibitory concentration (IC_{50}) value of 78.6 μM was observed (FIG. 2).

Isovitexin Inhibition of PGE_2 Production and COX-2 Expression

The effect of isovitexin on PGE_2 production and COX-2 protein expression following LPS stimulation was examined 18 h after treatment with LPS (50 ng/mL). PGE_2 production was reduced in a concentration-dependent manner with an IC_{50} of 80.0 μM (FIG. 3A). The inhibition was not due to general cellular toxicity (data not shown). LPS-induced COX-2 protein expression was also suppressed by isovitexin in a concentration-dependent manner after treatment for 18 h. The relative protein levels of COX-2 in the presence of 20, 40, 60, 80, and 100 μM isovitexin were 1.02,

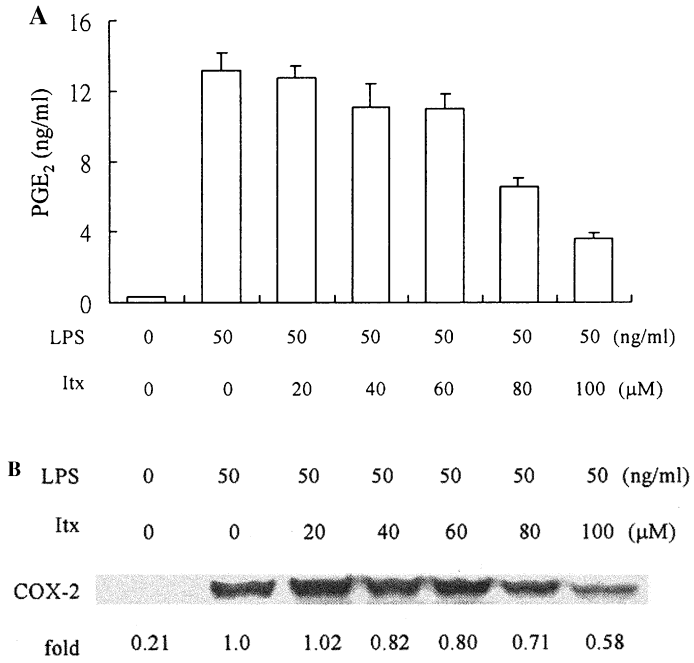

FIGURE 3. Effects of isovitexin on LPS-induced PGE_2 generation and COX-2 expression. (**A**) Cells were treated with LPS (50 ng/mL) in the presence or absence of isovitexin (Itx) for 18 h. Amount of PGE_2 released into culture medium was determined. Data are presented as means ± standard error of the mean ($n = 3$). (**B**) Values for expression of COX-2 protein upon various treatments for 18 h were quantities after resolving in 8.0% SDS- PAGE and Western blot analysis. Relative level of COX-2 expression observed with LPS alone was set at 1.0.

0.82, 0.80, 0.71, and 0.58, respectively, in reference to a value of 1.00 in samples treated with LPS alone (FIG. 3B).

APossible Mechanism for Isovitexin Inhibition of LPS Activity

Macrophages release various mediators in cellular inflammatory response. Among those released are ROS including H_2O_2, nitric oxide, superoxide, and hydroxyl radical. The intracellular ROS production is associated with several cellular events including the activation of NAD(P)H oxidase, xanthine oxidase, and the cellular mitochondrial respiratory chain.[15,16] ROS thus formed are potent activators of NF-κB, an essential transcription factor in the inflammatory signal transduction pathway.[17] ROS plays an essential role in the initiation of the NIK/MAPK cascade to trigger a series of responses in which IκB is phosphorylated to free NF-κB from IκB inhibition.[18] The active form of NF-κB is translocated from cytoplasm to the nucleus to bind the cognate NF-κB binding site in the promoter regions of genes including COX-2.[19,20] COX-2 overexpression mediated by activation of NF-κB is a possible molecular mechanism in cellular transformation to neoplasia. Another consequence of excessive ROS generation is oxidative DNA damage leading to gene

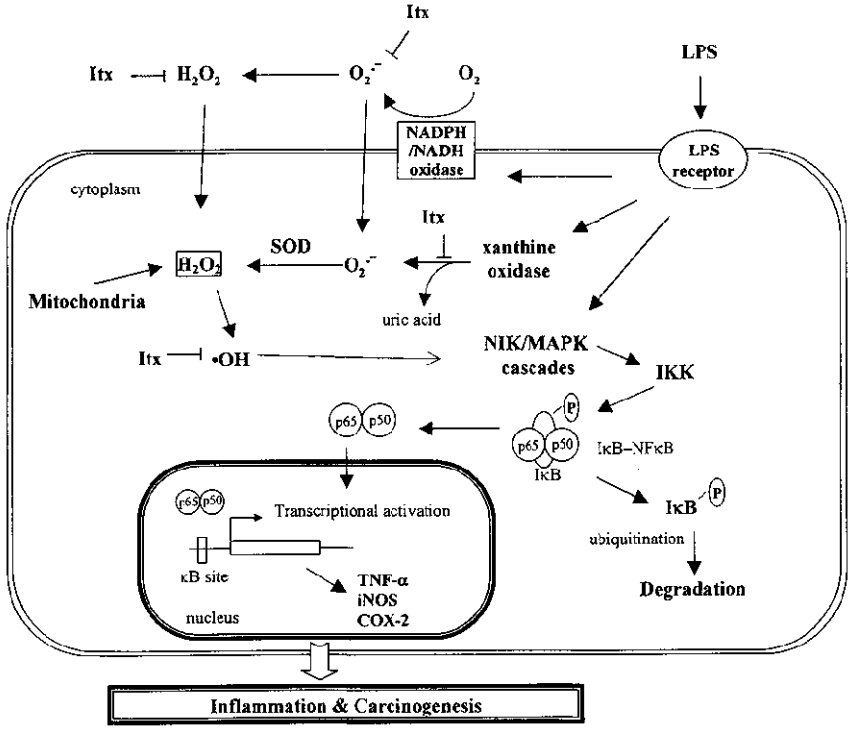

FIGURE 4. Possible mechanisms in which isovitexin (Itx) exerts its action against inflammation and carcinogenesis.

mutations and cancer formation. The inflammation-signaling transduction pathway mediated by LPS on macrophage is depicted in FIGURE 4.

In FIGURE 4, the roles of isovitexin in inhibiting inflammation and carcinogenesis are proposed. Isovitexin, with its potent antioxidant activity, is likely to suppress NF-κB activation. This contention is supported by the finding that the expression of TNF-α and COX-2, both transactivated by NF-κB, was suppressed by isovitexin. Reduced PGE_2 formation by isovitexin is likely due to its inhibition of COX-2 expression.

In summary, isovitexin is a potent antioxidant that inhibits TNF-α and COX-2 expression caused by LPS in mouse macrophage RAW264.7 cells. This effect of isovitexin is likely due to its inhibition of NF-κB activation via its antioxidant activity. Isovitexin also inhibits PGE_2 formation, likely secondary to its suppression of COX-2 expression. These antioxidant and anti-inflammatory effects of isovitexin may have implications in carcinogenesis. Isovitexin is a food phytochemical enriched in rice. Whether lower incidence of selected neoplasia such as breast and colon cancer in countries with rice as a major staple can be attributed to the antioxidant gradients in rice including isovitexin remains to be determined.

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