Isovitexin Suppresses Lipopolysaccharide-Mediated Inducible Nitric Oxide Synthase through Inhibition of NF-kappa B in Mouse Macrophages

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Abstract

Isovitexin exhibits potent antioxidant activities. In this study, the activity of nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-activated RAW264.7 macrophages after incubation with isovitexin was investigated. Isovitexin was able to reduce the production of hydrogen peroxide induced by LPS in mouse macrophage RAW264.7 cells. The cells incubated with isovitexin had markedly reduced LPS-stimulated NO production with an IC_{50} value of 58.5 μM. The expression of iNOS was also inhibited when the cells were treated with isovitexin. A transient transfection experiment showed that isovitexin suppressed the iNOS promoter and NF-κB-dependent transcriptional activities. It was also found to inhibit IKK kinase activity and prevent the degradation of IκBα in activated RAW264.7 cells. Additionally, Western blotting analysis revealed that isovitexin prevented the translocation of NF-κB from the cytoplasm to the nucleus. Our results indicate that its ROS scavenger and IKK inhibitory activities also contribute to the suppression of ROS-mediated NF-κB activity. These results suggest that isovitexin, a food phytochemical contained in dietary rice products, might have biological significance.

Key words
Isovitexin · iNOS · inflammation · ROS · IKK · I-kappaB · NF-kappaB

Introduction

Chronic increases in oxidative stress and nitric oxide (NO) production in cells are key biochemical events that have been linked to cancer and inflammation. Overproduction of reactive oxygen species (ROS) can lead to a wide range of toxic, oxidative reactions resulting in enormous physiological and pathological damage [1]. Prolonged NO generation has attracted attention because of its relevance in epithelial carcinogenesis. It also plays important roles in inflammatory responses [2]. Several studies have shown that antioxidant agents are able to decrease the occurrence of inflammation and cancer by decreasing oxidative stress, prostaglandin secretion, and nitric oxide production in cells via the NFκB pathway [3].

NO is released by a family of enzymes, constitutive NO synthase and an inducible NO synthase (iNOS), namely. NO can stimulate tumor growth and metastasis by promoting the migratory and invasive abilities of tumor cells, which may also be triggered by activation of cyclooxygenase-2 (COX-2). Suppression of enzyme induction and of the activities of iNOS/COX-2 represent a new paradigm for the prevention of carcinogenesis in several organs. Thus, selective inhibitors of iNOS may have a therapeutic role in certain cancers [2].

The NF-κB family of transcription factors regulates the expression of many genes, including the iNOS gene involved in immune and inflammatory responses. In unstimulated cells, inactive NF-κB exists in the cytosol in a latent form bound to its inhibitory

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Received October 5, 2004 · **Accepted** April 25, 2005

Bibliography
Planta Med 2005; 71: 748–753 · © Georg Thieme Verlag KG Stuttgart · New York
DOI 10.1055/s-2005-871287 · Published online August 11, 2005
ISSN 0032-0943

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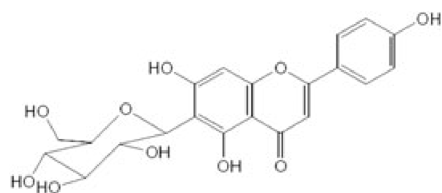

Fig. 1 Structure of isovitexin.

protein, IκB. Upon stimulation of the immune cells, IκB is phosphorylated and degraded proteolytically. As a result, NF-κB is activated and can then translocate into the nucleus for activation of target enhancers. It has been proposed that reactive oxygen species are involved in the activation of NF-κB via regulation of various redox-sensitive protein kinases or tyrosine kinases [4]. An increase in oxidative stress can promote inflammatory cytokine production from antigen presenting cells (APC) by the activation of redox-sensitive signal transduction pathways such as the mitogen-activated protein kinase (MAPK) and NF-κB [5]. Antioxidants such as (-)-epigallocatechin 3-gallate (EGCG), resveratrol, aspirin and naturally occurring flavonoids [6] have been reported to suppress NO production. Their inhibitory mechanisms are based on their ability to inhibit activation of NF-κB. However, the contribution of redox regulation to NF-κB activation is still a subject of intense debate because of many conflicting reports [7].

Rice possesses special dietary importance in Asia where the incidence of breast and colon cancer is markedly lower than in the West. Some investigations on potentially beneficial effects of specific rice constituents that exhibit preventive or ameliorative effects on malignant diseases have been performed. It has been reported that rice constituents counteract chemically-induced mutagenicity, tumor promotion, and carcinogenicity [8]. Isovitexin and related flavonoids were isolated from the rice hull of *Oryza sativa* and identified to have strong antioxidant activity [9]. Furthermore, xanthine oxidase inhibition [10], DNA protection, and prevention of heavy-metal-induced cell damage by isovitexin were described in our laboratory [11]. Here, we further evaluate the pharmacological effect of isovitexin (Fig. **1**) on the production of NO in LPS-activated mouse macrophages. The molecular mechanisms involved in the reduction of iNOS expression and of NF-κB activation in LPS-stimulated RAW 264.7 cells were also explored.

Materials and Methods

Materials

LPS (lipopolysaccharide, *Escherichia coli* 026:B6), sulfanilamide, and naphthylethylenediamine dihydrochloride were purchased from Sigma Chemical Company (St. Louis, MO). RT-PCR reagents were purchased from Promega (Madison, WI). The mouse iNOS promoter plasmid was generously provided by Dr. Charles J. Lowenstein [12]. The plasmid construct contains a 1.75 kb *Hinc* II restriction fragment upstream of the macrophage NOS gene, which was cloned into the GeneLight luciferase vector system (Promega). The plasmid pNFκB-Luc was purchased from Stratagene Corp. (La Jolla, CA) and phRL-TK from Promega Corp. (Madison, WI). Isovitexin (Fig. **1**) was purified and identified from rice hull [11]. We isolated isovitexin from rice hull of *Oryza sativa* with a yield of 2.3 mg/100 g of hull.

Cell culture

The mouse monocyte-macrophage cell line RAW 264.7 (ATCC TIB-71) was cultured in DMEM containing 10% heat inactivated fetal bovine serum (GIBCO Life Technologies, Grand Island, NY). Except for the transient transfection assay, cells were plated in dishes at a density of 4×10^6/mL for 18 – 24 h before activation by LPS (100 ng/mL). Test compounds were co-treated with LPS, and the final DMSO (as vehicle) concentration was less than 0.2% (v/v).

IκB kinase (IKK) assay

Whole cell extracts were lysed with lysis buffer (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 PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin) for 30 min at 4 °C. The prepared enzyme (150 μg) was incubated with a GST-IκBα fusion protein (Santa Cruz Biotechnology) as a substrate in kinase buffer containing HEPES (20 mM pH 7.7), 10 mM $MgCl_2$, 10 μM ATP, [γ-^{32}P]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM sodium orthovanadate, 1 mM benzamidine, 2 μM PMSF, 10 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mM DTT. The reaction was terminated by addition of 5X SDS-polyacrylamide gel electrophoresis (PAGE) sample-buffer and by boiling for 10 min. Proteins were separated on an 8% SDS-PAGE, and the gels were dried and visualized by autoradiography at –70 °C [13].

Flow cytometric detection of hydrogen peroxide

RAW 264.7 cells (2×10^5 cells/mL) were stained with 100 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA) in the dark for 30 min, then analyzed using a FACScan (Becton Dickinson). 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 [11].

Determination of nitrite

The nitrite accumulating in the culture medium was measured as an indicator of NO production according to the Griess reaction [14]. Cells were stimulated with LPS (50 ng/mL) in the presence or absence of various concentrations of test compounds for 24 h. 100 μL of each media supernatant were mixed with 50 μL 1% sulfanilamide (in 5% phosphoric acid) and 50 μL 0.1% naphthylethylenediamine dihydrochloride (in dH_2O) at room temperature. Absorbance at 550 nm was measured with an $NaNO_2$ serial dilution standard curve, and nitrite production was determined.

Western blots

Cellular protein (for iNOS, β-actin, NF-κB, IκBα) was prepared using Gold lysis buffer. Cytosolic and nuclear fractions were prepared according to a modified procedure of Xie et al. [15]. 30 – 50 μg proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon®, Millipore, Bedford, MA, USA). The membrane was incubated with primary antibody at room temperature for 2 h and then incubated with horseradish peroxidase-conjugated secondary IgG antibody. The immunoreactive bands were visualized with enhanced chemiluminescent reagents (ECL, Amersham, Buckinghamshire,

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UK). Data were quantified using a densitometer (Alpha Innotech IS-1000 Digital Imaging System, California, US).

Reverse transcription-polymerase chain reaction

RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction [16]. 5 μg total RNA were reverse-transcribed (RT) into cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase and oligo $(dT)_{18}$ primer by incubating the reaction mixture (15 μL) at 37 °C for 90 min. The polymerase chain reaction (PCR) was performed as described previously (primers: iNOS; forward: 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and reverse: 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′; G3PDH: 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′) [12]. Samples (5 μL) were electrophoresed on a 2% agarose gel and visualized by staining with ethidium bromide (497-bp iNOS fragment and 983-bp GAPDH fragment).

Transient transfection and luciferase assay

RAW 264.7 cells were transfected with the pNF-κB-Luc or pGL2-iNOS plasmid reporter genes using LipofectAMINE™ (Gibco BRL) and phRL-TK (Promega Corp., Madison, WI) as internal control. After 48 h incubation, equal numbers of cells were plated in 12-well tissue culture plates for 12 h [17]. Cells were then treated with LPS (50 ng/mL) alone or with isovitexin (50 μM) for 3 h. Cells were harvested in 150 μL of lysis buffer (0.5 M HEPES pH 7.8, 1% Triton-X-100, 1 mM $CaCl_2$, and 1 mM $MgCl_2$). Aliquots of 100 μL of cell lysate were used to assay luciferase activity with a Luc-Lite™-M luciferase reporter gene assay kit (Packard Instrument Co., Meriden, CT). Luciferase activity was measured on a TopCount Microplate Scintillation and Luminescence Counter (Packard 9912V1) in single photon counting mode for 3 sec/well. Luciferase activities of reporter plasmids were normalized to luciferase activities of the internal control plasmid. Data represent one of three similar experimental results.

Statistical analysis

Data were presented as means ± SE for the indicated number of independently performed experiments. Statistical analysis was done by Student's *t* test.

Results and Discussion

The amount of intracellular hydrogen peroxide in LPS-activated Raw 264.7 cells co-incubated with isovitexin was examined. The results are shown in Fig. **2**. A green DCH fluorescence dye generated upon oxidation by hydrogen peroxide was used for measuring the cellular hydrogen peroxide concentration. Fluorescence intensities were determined with flow cytometry. RAW 264.7 cells incubated with isovitexin prior to LPS treatment exhibited lower concentrations of hydrogen peroxide than untreated cells (Fig. **2**). The FL1-H intensity of untreated cells peaked after 30 min of LPS treatment (100 ng/mL) while the LPS-induced intensity of isovitexin-treated cells (5, 10, 20 μM, respectively) was suppressed in a dose-dependent manner. Therefore, isovitexin possesses the potential to reduce oxidative stress in LPS activated RAW 264.7 macrophage cells.

The overproduction of NO is a key biochemical event during inflammation. Many anti-inflammatory agents suppress NO production *in vivo*. The influence of NO production in isovitexin incubated, LPS-activated macrophage was examined. The results are shown in Fig. **3**. We analyzed nitrite production as the indicator of NO release in the LPS-activated macrophage. The nitrite concentrations in the culture media from LPS-stimulated (50 ng/mL) cells treated with or without isovitexin were measured. The macrophages treated with isovitexin were found to produce less NO in a dose-dependent manner with an IC_{50} value of 58.5 μM (Fig. **3A**). The cell viability assay confirmed that the inhibition by isovitexin was not due to general cellular toxicity (data not shown). The isovitexin dissolved in DMSO did not interfere with the Griess reaction. The iNOS protein expression following isovitexin treatment in activated macrophage cells was also evaluated (Fig. **3B**). RAW 264.7 cells maintained under normal conditions express hardly detectable levels of iNOS protein (Fig. **3B**, lane 1). After stimulation with LPS, the amount of expressed iNOS protein increased (Fig. **3B**, lane 2). Macrophages treated with isovitexin exhibited reduced iNOS protein expression, which was dose-dependent (Fig. **3B**, lanes 3 – 5). The relative amounts of iNOS protein in macrophages treated with 25, 50, 75, and 100 μM isovitexin were 0.86, 0.64, 0.38, and 0.13, respectively, compared to 1 for LPS alone. The IC_{50} value for iNOS protein expression was similar to that of the nitrite formation reduction. To further investigate whether the suppression of iNOS protein expression by isovitexin was caused by reduction of iNOS mRNA expression, an RT-PCR analysis for total mRNA samples, extracted from RAW 264.7 cells after 12 h, was carried out. The amplification of cDNA with primers specific for mouse iNOS and GAPDH (as control gene) was analyzed. The results are shown in Fig. **3C**. They show that iNOS mRNA expression was suppressed

LPS	0	100	100	100	100 (ng/ml)
Itx	0	0	5	10	20 (μM)

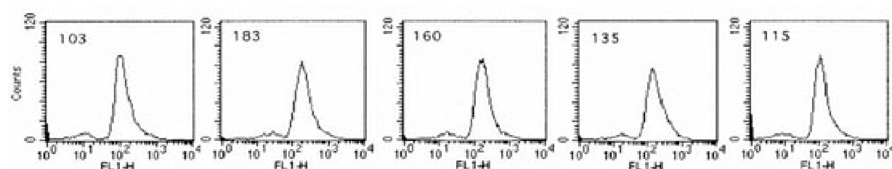

Fig. **2** Isovitexin (Itx) suppresses the LPS-induced production of hydrogen peroxide. RAW 264.7 cells were untreated or treated with LPS (100 ng/mL), or LPS and isovitexin (5 – 20 μM) for 30 min. Cells were stained with DCFH-DA and subjected to flow cytometry. The peaks of FL1-H fluorescence intensity are indicated for each treatment.

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in isovitexin-treated, LPS-activated macrophages. To further analyze isovitexin's modulation of iNOS expression, transient transfection was employed using mouse the iNOS promoter-driven luciferase construct as a model. The cells were transfected with 2.5 μg of pGL2-iNOS and phRL-TK, a control plasmid containing the gene coding for luciferase driven by the TK promoter, and then treated with LPS (50 ng/mL) with isovitexin (50 μM) or without for 3 h. Cells were harvested, and the levels of luciferase activity were determined. Luciferase activities of reporter plasmids were normalized to the luciferase activity of the internal control plasmid. LPS-induced iNOS promoter activity was inhibited by isovitexin as shown in Fig. **3D**. Luciferase activity was stimulated up to 10.81-fold in LPS-activated cells if compared to control cells. Isovitexin treated macrophages showed a stimulated luciferase activity of up to 3.47-fold when compared to control. These data suggest that isovitexin modulates iNOS expression at the transcriptional level, thereby inhibiting the production of NO in LPS-stimulated RAW 264.7 cells.

IκBα is an important regulatory protein that inhibits the function of NF-κB by binding to NF-κB. Upon LPS-mediated activation of macrophages, IκBα is hyperphosphorlated by IκBα kinase and is subsequently degraded. In order to elucidate if isovitexin suppresses IKK activity in activated macrophages, RAW 264.7 cells were treated with LPS (100 ng/mL) in the presence or absence of isovitexin (Itx) for 30 min. IKK kinase activity was then measured using GST-IκBα as substrate. ^{32}P-GST-IκBα was separated on an 8% SDS-PAGE. The amount of ^{32}P-GST-IκBα produced by IKK from untreated RAW 264.7 cells was barely detectable (Fig. **4A**, lane 1). Upon macrophage stimulation with LPS, the levels increased (Fig. **4A**, lane 2). Macrophages treated with isovitexin (20, 40, 60 μM, respectively) produced less ^{32}P-GST-IκBα in the kinase assay if compared to cells stimulated without the com-

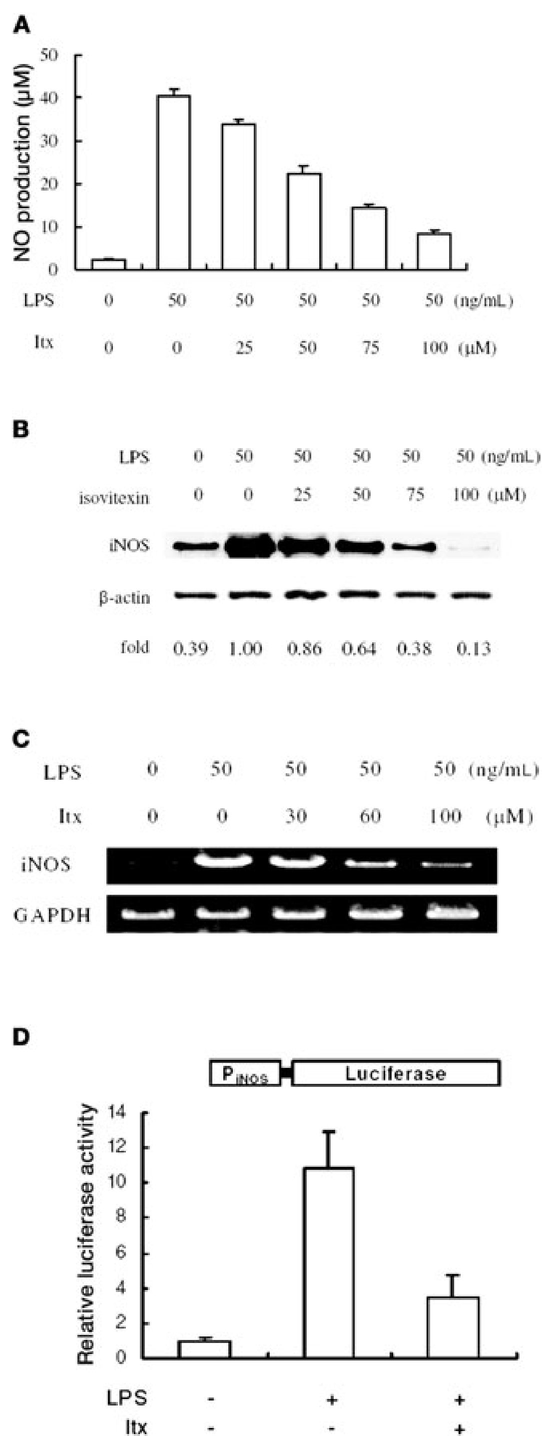

Fig. **3** Effects of isovitexin on LPS-induced NO production and iNOS expression in RAW 264.7 cells. **A** Cells were treated with LPS (50 ng/mL) in the presence or absence of isovitexin (Itx) for 18 h. Amounts of nitrite released into the culture medium were determined by using the Griess reagent. Data are presented as means ± SE (n = 3). **B** iNOS protein levels of the cells resulting from various treatments for 18 h were quantitated after separation of the proteins on an 8.0% SDS-PAGE and Western blot analyses. β-Actin was an internal expression control. The relative level observed with LPS alone is set at 1.0. **C** iNOS RNA levels of the cells following various treatments for 12 h were quantitated from RT-PCR analyses resolved on a 1% agarose gel. GAPDH was an internal expression control. **D** Effects of isovitexin on LPS-induced iNOS promoter activity in RAW 264.7 cells. The cells were transfected with pGL2-iNOS and phRL-TK, respectively, and then treated with LPS (50 ng/mL) with or without isovitexin (50 μM) for 3 h. Cells were harvested, and the levels of luciferase activity were determined as described in Materials and Methods. Data are presented as means ± SE (n = 3).

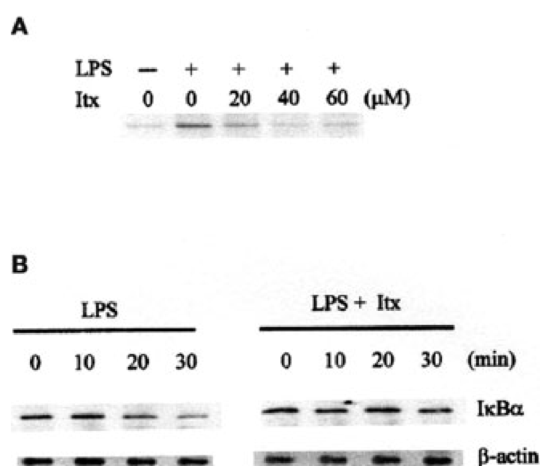

Fig. **4** Inhibitory effects of isovitexin (Itx) on IκBα phsphorylation and degradation. **A** Isovitexin decreased IKK activity in LPS-activated Raw 264.7 cells. Cells were treated with LPS (100 ng/mL) and isovitexin (20, 40, 60 μM, respectively) and then incubated for 30 min. The whole cell lysate (150 μg) was incubated with GST-IκBα fusion protein and [γ-^{32}P]ATP. The resulting ^{32}P-GST-IκBα was resolved on an 8% SDS-PAGE. The gel was dried and visualized after autoradiography at –70 °C. **B** Isovitexin decreased the IκBα degradation rate upon LPS induction. Cells were treated with LPS (50 ng/mL) and isovitexin (50 μM) and then incubated for 0 – 30 min. Cytosolic fractions were prepared and analyzed for the content of IκBα by Western blot.

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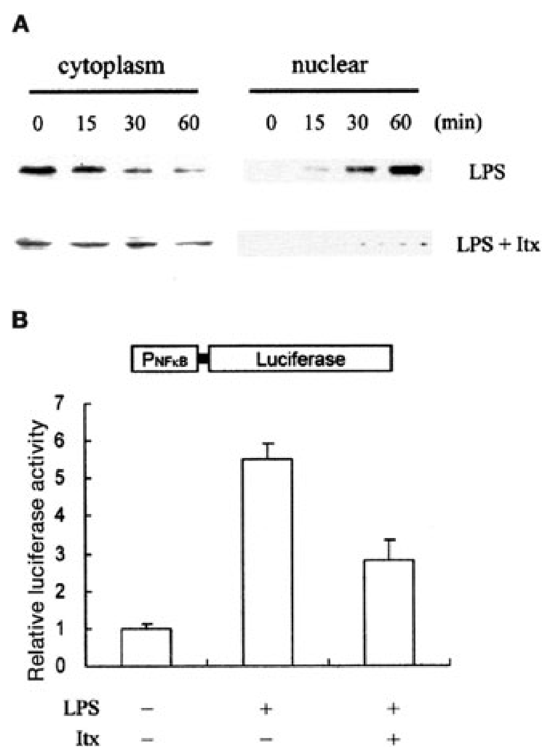

Fig. **5** Suppression of LPS-induced NF-κB activation by isovitexin. **A** Isovitexin (Itx) reduced NF-κB nuclear levels. Cells were treated with LPS (50 ng/mL) and isovitexin (50 μM) and then incubated for 0 – 60 min. Cytosolic and nuclear fractions were prepared and analyzed for the content of NF-κB subunit p65 by Western blot. **B** Effects of isovitexin on LPS-induced NF-κB promoter activity in RAW 264.7 cells. The cells were transfected with pNFκB-Luc and phRL-TK, respectively, and then treated with LPS (50 ng/mL) with or without isovitexin (50 μM) for 3 h. Cells were harvested, and the levels of luciferase activity were determined as described in Materials and Methods. Data presented are means ± SE (n = 3).

pound (Fig. **4A**, lanes 3 – 5). We also examined the IκBα protein levels in activated macrophages. The cytosolic fraction of the IκBα protein in LPS-activated cells treated with or without isovitexin (50 μM) was prepared at various timepoints (0, 10, 20, 30 min, respectively), and the amount of IκBα protein was determined by immunoblot analysis. In the LPS-activated cells, the amounts of IκBα diminished after 30 min (Fig. **4B**, left) while isovitexin-treated cells maintained fairly constant amounts of IκBα protein (Fig. **4B**, right). The results show that isovitexin can suppress IKK activity and prevent IκBα protein degradation in LPS-activated macrophage cells.

To determine whether isovitexin affected the translocation of NF-κB into the nucleus, nuclear and cytosolic fractions of isovitexin-treated, LPS-activated cells were prepared at various timepoints (0, 15, 30, 60 min, respectively). Each fraction was examined for the presence of the NF-κB p65 subunit with Western blot analysis. The results are shown in Fig. **5A**. In the LPS-induced macrophages, nuclear p65 significantly increased after 60 min. In contrast, cytoplasmic p65 decreased (Fig. **5A**, upper panel). The isovitexin-treated, LPS-stimulated macrophages exhibited little nuclear p65; most of it was retained in the cytoplasm (Fig. **5A**, lower panel). The plasmid pNF-κB-Luc is an NF-κB transcription reporter containing the firefly luciferase gene driven by the k enhancer element (κB site). It provides a direct assay for measuring induction of the NF-κB pathway. LPS promotes the phosphorylation and subsequent dissociation of the IκB inhibitor protein from the inactive NF-κB complex, allowing liberated NF-κB to translocate into the nucleus. Once active and inside the nucleus, NF-κB binds to the κB site on the DNA and activates transcription of the luciferase gene. To further investigate the effect of isovitexin on NF-κB activation, pNFκB-Luc and phRL-TK, a control plasmid containing the gene coding for luciferase driven by the TK promoter, were transiently expressed in RAW 264.7 cells. The luciferase activities in LPS-activated cells were stimulated up to 5.51-fold when compared to non-activated cells. Luciferase activity in isovitexin-treated, LPS-induced cells was stimulated 2.84-fold when compared to the non-stimulated control (Fig. **5B**). The NF-κB promoter activity in isovetixin-treated macrophages was inhibited due to a lack of the NF-κB nuclear translocation function.

ROS released by phagocytic cells are a key mediator in the process of inflammation and cancer. Schreck et al. proposed that ROS were the common second messenger for activation of NF-κB [18]. In another study, H_2O_2 was implicated in platelet-derived growth factor signal transduction involving p42/p44 mitogen-activated protein kinase and JNK activation. A model has therefore been proposed where diverse agents can activate NF-κB by causing oxidative stress [19]. Our results demonstrate that isovitexin possesses antioxidant activity and blocks the activation of NF-κB by interfering with the phosphorylation of IκB. As a result, IκB remains bound to NF-κB and prevents NF-κB translocation from the cytoplasm to the nucleus. IKK activity analysis suggests that the inhibition of NF-κB activity by isovitexin results from the inhibition of IκBα phosphorylation and of its degradation resulting in reduced translocation of NF-κB. However, it is also commonly believed that ROS are not activators, but rather inhibitors of NF-κB. In order to clarify whether or not ROS act as second messengers leading to NF-κB activation in response to extracellular stimuli, Hayakawa et al. established cell lines that enabled them to control the endogenous ROS during NF-κB activation. The results reveal that endogenous ROS produced through Rac/NADPH oxidase do not mediate NF-κB signaling [7]. Zhang et al. discussed the conflicting data regarding the interplay between ROS and NF-κB activation [20]. NF-κB and JNK share several common signaling pathways for their activation in response to cytokines or growth factors. ROS may act as troublemakers between NF-kB and JNK by inhibiting one but promoting the other, thereby creating a new form of cross-talk between these two important stress-responsive systems. Currently, it appears to be difficult to determine why ROS mediates two such opposite effects in NF-κB activation. More detailed and extensive study will be required to resolve the controversy.

Acknowledgements

This study was supported by the National Science Council NSC93 – 2113-M-038 – 004, NSC93 – 2113-M-038 – 004, and TMU93-AE1-B-10.

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