Daedalea gibbosa substances inhibit LPS-induced expression of iNOS by suppression of NF-κB and MAPK activities in RAW 264.7 macrophage cells

NILI RUIMI[1,2], HAITHEM RWASHDEH[1], SOLOMON WASSER[2], BADIREENATH KONKIMALLA[3], THOMAS EFFERTH[3], MONICA BORGATTI[4], ROBERTO GAMBARI[4] and JAMAL MAHAJNA[1]

[1]Cancer Drug Discovery Program, Migal, Galilee Technology Center, P.O. Box 831, Kiryat Shmona; [2]Department of Evolutionary and Environmental Biology, Faculty of Science and Science Education and Institute of Evolution, University of Haifa, Mount Carmel, Haifa 31905, Israel; [3]German Cancer Research Center, C015, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany; [4]BioPharmaNet, Department of Biochemistry and Molecular Biology, Ferrara University, Ferrara, Italy

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Abstract. Nitric oxide (NO) is a radical molecule produced by iNOS and plays a role in various physiological and pathophysiological conditions including inflammatory diseases and cancer. In the present study, organic extract of *Daedalea gibbosa* was effective in inhibiting NO and PGE_2 production in RAW 264.7 cells. The extract of *D. gibbosa* was chemically fractionated leading to the isolation of three active fractions (F5-F7) that were effective in inhibiting NO and iNOS production. In addition, F6 and F7 significantly inhibited the iNOS transcript, while F5 did not cause a reduction in the iNOS transcript. Furthermore, the active fractions showed a differential effect on levels of phospho-p38, phospho-JNK, and phospho-IKBα. Phopsho-p38 was moderately inhibited by F5 and only F7 was significantly active in inhibiting phospho-IKBα. Interestingly, all active fractions significantly enhanced levels of phospho-JNK. In addition, the three active fractions also showed differential inhibitory effects on NF-κB DNA binding activity.

Introduction

Excessive inflammation has become an acceptable critical factor in many human diseases, including inflammatory and autoimmune disorders, neurodegenerative conditions, infection, cardiovascular diseases, and cancer. Inflammation is mediated by a number of signaling molecules including cytokines, lymphokines, nitric oxide, and PGE_2. Nitric oxide (NO) is a gaseous, radical molecule that functions as both an intracellular and an extracellular messenger. Nitric oxide has been implicated in various physiological processes including vasodilatation, inhibition of platelet aggregation, neurotransmission, neural plasticity, and modulation of inflammatory and immunological functions. NO is generated from L-arginine by nitric oxide synthases (NOS), which exist in three isoforms: the inducible-NOS (iNOS), endothelial-NOS (eNOS), and neural-NOS (nNOS).

The expression of iNOS is differentially regulated in a cell-typed manner (1). Most mammalian cells require a mixture of cytokines usually composed of interferon γ, tumor necrosis factor α (TNFα), and interleukin-1ß (2,3) that synergize to obtain the maximal transcriptional activity of the iNOS promoter.

Macrophages that are ubiquitously distributed in healthy tissues are essential for the support of homeostasis and host defense against invaders. However, tumor-associated macrophages (TAM) are key regulators of the link between inflammation and cancer and express several protumoral functions, including secretion of growth factors and matrix-proteases, promotion of angiogenesis, and suppression of adaptive immunity (4). Macrophages are activated upon appropriate extra-cellular stimulation, most often by stress or pro-inflammatory cytokines including TNF-α and IL-1ß (5), and pathogens, such as bacterial components including LPS (6), leading to the activation NF-κB family of transcription factors and consequently results in the up-regulation of pro-inflammatory proteins, such as cyclooxygenase-2 (COX-2) (7) and inducible nitric oxide synthase (iNOS) (8). Activation of NF-κB requires the activation of the IKBα kinase (IKK), which phosphorylates serine residues of IKBα, the negative regulator of NF-κB. Phosphorylation of IKBα leads to its ubiquitination and subsequent degradation by the 26S proteasome, thereby releasing NF-κB dimers from the cytoplasmic NF-κB-IKBα complex and allowing translocation of NF-κB to the nucleus.

Correspondence to: Dr Jamal Mahajna, Cancer Drug Discovery Program, P.O. Box 831, Kiryat Shmona, Israel
E-mail: jamalm@migal.org.il

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Numerous studies have been carried out on natural bioactive substances with fungal origin able to modulate the NF-κB activation pathway (9) and iNOS expression (10-12). Occasionally, the chemical structures of the active moieties have been elucidated, and their exact mechanism of action was established. Limited information about the biological activity of *Daedalea gibbosa* mushroom is available. Recently, the ability of the mycelium organic extract to inhibit growth of CML cell lines by interfering with the Bcr-Abl kinase activity was reported (13).

In the present study, we report the identification of active fractions of the medicinal mushroom *D. gibbosa* having anti-inflammatory effects by the inhibition of iNOS. Interestingly, the active fractions significantly inhibited NO production and iNOS protein expression using different molecular mechanisms.

Materials and methods

Cell cultures. Murine macrophage cells RAW 264.7 (ATCC, VA, USA) was maintained in DMEM medium (Sigma, Rehovot, Israel) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% PenStrep (penicillin + streptomycin) (Biological Industries, Israel). The human breast cancer cell line MCF-7 (ATCC, VA, USA) was transfected with an iNOS promoter reporter plasmid, piNOS(7.2)Luc (14). Cells were maintained in RPMI-1640 medium with L-glutamine (Sigma) supplemented with 10% (FCS), 1% PenStrep, and 1 mg/ml G418 antibiotic (Sigma).

Materials and pharmaceutical inhibitors. All organic solvents used in the mushrooms extraction and fractionation procedures were obtained from Frutarom Co., Israel. Pharmaceutical inhibitors used in the study such as IMD-0354 (15), curcumin (16), JSH-23 (17), SP600125 (18), SB202190 (19), PD98059 (20), and 1400W (21) were purchased from Calbiochem, CA, USA.

Sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) and Western blot analysis. RAW 264.7 cells ($1x10^5$) were plated in a 6-well plate and maintained at 37˚C. After 24 h, the growth medium was substituted with a low serum medium (1% FCS), and LPS (1 μg/ml) was added alongside the appropriate extracts or fractions as indicated in each experiment. DMSO treatment alone was used as a negative control; JSH-23 and SB20290 (10 μM), inhibitors of NF-κB and p38, respectively (Calbiochem), were used as positive inhibitory controls. After an additional 24 h, cells were collected, washed, and total cell lysates were prepared (9). Equal amounts of proteins (25-40 μg) were separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane (Schleicher & Schuell BioScience GmbH, Germany), and subjected to Western blot analysis (9) using anti-pIκBα and anti-iNOS antibodies (Santa Cruz Biotechnology, Inc., CA, USA) as well as anti-pSAPK/JNK and anti-phospho-p38 antibodies (Cell Signaling Technology Inc., MA, USA) according to the manufacturer's instructions. The equality of sample loading in all lines was confirmed by stripping and re-blotting with α-tubulin antibody (Santa Cruz Biotechnology Inc.).

Measurement of nitrite levels by Griess reagent. NO production by RAW 264.7 cells was determined by measuring the nitrite contents in the culture media. Cells were plated in a 6-well plate ($1x10^5$ cells/well) and after 24 h the growth medium was substituted with a low serum medium (1% FCS). LPS at 1 μg/ml was added in the presence and absence of *D. gibbosa* extract or fractions and samples were collected after additional 24 h. Positive inhibitory controls include curcumin, JSH-23, SB202190, and 1400W. Nitrite contents of the culture media were determined using Griess reagent kit (Sigma) according to the manufacturer's instructions. Concentrations of nitrite were calculated by using a known $NaNO_2$ concentration in a standard curve.

PGE_2 determination. PGE_2 production by RAW 264.7 cells was determined by measuring the PGE_2 contents in the culture media. Cells were plated as before and PGE_2 contents in the culture media were determined using Prostaglandin E_2 Immunoassay (R&D Systems, USA) according to the manufacturer's instructions, and calculated using a pure PGE_2 in a standard curve.

RT-PCR of iNOS. RAW 264.7 cells were starved 24 h before treatment with LPS (1 μg/ml) in the presence and absence of *D. gibbosa* extract or fractions. Positive inhibitory controls include JSH-23, SB202190, SP600125, PD98059, and 1400W. After 24 h of incubation, total RNA was extracted from the cells using Tri Reagent (Sigma). Single-stranded complementary DNA (cDNA) was synthesized from the total RNA and the resulting cDNA was amplified with a polymerase chain reaction (PCR) kit (Bioline, USA) with the following primers: mouse iNOS forward: 5'-ACGAGGTGTTCAGCGT GCTCCACG-3'; mouse iNOS reverse: 5'-CCACAATAGT ACAATACTACTTGG-3'. A total of 30 cycles of amplification were performed with an initial incubation at 94˚C for 2 min and a final extension at 72˚C for 15 min; each cycle consisted of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec and an extension at 72˚C for 2:30 min. To ensure the use of equal amounts of cDNA from each sample in PCR, the aliquots of the reverse transcription products were used in PCR with the following primers for the housekeeping gene GAPDH: mouse GAPDH forward: 5'-GGCAT TGCTCTCAATGACAA-3'; mouse GAPDH reverse: 5'-TGT GAGGGAGATGCTCAGTG-3'. A total of 5 μl of the PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

Luciferase reporter gene assay. MCF-7 transfected cells ($2x10^4$) were seeded in 200 μl of medium, using 96-well plates. After 24 h, *D. gibbosa* organic crude extract and fractions were added. Control wells include solvent-treated wells (1% of DMSO) and IKK inhibitors, IMD-0354, and curcumin (Calbiochem). After 24 h, luciferase levels were determined according to the manufacturer's instructions using a microplate luminometer (Perkin Elmar, Singapore).

Electrophoretic mobility shift assay. The electrophoretic mobility shift assay (EMSA) was performed using a double-stranded synthetic oligonucleotides mimicking the NF-κB binding sites (22,23). The synthetic oligonucleotides (Sigma-

Genosys, UK) were 5'-end labelled using [γ-^{32}P]ATP and T4 poly-nucleotide kinase (MBI Fermentas, Italy). Binding reactions were set up as described by Bezzerri *et al* (22) in a total volume of 20 μl containing binding buffer plus 5% glycerol, 1 mM DTT, 0.75 μg of the non-specific competitor poly (dI:dC)·poly(dI:dC), 4 μg of crude nuclear extracts isolated from human K562 cells, increasing amounts of *D. gibbosa* active fractions (F5-F7), and 0.25 ng of labeled oligo-nucleotide. In these experiments, the *D. gibbosa* active fractions were pre-incubated for 20 min with nuclear extracts, before the addition of the labelled target DNA. After 20 min of binding at room temperature with the ^{32}P-labelled DNA probe, the samples were electrophoresed at constant voltage (200 V) under low-ionic strength conditions (0.25X TBE buffer pH 8.3:22 mM Tris borate, 0.4 mM EDTA) on 6% polyacrylamide gels. Gels were dried and exposed for auto-radiography with intensifying screens at -80˚C.

microRNA profiling

RNA isolation and analysis. Total RNA from murine RAW 264.7 cells treated with LPS, or with LPS and F5, were isolated using Trizol according to the manufacturer's instructions. The quality and quantity of total RNA was validated by RNA Nanochip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Germany) and NanoDrop spectrophotometer (NanoDrop Technologies, DE, USA).

Probe labeling and illumina BeadArray hybridization. The miRNA expression was carried out by DASL® assay (cDNA-mediated annealing, selection, extension and ligation) (24). Total RNA (200 ng) was first poly-adenylated using Poly-A Polymerase then heat inactivated at 70˚C for 10 min. The introduced poly-A tail was then used as a priming site for cDNA synthesis, incubated at 42˚C for 60 min. The primer used for cDNA synthesis was biotinylated and contained a universal PCR primer sequence. After cDNA synthesis, miRNAs were individually interrogated using specific oligo-nucleotides. A single miRNA-specific oligo (MSO) is designed for each mature miRNA sequence, which consists of three parts: at the 5'-end is another universal PCR priming site; in the middle is an address sequence used for capturing the product on the array, and; at the 3'-end is a miRNA-specific sequence. The second universal PCR priming site is shared among all MSOs, and each address sequence is associated uniquely with each of the miRNA targets. As controls, central mismatch probes for miRNAs mmu-let-7a, let-7c, let-7f, miR-152 and miR-182, and 3'-end mismatch probes for small nuclear RNAs, RNU24 and RNU66, were used. The subsequent assay process and array hybridization were performed as described previously (24).

All mouse miRNAs were assayed simultaneously. After binding and washing, the annealed MSOs were extended through the cDNA primer, forming an amplifiable product. The extended oligos were eluted from the streptavidin beads and added to a PCR reaction, in which one of the universal primers was Cy3 labeled and the other universal primer was biotinylated. The PCR products were captured on streptavidin paramagnetic beads, washed and denatured to yield single-stranded fluorescent molecules to hybridize to the arrays. The universal arrays used for fluorescent reporting consist of capture oligos immobilized on beads and randomly assembled onto the ends of fiber optic bundles, which are arranged in a matrix to match a 96-well plate (Sentrix® Array Matrix) (25). Arrays were scanned on the BeadArray reader, and automatic image registration and intensity extraction software were used to derive intensity data per bead type corresponding to each miRNA (26).

Microarray data analysis. The array intensity data were imported into Beadstudio version 3.1 (Illumina), a software package that permits visualization and normalization of the data. We used the quantile normalization method for all the analyses reported here (27).

Preparation of D. gibbosa mushroom extract. *D. gibbosa* pure mycelia culture was obtained from the Culture Collection (HAI) of the Institute of Evolution, University of Haifa, Israel. Mushroom mycelium was grown in submerged conditions, then the culture liquid was extracted with ethyl acetate, evaporated, and the dry crude extract was dissolved in dimethyl sulfoxide as previously described (9).

Liquid chromatography. The crude ethyl acetate extract of *D. gibbosa* (2.77 g) was dissolved in 2 ml of ethyl acetate and loaded on a silica gel column (40x3.5 cm). The column was washed with the following mobile phases (700 ml each) and 8 fractions were collected: F1 (150 mg) eluted with 100% hexane, F2 (632 mg) eluted with 50% dichlorometane (DCM) and 50% hexane, F3 (242 mg) eluted with 100% DCM, and F4 (184 mg) eluted with 2% methanol in DCM, F5 (548 mg) eluted with 5% methanol in DCM, F6 (460 mg) eluted with 10% methanol in DCM, F7 (128 mg) eluted with 20% methanol in DCM, and F8 (310 mg) eluted with 100% methanol. Fractions were dried, dissolved in DMSO, and kept at -20˚C.

Results

Fungal extracts inhibit LPS-induced NO production in RAW 264.7 macrophage cells. In the present study, 15 fungal species, belonging to different taxonomic and ecological groups, were selected and evaluated for their abilities to affect the function and expression of iNOS as previously described (9). From the 15 fungal extracts tested, 6 extracts were found to inhibit the iNOS promoter activity in MCF-7 breast cancer cell line, carrying a luciferase reporter gene under the control of iNOS promoter (data not shown). The positive extracts were tested for their abilities to inhibit the LPS-induced NO production in RAW 264.7 macrophage cells. Cells were stimulated with LPS (1 μg/ml) for 48 h, and treated with the different extracts, as described in Materials and methods. The results, presented in Fig. 1, show that three extracts, AA04, MO04, and FF04, prepared from *Agrocybe aegerita*, *Marasmius oreades*, and *Fomes fomentarius*, respectively, had minimal inhibitory effects on NO production, compared to the levels of nitrite measured in the growth medium of cells treated with LPS alone. In contrast, three extracts, DG04, PM14, and TZ04, prepared from *Daedalea gibbosa* (DG04), *Pleurotus pulmonarius* (PM14) and *Trametes zonata*

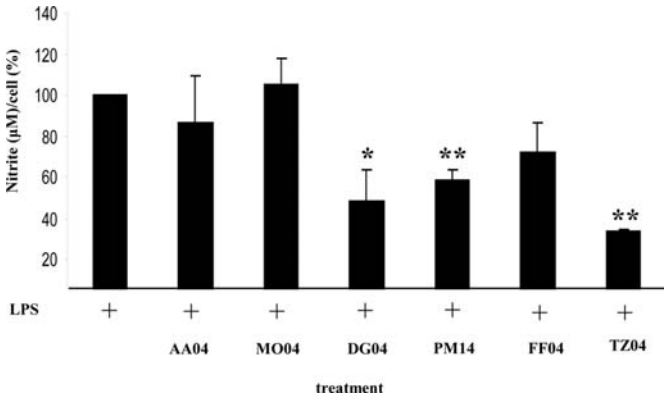

Figure 1. Effect of selected mushroom extracts on LPS-induced NO production in RAW 264.7 macrophage cells. RAW 264.7 cells were stimulated with LPS (1 μg/ml) and treated with six selected extracts (25 μg/ml) for 48 h. At the end of the incubation time, nitrite concentration in the cell growth medium was determined using Griess Reagent as described in Materials and methods. Data represent three repeats with comparable outcome. *P<0.05, **P<0.01.

(TZ04), significantly inhibited the LPS-induced production of NO, causing a reduction of 52, 41 and 66% in the presence of DG04, PM14 and TZ04 extracts, respectively.

Fungal extracts inhibit LPS-induced iNOS protein expression in RAW 264.7 macrophage cells. In an attempt to establish whether the inhibitory effect of the extracts on NO production is mediated by the inhibition of iNOS protein expression, five extracts were tested for their effect on iNOS protein expression using immunoblotting. Data shown in Fig. 2 illustrate that while all tested extracts caused a dose-dependent reduction in LPS-induced iNOS protein expression in RAW 264.7 macrophage cells with varying potency, two extracts, DG04 and MO04, at 25 μg/ml, showed the most potent effect in inhibiting LPS-induced iNOS protein expression in these cells. The culture liquid extract of *Daedalea gibbosa*, DG04, was defined as the most potent and selected for further evaluation.

D. gibbosa crude organic extract, DG04, inhibits the LPS-induced PGE_2 production in RAW 264.7 murine macrophage cells. It is well-established that LPS treatment of RAW 264.7 macrophage cells causes the stimulation of many pro-inflammatory molecules such as various lymphokines, cytokines, nitric oxide (NO), and prostaglandin E_2 (PGE_2) (28). Thus, we also monitored levels of PGE_2 in LPS-treated RAW 264.7 macrophage cells in response to the presence of *D. gibbosa* crude organic extract. Results presented in Fig. 3 show that treatment with LPS caused a significant induction of PGE_2 (Fig. 3A) and NO (Fig. 3B) production. In addition, levels of LPS-induced PGE_2 (Fig. 3A) and NO (Fig. 3B) were significantly abrogated in the presence of curcumin and JSH-23, arguing that production of both substances are regulated by the NF-κB pathway. As expected, 1400W, an iNOS selective enzymatic inhibitor, was a potent inhibitor of NO production (Fig. 3B) with minimal effect on PGE_2 production (data not shown). Presence of the crude extract of *D. gibbosa*, DG04, inhibited both the LPS-induced PGE_2 (Fig. 3A) and NO (Fig. 3B) production in RAW 264.7 macrophage cells, arguing that *D. gibbosa* organic extract contains substances that interfere with the regulation of NO and PGE_2 production.

The crude extract of D. gibbosa, DG04, inhibits iNOS transcription in LPS-induced RAW 264.7 macrophage cells. In order to establish whether the inhibitory effect of DG04 on NO production and iNOS protein expression in RAW 264.7 macrophage cells is mediated by its effect on iNOS gene transcription, we examined the effect of *D. gibbosa* organic extract on iNOS transcript using RT-PCR. RAW 264.7 macrophage cells were treated with LPS in the presence and absence of the *D. gibbosa* crude organic extract and several pharmaceutical inhibitors relevant to iNOS regulation. Results shown

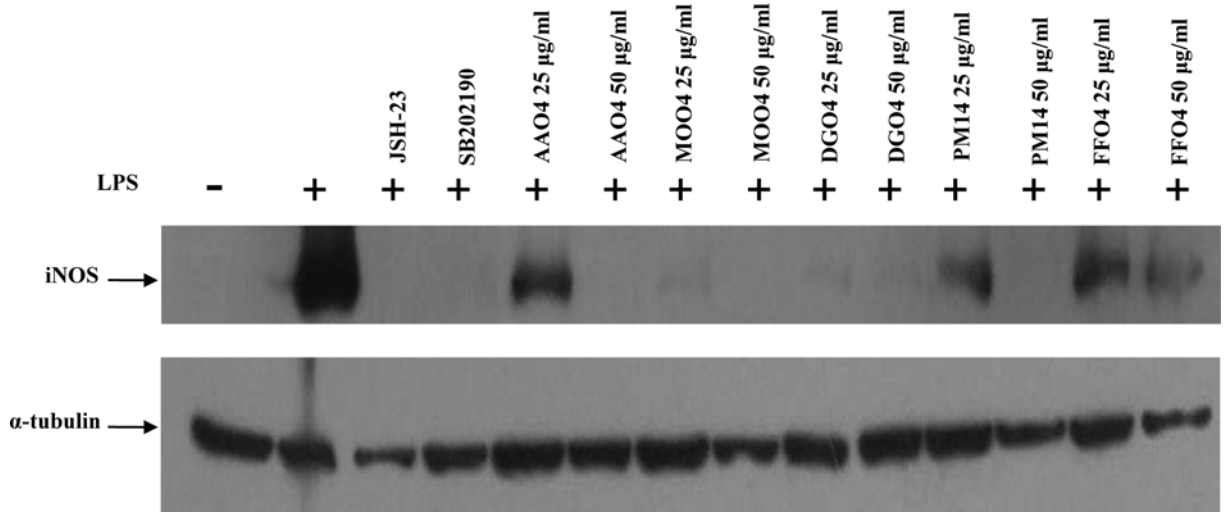

Figure 2. Effect of selected fungal extracts on LPS-induced iNOS protein expression in RAW 264.7 macrophage cells. RAW 264.7 cells ($1x10^5$ cells/well) were incubated for 24 h with or without LPS (1 μg/ml) and in the presence of selected fungal extracts at 25 and 50 μg/ml. Levels of iNOS and α-tubulin were monitored as described in Materials and methods.

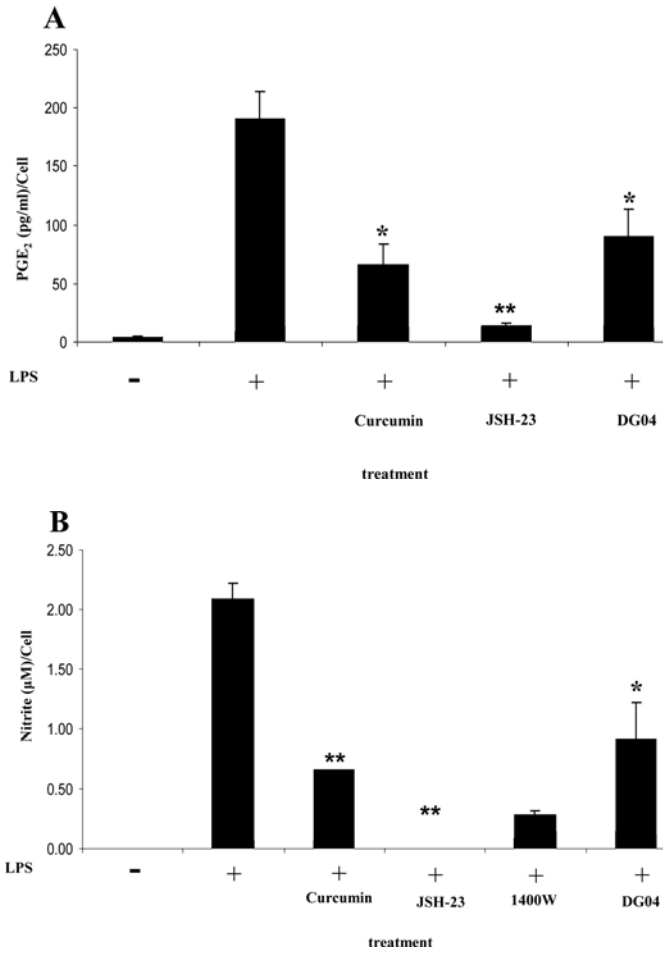

Figure 3. Effect of the crude extract of *D. gibbosa*, DGO4, on PGE_2 (A) and NO (B) production in RAW 264.7 macrophage cells. Cells were stimulated with 1 μg/ml LPS and treated with IKK inhibitor (curcumin), NF-κB inhibitor (JSH-23), iNOS enzymatic activity inhibitor (1400W) and 25 μg/ml of the crude extract, DG04, for 24 h. Nitrite and PGE_2 concentration in the cell growth medium was determined using Griess reagent and Prostaglandin E_2 immunoassay kit, respectively, as described in Materials and methods. Values represent two independent experiments. *P<0.05, **P<0.01.

in Fig. 4 illustrate that expression of iNOS is up-regulated by LPS. Furthermore, co-treatment with 10 μM of either JSH-23 (NF-κB inhibitor), SB20190 (p38 inhibitor), or SP600125 (JNK inhibitor) resulted in a significant reduction of the steady-state mRNA levels of iNOS, arguing that LPS induction activates NF-κB, p38, and JNK pathways leading to an accumulation of iNOS transcript. Similarly, the crude organic extract of *D. gibbosa* significantly inhibited the levels of LPS-induced iNOS transcript in RAW 264.7 macrophage cells in a dose-dependent manner, indicating that *D. gibbosa* organic extract mediated its effect by modulating the transcription of the iNOS gene. In contrast, presence of ERK inhibitor, PD98059, and the iNOS selective enzymatic inhibitor, 1400W, showed a minimal effect on the level of iNOS transcript.

Effect of D. gibbosa fractions on iNOS promoter activity. The crude organic extract of *D. gibbosa*, DG04, was chemically fractionated through liquid chromatography, as described in Materials and methods. Eight fractions, named F1-F8, were obtained. With the aim at identifying active fractions, the crude organic extract (DG04), along with the eight fractions, F1-F8, were evaluated for their abilities to inhibit iNOS promoter activity in MCF-7 breast cancer cell line, carrying a luciferase reporter gene under the control of iNOS promoter (14). Results presented in Fig. 5 show that IMD-0354 (IKKß selective inhibitor) as well as curcumin (NF-κB inhibitor) caused a significant inhibition of iNOS promoter-mediated reporter activity, indicating that the reporter expression is regulated by the NF-κB pathway. Similarly, the crude organic extract of *D. gibbosa* caused a significant reduction in levels of reporter activity. Three fractions, F5-F7, caused a significant reduction in the reporter levels and were assigned as *D. gibbosa* active fractions.

Effect of D. gibbosa fractions on LPS-induced NO production and iNOS protein expression in RAW 264.7 macrophage cells. Next, the eight fractions, F1-F8, were tested for their abilities to inhibit LPS-induced NO production and iNOS

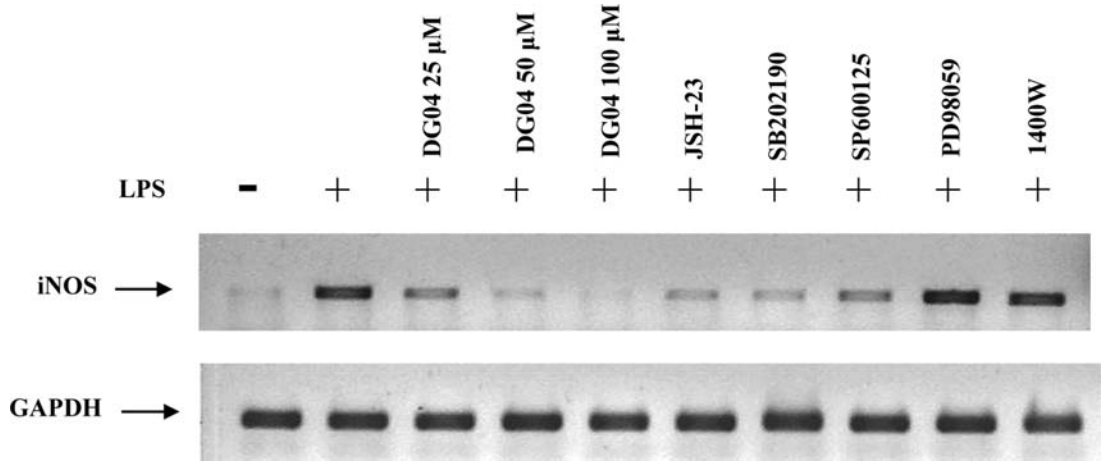

Figure 4. Effect of the crude extract of *D. gibbosa*, DGO4, on LPS-induced iNOS transcription in RAW 264.7 macrophage cells. Cells were stimulated with LPS (1 μg/ml) and treated with 10 μM of either NF-κB inhibitor (JSH-23), p38 inhibitor (SB202190), JNK inhibitor (SP600125), ERK inhibitor (PD98059), or iNOS enzymatic activity inhibitor (1400W) and the crude extract, DG04, at three different concentrations for 24 h. Levels of iNOS transcript were determined using RT-PCR and compared to levels of the housekeeping gene, GAPDH, as described in Materials and methods.

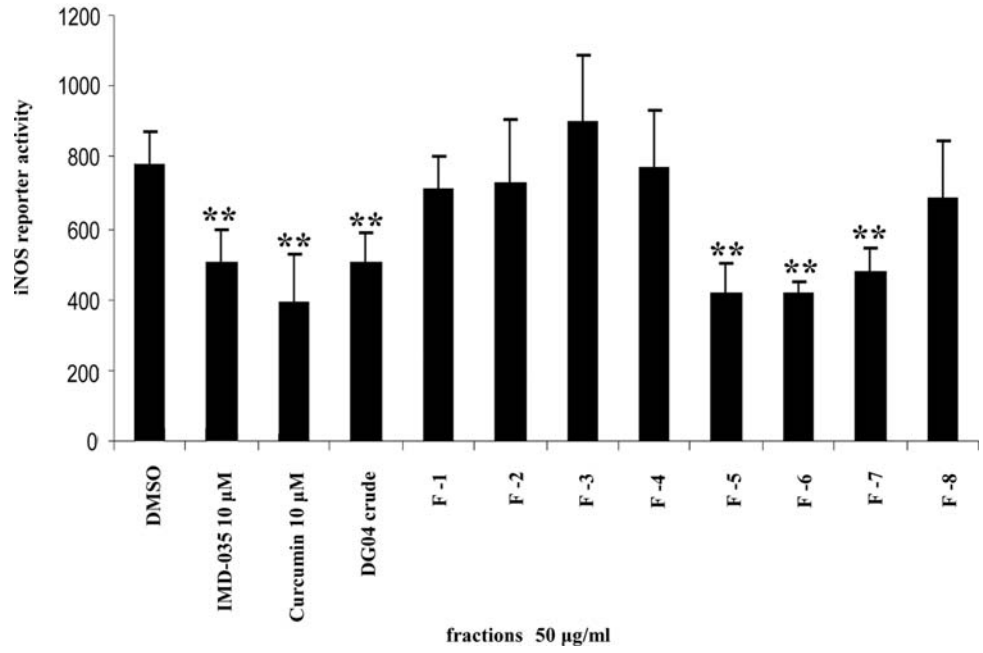

Figure 5. Effect of *D. gibbosa* fractions on iNOS-Luciferase expression in MCF7 breast cancer cell line. Stably transfected MCF-7 cells with iNOS-Luciferase were treated with *D. gibbosa* organic crude extracts and *D. gibbosa* fractions for 24 h. Positive controls included IMD-035 and curcumin. Level of reporter activity was determined as described in Materials and methods. $^{*}P<0.05$, $^{**}P<0.01$.

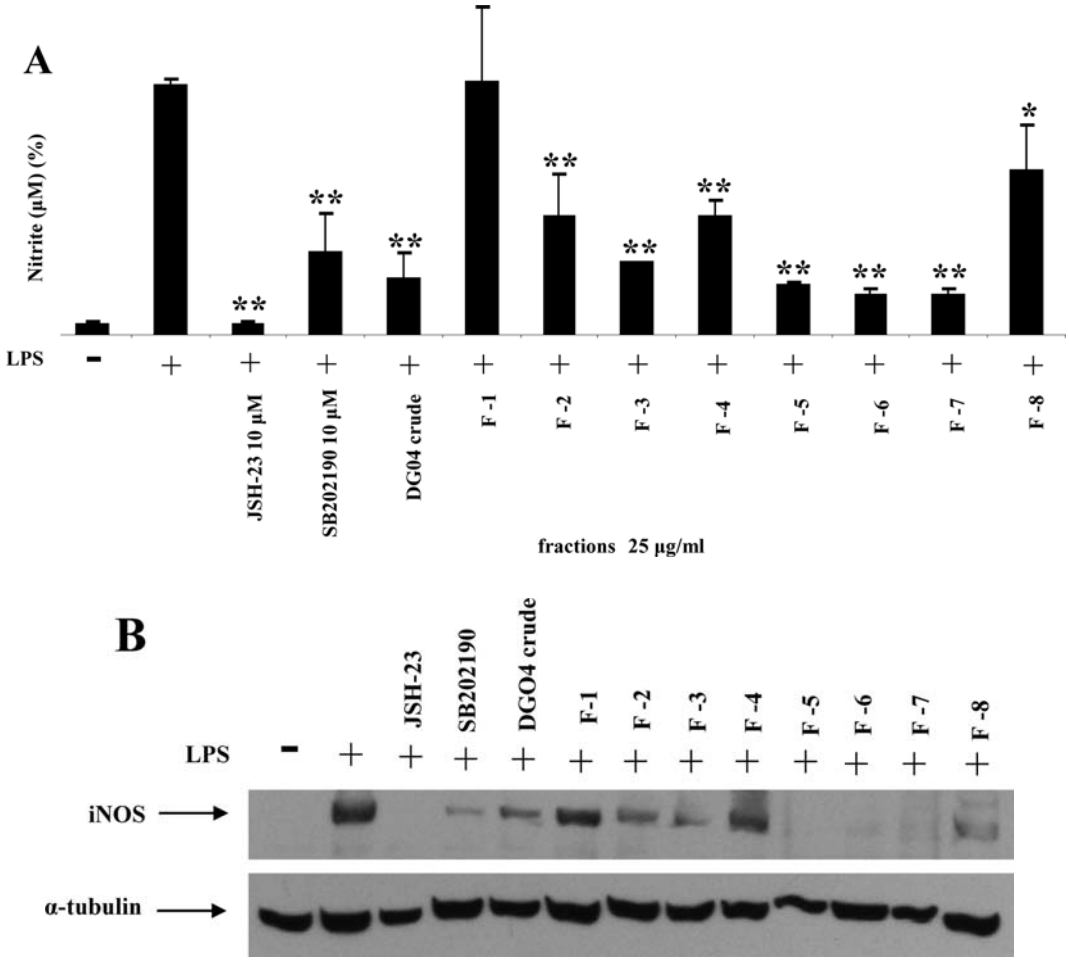

Figure 6. Effect of *D. gibbosa* fractions on LPS-induced NO production (A) and iNOS protein expression (B) in RAW 264.7 macrophage cells. Cells were stimulated with LPS (1 μg/ml) and treated with NF-κB inhibitor (JSH-23), p38 inhibitor (SB202190) and *D. gibbosa* crude extract and fractions (25 μg/ml). (A) Nitrite concentration in the cell growth medium was determined using Griess reagent. (B) Levels of iNOS protein were determined using immunoblotting and compared to levels α-tubulin as described in Materials and methods. $^{*}P<0.05$, $^{**}P<0.01$.

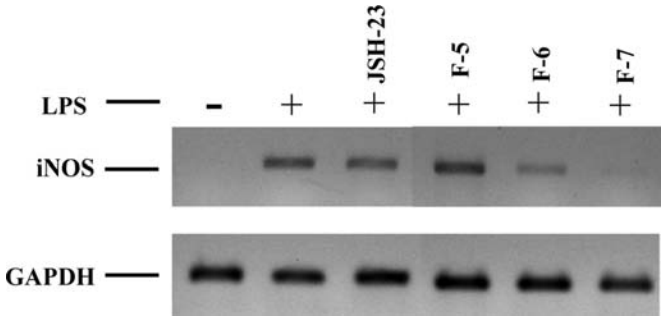

Figure 7. Effect of *D. gibbosa* active fractions F5-F7 on LPS induced iNOS transcription in RAW 264.7 macrophage cells. Cells were stimulated with LPS (1 μg/ml) and treated with NF-κB inhibitor (JSH-23) and the fractions DG04-5-DG04-7 (25 μg/ml), for 24 h. Levels of iNOS transcript were determined using RT-PCR and compared to levels of the housekeeping gene, GAPDH, as described in Materials and methods.

protein expression in RAW 264.7 macrophage cells. Cells were stimulated with LPS (1 μg/ml) and treated with the different fractions at 25 μg/ml for 24 h, as described in Materials and methods. Results, presented in Fig. 6, show that NO production is significantly up-regulated by LPS, and the presence of 10 μM of JSH-23 (NF-κB inhibitor) or SB202190 (p38 inhibitor) abrogated LPS-induced production of NO. Treatment of RAW 264.7 macrophage cells with *D. gibbosa* organic crude extract caused a significant reduction in NO production. Interestingly, all fractions caused a reduction in LPS-induced NO production in RAW 264.7 macrophage cells with varying potency, except for fraction F1. However, three fractions (F5-F7) showed the most potent effect in inhibiting LPS-induced NO production in these cells (Fig. 6A). Comparable data were obtained when we followed levels of iNOS protein in LPS-induced RAW 264.7 macrophage cells (Fig. 6B) by immunoblotting. Altogether, our results illustrated that fractions F5-F7 are the most active fractions, and therefore we will focus on them.

Effect of fractions F5-F7 on iNOS transcription in LPS stimulated RAW 264.7 macrophage cells. Our previous data have identified fractions F5-F7 as the most active fractions of *D. gibbosa* organic extract, causing significant inhibition of NO production and iNOS protein expression. The effect of the *D. gibbosa* active fractions on the levels of iNOS transcript in LPS-treated RAW 264.7 macrophage cells was followed. Results shown in Fig. 7 illustrate that *D. gibbosa* active fractions, F6 and F7, caused a reduction in iNOS transcript, indicating that their activity is mediated through interference with iNOS transcription. In contrast, *D. gibbosa* active fraction F5 showed minimal effect on iNOS transcription, while causing a dramatic reduction in NO production and iNOS protein expression (Fig. 6), arguing for a potential post-transcription regulation of iNOS expression caused by substances found in *D. gibbosa* active fraction F5.

miRNA profiling of RAW 264.7 macrophage cells treated with F5 fraction. Prior to miRNA profiling, a set of quality control analysis such as polyadenylation with poly-A-polymerase, hybridization intensity, annealing control, single mismatch control, extension control for 3'-mismatch and cross contamination were performed. Polyadenylation control oligos detect transcripts of a set of highly expressed housekeeping genes that already contain a stretch of polyA sequence and were independent of the polyadenylation process. In contrast, microRNA target amplification is dependent on successful polyadenylation. Total RNA was isolated from RAW 264.7 macrophage cells treated with LPS and F5 fraction for 4 h and miRNA profiling was conducted, as described in Materials and methods. Data analysis with appropriate cut-off scores show that there is an effect of LPS and LPS + F5 fraction on miRNA expression in RAW 264.7 cells (data not shown). Levels of different miRNAs were significantly down-regulated upon treatment with LPS such as miR-495, miR-450b-5p, miR-449c, miR-218-2*, miR-501-3p, miR-34c, miR-124, miR-691 and miR-677, miR-201, miR-669k, and miR-466b-5p are shown in Fig. 8.

Levels of miR-495, miR-450b-5p, and miR-449c that were down-regulated in response to LPS did not change following treatment with F5 fraction (Fig. 8A). However, the presence of F5 fraction partially abrogated the LPS-induced increase in miRNAs such as miR-218-2*, miR-501-3p, miR-34c, miR-124, miR-691, and miR-677 (Fig. 8B) and completely abrogated levels of miR-201, miR-669k, and miR-466b-5p (Fig. 8C).

Effect of fractions F5-F7 on LPS-induced phosphorylation of IKBα, p38, and JNK in RAW 264.7 macrophage cells. LPS is known to activate the NF-κB and MAPK pathways in macrophages (1), which is mediated by enhanced phosphorylation of IKBα, p38, and JNK leading to the activation of the NF-κB and MAPK pathways, respectively. Our previous results pointed toward the involvement of these pathways in the regulation of iNOS expression in RAW 264.7 macrophage cells, in response to LPS (data not shown). In an attempt to initiate elucidation of the mechanism of action of the active fractions, we tested their effect on IKBα, JNK, and p38 phosphorylation. Our preliminary data showed that 20-25 min of treatment with LPS caused the highest level of IKBα, p38, and JNK phosphorylation in treated RAW 264.7 macrophage cells. Thus, RAW 264.7 macrophage cells were stimulated with LPS (1 μg/ml) for 20-25 min, as described in Materials and methods in the presence and absence of *D. gibbosa* active fractions.

Results summarized in Fig. 9 show that LPS treatment caused a significant increase in levels of phopsho-IKBα (Fig. 9A), phopsho-p38 (Fig. 9B), and phospho-JNK (Fig. 9C). JSH-23 and SB202190 had minimal effect on levels of LPS-induced phopsho-IKBα (Fig. 9A). *D. gibbosa* crude organic extract, as well as active fractions F5 and F6, showed minimal inhibitory effect on levels of LPS-induced phospho-IKBα (Fig. 9A). In contrast, the presence of *D. gibbosa* active fraction F7 caused a significant inhibition of the levels of LPS-induced phospho-IKBα (Fig. 9A). LPS-induced phopsho-p38 and phospho-JNK were partially inhibited by SB202190 and SP600125, respectively (Fig. 9B), which correlated with published data, suggesting autophosphorylation activity of p38 MAPK (29). The *D. gibbosa* organic crude extract showed minimal effect on LPS-induced phospho p38 (Fig 9B). Only *D. gibbosa* active fraction F5 showed moderate inhibition of LPS-induced p38 phosphorylation (Fig. 9B).

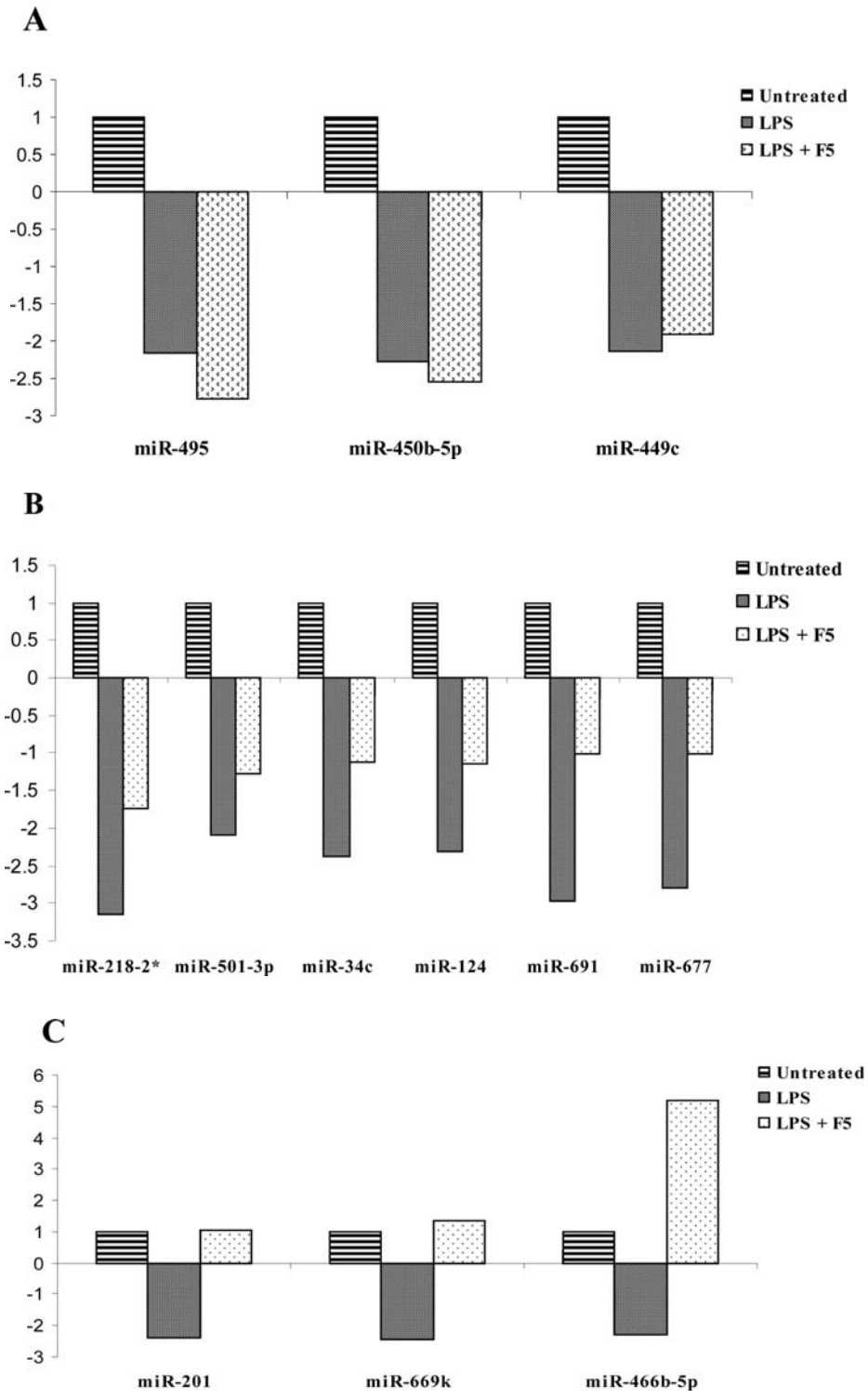

Figure 8. miRNA profiling of RAW 264.7 macrophage cells treated with F5 fraction. miRNA that were significantly down-regulated by presence of LPS are selected. Experiment and data analysis were performed as described in Materials and methods. Levels of miRNA that were down-regulated by LPS with minimal (A), moderate (B), and significant (C) effects in the presence of F5.

In addition, Fig. 9C shows that LPS significantly increased levels of phospho-JNK. The presence of curcumin, as well as SP200125, caused a significant inhibition of LPS-induced phospho-JNK (Fig. 9C), arguing that SP600125 is capable of inhibiting autophosphorylation of JNK. In contrast, SB202190 caused augmentation of phospho-JNK levels, in accordance with Muniyappa and Das (30), which demonstrated the phosphorylation and activation of JNK caused by SB2020190 in multiple cell lines. However, the presence of *D. gibbosa* organic crude extract resulted in minimal effect on levels of LPS-induced phospho-JNK. Interestingly, the presence of *D. gibbosa* active fractions F5-F7 resulted in augmenting levels of phospho-JNK (Fig. 9C) similarly to the presence of SB202190, a p38 inhibitor. Thus, inhibition of iNOS

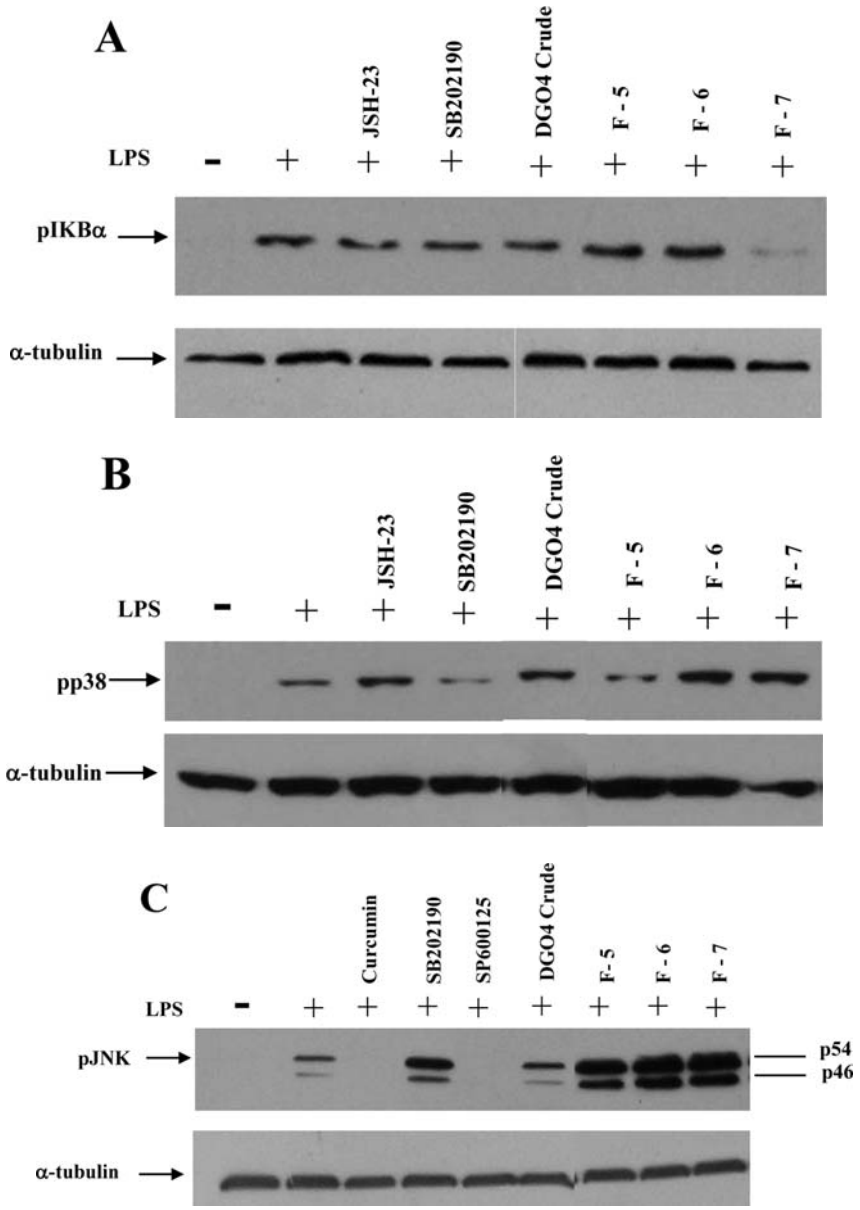

Figure 9. Effect of *D. gibbosa* active fractions F5-F7 on LPS-induced phosphorylation of IKBα (A), p38 (B) and JNK (C) in RAW 264.7 macrophage cells. Cells were stimulated with LPS (1 μg/ml) and treated with either JSH-23 (NF-κB inhibitor), curcumin (NF-κB inhibitor), SB202190 (p38 inhibitor), or SP600125 (JNK inhibitor) as well with *D. gibbosa* active fractions F5-F7 (25 μg/ml) for 20 min. Levels of phospho-IKBα, phospho-p38 and phospho-JNK were monitored by immunoblotting using phopsho-specific antibodies as described in Materials and methods.

expression by *D. gibbosa* active fractions is probably not mediated through the JNK pathway.

Effect of D. gibbosa active fractions on NF-κB binding activity. As shown in Fig. 9, the active fraction F6 modulated iNOS transcription with minimal effects on IKK and p38 activity. Therefore, in order to investigate the possibility that F6 affects NF-κB DNA binding activity, EMSA analysis was performed. The results obtained are presented in Fig. 10 and demonstrate that while the presence of F4 had no effect on NF-κB/DNA interactions, treatment with the active fractions, F5 and F7, caused a reduction in NF-κB DNA binding activity, while the addition of F6 to the EMSA reaction was found to moderately inhibit NF-κB DNA binding activity. The IC_{50} of the active fractions F5, F6, and F7 was 20, 50, and 15 μg reaction, respectively. These results suggest that inhibition of iNOS expression by *D. gibbosa* active fractions, was involved in part in the attenuation of NF-κB DNA binding activity.

Discussion

An increasing number of studies demonstrated the ability of mushroom extracts or mushroom-derived moieties to exhibit anti-inflammatory effects (31-38). Anti-inflammatory activity was mediated by polysaccharides (38) or by small molecules such as cordycepin (34), grifolin (33,37), ergosterol peroxide (35), cycloepoxydon (32), panepoxydone (31), and caffeic acid phenethyl ester (CAPE) (39,40). Anti-inflammatory activity of mushroom-derived moieties have been reported to be mediated by NF-κB (31,32,34,35) in association with MAPK (35). Mushroom substances were reported to affect expression of iNOS and COX-2 in LPS-induced macrophages (31,33,34).

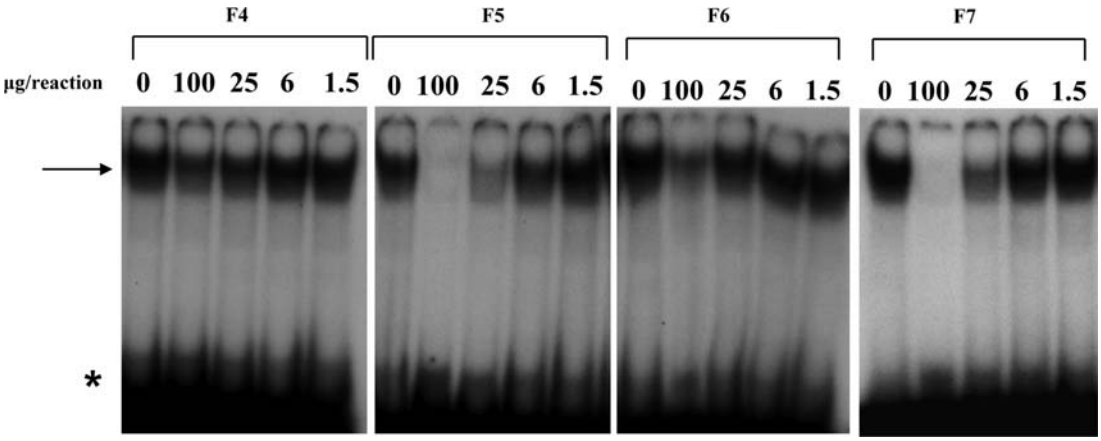

Figure 10. Effect of *D. gibbosa* active fractions on NF-κB binding activity. Nuclear extract, in the absence or presence of different concentrations (1.5, 6, 25, and 100 µg/reaction) of *D. gibbosa* fractions, F4-F7, were prepared and analyzed for NF-κB binding to DNA by EMSA, as described in Materials and methods. The asterisk indicates the position of the free probe. The arrow indicates the position of the NF-κB bound probe.

The present study aimed at identifying active fungal substances able to inhibit iNOS expression. Fifteen fungal species, belonging to different taxonomic and ecological groups, were selected and evaluated for their abilities to affect the expression of LPS-induced iNOS from RAW 264.7 murine macrophage cells.

Initially, we tested signaling pathways that are involved in mediating the LPS-induced iNOS expression in RAW 264.7 murine macrophage cells. Our results, agreed with published data (41,42), showing the involvement of NF-κB, and the two MAPKs, p38 and JNK, in the regulation of the LPS-induced expression of iNOS.

From the 15 fungal extracts tested, we found that 6 extracts inhibited the iNOS promoter activity in MCF-7 breast cancer cell line, carrying a luciferase reporter gene under the control of iNOS promoter, and the LPS-induced iNOS protein expression and NO production in RAW 264.7 cells. Among the different mushrooms, the culture liquid extract of *Daedalea gibbosa* was defined as the most potent.

Little is known about the biological activity of *D. gibbosa*. Recently, the ability of *D. gibbosa* mycelium extract to interfere with the Bcr-Abl kinase activity, leading to the inhibition of CML cell lines growth was reported (13). Previous work from our laboratory demonstrated that a crude ethyl acetate extract of *D. gibbosa* culture liquid strongly inhibited the NF-κB reporter activity in MCF-7 breast cancer cell line, carrying a luciferase reporter gene under the control of NF-κB -responsive promoter (9).

The crude liquid extract of *D. gibbosa* was potent in modulating PGE_2 and NO production from LPS-induced RAW 267.4 macrophage cells, arguing that the *D. gibbosa* liquid extract interfered with a common pathway that mediated the expression of pro-inflammatory genes, such as iNOS and COX-2. Similarly, other mushroom extracts, such as *Phellinus linteus*, were reported to modulate iNOS expression in LPS-induced macrophage, mediated by NF-κB and MAPK pathways (43,44).

With the aim of obtaining active moieties, the crude organic extract of *D. gibbosa* was chemically fractionated using liquid chromatography to obtain eight fractions. While most of the fractions caused a reduction in LPS-induced NO production in RAW 264.7 cells with varying potency, three fractions (F5-F7) showed the most potent effect. Furthermore, comparable data were obtained when we followed levels of iNOS protein in LPS-induced RAW 264.7 cells, and when we examined the fractions' ability to inhibit the iNOS reporter activity in the MCF-7 cell line. Initial examining of the mode of actions revealed that fraction F6 and F7 inhibited the accumulation of steady-state levels of iNOS transcript, indicating that the two fractions are directly involved in regulating the transcription of iNOS gene. In contrast, presence of fraction F5 did not cause a reduction in iNOS transcript and yet caused a dramatic reduction in iNOS protein and in NO production, arguing for potential post-transcription regulation of the iNOS expression caused by substances found in this fraction.

To elucidate the action mechanisms of the fractions, the effect of the fractions F5-F7 on the levels of pIKBα, pJNK, and pp38 in RAW 264.7 cells were monitored. Results shown in Fig. 9 demonstrated that the presence of fraction F7 caused a significant reduction in the phosphorylation levels of IKBα, arguing that this fraction might inhibit the activity of the IKK complex. However, fractions F5 and F6 showed minimal activity in inhibiting phosphorylation of IKBα. Furthermore, results shown in Fig. 9 demonstrate that fraction F5 caused a reduction in the levels of phospho-p38, indicating that activity of fraction F5 is mediated, at least in part, through the p38 MAPK pathway. While we provided evidence that fraction F5 affects the MAPK signaling pathway, it is unclear whether it affects p38 activity or its activation by modulating upstream signaling kinases such as MEK 3/6.

Our data and others (41) showed that the JNK signaling pathway is also involved in modulating the LPS-induced iNOS expression. Presented data showed that inhibition of p38 by SB20190 caused a significant augmentation of levels of pJNK, which is similar to published data (45). None of the active fractions F5-F7 inhibited phosphorylation levels of JNK. However, they were equally potent in the augmentation of pJNK levels. It is unclear yet whether substances that inhibited the activity of IKK (in F8) and p38 (in F5) were

also involved in the augmentation of pJNK in a similar fashion to SB20190. Fraction F6, which significantly inhibited iNOS transcription, showed a very minimal effect on IKK and MAPK activity. It is possible that F6 affects iNOS transcription by affecting downstream target molecules that were not the subject of our current study.

In an attempt to elucidate the mechanism of action of F6, we followed the ability of F6 and other active fractions to affect NF-κB DNA binding activity. Results presented in Fig. 10 demonstrate that fractions F5 and F7 significantly inhibited NF-κB DNA binding activity, which means that F5 and F7 contain substances that also affect iNOS transcription, probably by interfering with NF-κB DNA binding activity. In contrast, the presence of F6 showed minimal activity in modulating NF-κB DNA binding activity. Furthermore, data presented in Fig. 9 showed that substances found in F6 were not active in modulating either IKK or p38 phosphorylation. Thus, the mode of action of substances found in F6 in regulating iNOS expression is still unknown.

The active fraction F5 was shown to inhibit p38 phosphorylation (Fig. 9B) and therefore its activity, but not the activity of IKK complex (Fig. 9A). In addition, fraction F5 inhibited NF-κB DNA binding activity (Fig. 10). Thus, one might speculate that inhibition of p38 by F5 might lead to compromise activity of NF-κB, since it is well documented that p38 regulates the association between p65 and p300 and the acetylation of p65-K310 (46,47). Inhibition of p65 acetylation by p38 is expected to inhibit NF-κB transcription activity and, consequently, levels of iNOS transcript. Our results, presented in Fig. 7, do not support this hypothesis. The presence of F5 had minimal effect on iNOS transcription, and data in Fig. 7 argue for post-translation activity of F5 that may be mediated, in part, by MAPK p38. One possible mechanism might be that translation of iNOS is modulated by miRNA in a similar fashion to other pro-inflammatory genes (48).

Substances found in fraction F5 may affect expression of LPS-induced miRNA through the MAPK p38 pathway. We hypothesize that LPS affects iNOS expression in a transcriptional and a post-transcriptional manner. LPS-mediated post-transcription modulation might be mediated by miRNA that regulate translation of iNOS and other inflammatory mediators (49). Our preliminary miRNA profiling data identified a number of miRNA that are significantly down-regulated upon LPS treatment, such as miR-218-2*, miR-501-3p, miR-34c, miR-124, miR-691, miR-677, miR-201, miR-669k, and miR-466b-5p (Fig. 8). Active fraction F5 might inhibit iNOS translation by abrogating the LPS-induced down-regulation of miRNA such as miR-201, miR-669k, and miR-466b-5p (Fig. 8C). Experiments are underway to examine this possibility.

Substances found in the *D. gibbosa* liquid culture exhibited diverse biological activities leading to the inhibition of NO and PGE_2 production, and thereby exhibited an anti-inflammatory effect. Our results illustrated the biological effects of natural products that might be mediated by a variety of substances capable of interfering in a synergistic or additive way with multiple signaling pathways, leading to the desired pharmaceutical effect. The multiple activity of *D. gibbosa* fractions are expected, due to the fact that the different fractions analyzed might exhibit a different chemical composition. Identification of the chemical composition of the fractions will be a key step to possibly associate specific biological activity to single molecules or to determine possible cooperative biological effects.

The present study demonstrated the potential of the medicinal mushroom *D. gibbosa* as a source of bioactive substances to be utilized for pharmaceutical purposes including cancer and anti-inflammatory therapeutics.

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