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Dipyridamole activation of mitogen-activated protein kinase phosphatase-1 mediates inhibition of lipopolysaccharide-induced cyclooxygenase-2 expression in RAW 264.7 cells

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Abstract

Dipyridamole is a nucleoside transport inhibitor and a non-selective phosphodiesterase inhibitor. However, the mechanisms by which dipyridamole exerts its anti-inflammatory effects are not completely understood. In the present study, we investigated the role of mitogen-activated kinase phosphatase-1 (MKP-1) in dipyridamole's anti-inflammatory effects. We show that dipyridamole inhibited interleukin-6 and monocyte chemoattractant protein-1 secretion, inducible nitric oxide synthase protein expression, nitrite accumulation, and cyclooxygenase-2 (COX-2) induction in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages. Dipyridamole inhibited the nuclear factor kappa B (NF-κB) signaling pathway as demonstrated by inhibition of the inhibitor of NF-κB (IκB) phosphorylation, IκB degradation, p65 translocation from the cytosol to the nucleus, and transcription of the reporter gene. Dipyridamole also inhibited LPS-stimulated p38 mitogen-activated protein kinase (p38 MAPK) and IκB kinase-beta (IKK-β) activities in RAW 264.7 cells. A p38 MAPK inhibitor, SB 203580, inhibited LPS-stimulated COX-2 expression and IKK-β activation suggesting that LPS may activate the NF-κB signaling pathway via upstream p38 MAPK activation. Furthermore, dipyridamole stimulated transient activation of MKP-1, a potent inhibitor of p38 MAPK function. Knockdown of MKP-1 by transfecting MKP-1 siRNA or inhibition of MKP-1 by the specific inhibitor, triptolide, significantly reduced the inhibitory effects of dipyridamole on COX-2 expression induced by LPS. Taken together, these data suggest that dipyridamole exerts its anti-inflammatory effect via activation of MKP-1, which dephosphorylates and inactivates p38 MAPK. Inactivation of p38 MAPK in turn inhibits IKK-β activation and subsequently the NF-κB signaling pathway that mediates LPS-induced cyclooxygenase-2 expression in RAW 264.7 cells.

Keywords: Lipopolysaccharide; Nitric oxide; Mitogen-activated kinase phosphatase-1; Cyclooxygenase-2; Signal transduction; RAW 264.7 macrophage; Anti-inflammatory effect

1. Introduction

Dipyridamole is a drug that is frequently used in nephrology clinics because it improves proteinuria in a variety of glomerulonephritis (Camara et al., 1991). Dipyridamole co-formulated with aspirin significantly reduces proteinuria in patients with membranous proliferative glomerulonephritis (Harmankaya et al., 2001), and reduces the risk of a second stroke in patients with prior stroke/transient ischemic attack (Lenz and Hilleman, 2000). Furthermore, dipyridamole is a useful agent when combined with warfarin in certain patient groups, such as those with prosthetic heart valves (Gibbs and Lip, 1998).

Dipyridamole is a non-selective phosphodiesterase (PDE) inhibitor, which inhibits degradation of cAMP and cGMP (Beavo, 1995), and an inhibitor of nucleoside transport (Hammond et al.,

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1985). Dipyridamole also inhibits platelet function by blocking adenosine reuptake and degradation (Stafford et al., 2003). Increasing evidence has indicated that many phosphodiesterases possess anti-inflammatory effects. At least 11 phosphodiesterase isozymes have been cloned. The rank order of the inhibition of phosphodiesterases by dipyridamole is PDE5>PDE4>PDE2> PDE1 and 3 (Beavo, 1995). Recently it was shown that dipyridamole inhibits the nuclear factor kappa B (NF-κB)-mediated inflammatory effect (Weyrich et al., 2005).

NF-κB comprises a family of transcription factors including subunit members of p50, p65 (Rel A), c-Rel, p52, and Rel B, that are involved in the inducible expression of a variety of genes that regulate inflammatory responses (Baldwin, 1996). NF-κB exists in a latent form in the cytoplasm of unstimulated cells comprising a transcriptionally active dimer bound to the inhibitory protein, the inhibitor of NF-κB (IκB). The multiple forms of IκB are IκB-α, -β, -γ (p105), -δ (p100), -ε, and Bcl-3 (Baldwin, 1996). A kinase complex known as IκB kinase (IKK) rapidly phosphorylates IκB-α. The IKKs are part of a large multiprotein complex called the IKK signalsome, which is important for NF-κB activation. The IKK isoforms, IKK-α and IKK-β, phosphorylate IκB, which leads to IκB degradation and the translocation of NF-κB from the cytosol to the nucleus (Brown et al., 1995; Chen et al., 1995). IKK kinase activity is stimulated when cells are treated with cytokines or by overexpression of the kinases, MAPK/Erk kinase kinase-1 and NF-κB-inducing kinase (Malinin et al., 1997; Yin et al., 1998). Recently, dipyridamole has been shown to attenuate NF-κB translocation and block MMP-9 and monocyte chemoattractant protein-1 (MCP-1) generated by lipopolysaccharide (LPS)-treated monocytes (Weyrich et al., 2005).

Activation of the NF-κB signaling pathway is closely linked to activation of mitogen-activated protein kinases (MAPKs). The p38 MAPK signaling pathway is involved in LPS-induced cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expressions in macrophages (Chen and Wang, 1999; Dean et al., 1999; Paul et al., 1999). Activation of MAPKs requires phosphorylation of both the threonine and tyrosine residues, and phosphorylation is strictly controlled in part by a family of about 12 dual-specificity phosphatases or MAPK phosphatases, which inactivate MAPKs by dephosphorylation of both the threonine and tyrosine residues within the activation motif (Camps et al., 2000). Glucocorticoids inhibit the expression of proinflammatory genes and are widely used in the treatment of inflammatory diseases. Several lines of evidence indicate that glucocorticoids mediate anti-inflammatory effects via induction and activation of mitogen-activated protein kinase phosphatase 1 (MKP-1) (Clark, 2003; Kassel et al., 2001). MKP-1 dephosphorylates and inactivates p38 MAPK (Lasa et al., 2002), and subsequently suppresses proinflammatory cytokine biosynthesis in LPS-stimulated macrophages (Chen et al., 2002).

In this study, we investigated the role of MKP-1 in dipyridamole inhibition of iNOS and COX-2 expressions in LPS-stimulated RAW 264.7 cells. We demonstrate that dipyridamole exerts potent inhibitory effects on LPS-induced interleukin-6 (IL-6), MCP-1, iNOS, and COX-2 expressions in RAW 264.7 macrophages via activation of MKP-1. We further linked MKP-1 activation to dephosphorylation and inactivation of p38 MAPK which regulates IKK activation and the NF-κB signaling pathway. Taken together, our data reveal that dipyridamole stimulates MKP-1 activation which negatively regulates p38 MAPK and NF-κB signaling pathways, and subsequently suppresses LPS-induced COX-2 expression in RAW 264.7 macrophages.

2. Materials and methods

2.1. Materials

Affinity-purified mouse polyclonal antibodies to COX-2, iNOS, IκB-α, IKK-β, and p65 NF-κB, protein A beads, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were obtained from Transduction Laboratory (Lexington, KY). All materials for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Hercules, CA). LPS, 2′-amino-3′-methoxyflavone (PD 98059), pyrrolidine dithiocarbamate (PDTC), and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB 203580) were purchased from Calbiochem (San Diego, CA). GST-IkB and activating transcription factor-2 (ATF-2) were from New England Biolabs (Beverly, MA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, sodium pyruvate, penicillin, and streptomycin were purchased from Life Technologies (Gaithersburg, MD). The 5-bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium (BCIP/NBT) substrate was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim (Mannheim, Germany). ELISA kits for IL-6 and MCP-1 were provided by the manufacturer (RayBiotech, Norcross, GA).

2.2. Culture of RAW 264.7 cells and preparation of cell lysates and nuclear extracts

The murine macrophage cell line of RAW 264.7 cells was cultured in DMEM supplemented with 2.44 g/l $NaHCO_3$, 2 mM L-glutamine, 1 mM sodium pyruvate, 10% FBS, and penicillin (100 U/ml)/streptomycin (100 mg/ml). Cultures were maintained in a humidified incubator in 5% CO_2 at 37 °C. Treatment of cells with either dipyridamole, specific inhibitors, a preparation of cell lysates, or nuclear extracts was as described previously (Wu et al., 2003). In studies of NF-κB translocation, both cytosolic and nuclear extracts were used; only cytosolic extracts were used for IκB-α degradation.

2.3. Determination of IL-6 and MCP-1

IL-6 and MCP-1 were quantified in supernatants of samples using ELISA kits according to instructions provided by the manufacturer (RayBiotech).

2.4. Measurement of nitrite formation

Cells were cultured in 35-mm Petri dishes and treated with dipyridamole or specific inhibitors as indicated followed by LPS and incubated in a humidified incubator at 37 °C. After

incubation, the supernatant of samples was removed and nitrite production was measured by adding 0.15 ml of cell culture medium to 0.15 ml of Griess reagent (Gross et al., 1991) in a 96-well plate, and incubating this in a dark place at 37 °C for 10 min. Absorbance was measured at 540 nm using a microplate reader.

2.5. Polyacrylamide gel electrophoresis (PAGE) and Western blotting

Electrophoresis was ordinarily carried out on different percentages of SDS-PAGE. Following electrophoresis, separated proteins on the gel were electrotransferred onto a polyvinyldifluoride (PVDF) membrane. Non-specific bindings were blocked with blocking buffer containing 5% fat-free milk powder in Tris buffer saline–Tween 20 for 1 h at room temperature, followed by incubation with a primary antibody in blocking buffer for 2 h. The PVDF membrane was then incubated with an alkaline phosphatase-conjugated secondary antibody for 1 h. Subsequently, Western blots were developed with bromo-chloro-indoryl phosphate/nitro blue tetrazolium (BCIP/NBT) as the substrate.

2.6. Immunoprecipitation and measurement of kinase activity

RAW 264.7 cells were lysed in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were then immunoprecipitated using an immobilized phospho-p38 MAPK (Thr180/Tyr182) monoclonal antibody or anti-IKK-β monoclonal antibody. Pellets obtained from immunoprecipitation were suspended and incubated with kinase buffer containing 25 mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$, then supplemented with 200 μM ATP and 2 μg of substrate protein. The ATF-2 fusion protein was used for p38 MAPK activity, and bacterially expressed GST-IκB-α (amino acids 5–55) was used for IKK-β activity. The reaction was stopped by the addition of sample buffer, and the sample was subjected to 10% PAGE. After the transfer, the PVDF membrane was incubated with buffer containing anti-phospho-ATF-2 (Thr71) or anti-phospho-IKK-β antibody, followed by buffer containing horseradish peroxidase-conjugated secondary antibody. For MKP-1 tyrosine phosphorylation, cell lysates (200 μg) were immunoprecipitated with the anti-MKP-1 antibody followed by Western blot analysis using phosphotyrosine-specific antibodies. Part of the immunoblots was cut and blotted with an anti-α-tubulin antibody to serve as equal loading control. Membranes were developed using the LumiGLO chemiluminescent reagent, and immunoreactive bands were visualized by autoradiography.

2.7. Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (Wu et al., 2003). Briefly, nuclear extracts were incubated with double-stranded oligonucleotides (CCT*GGGTTTCCCC*TTGAA*GGG*-

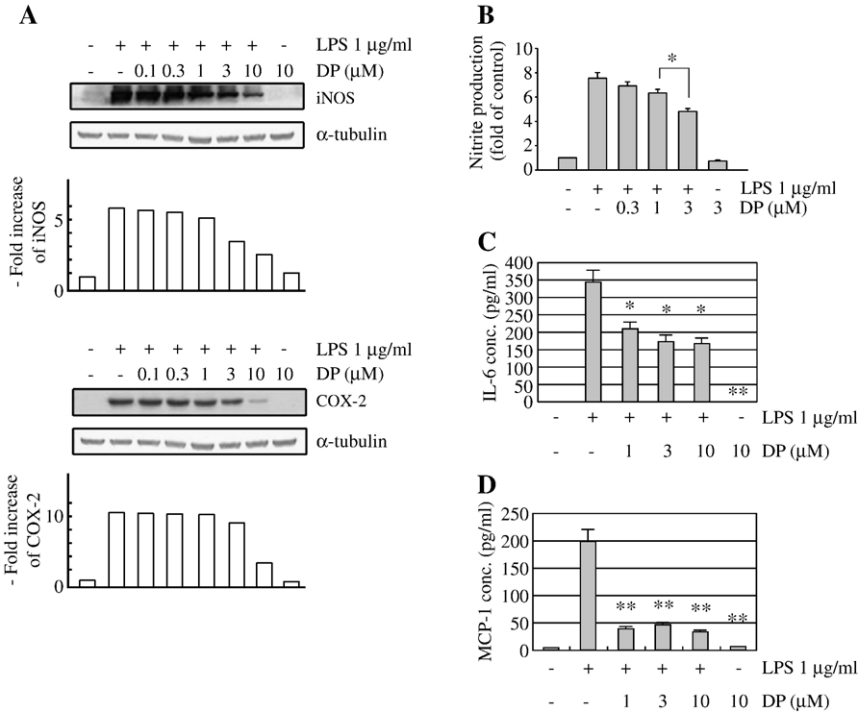

Fig. 1. Dipyridamole inhibited LPS-induced iNOS, COX-2, IL-6, and MCP-1 expressions in RAW 264.7 cells. Cells were incubated with different concentrations (0.1, 0.3, 1, 3, or 10 μM) of dipyridamole for 30 min before the addition of LPS (1 μg/ml) for 24 h. Cell lysates were subjected to Western blot analysis using iNOS- or COX-2-specific antibodies. The intensity of the protein bands of a typical experiment was quantified by densitometric scanning using a densitometer (Amersham Biosciences) and normalized with α-tubulin (A). Results are representative of three independent experiments. The supernatants of samples were removed and analyzed for nitrite accumulation (B) or IL-6 (C) and MCP-1 (D) accumulation. Data represents the mean ± S.E.M. of three independent experiments done in triplicate. *$P<0.05$.

*ATTTCCC*TCC) containing the NF-κB binding site (as underlined above). The oligonucleotides were end-labeled with [^{32}P]ATP. Extracted nuclear proteins (10 μg) were incubated with 0.1 ng [^{32}P]-labeled DNA for 15 min at room temperature in 25 ml binding buffer containing 1 mg poly (dI–dC). The mixtures were electrophoresed on 5% non-denaturing polyacrylamide gels. Gels were dried and imaged by autoradiography.

2.8. Immunofluorescent staining

For immunofluorescent staining, RAW 264.7 cells were plated on a 0.17-mm coverslip at a density of 1×10^6 cells/ml. After drug treatment, cells were washed with phosphate-buffered saline (PBS) twice, and fixed by 1 ml methanol: acetone (1:1) for 5 min at −20 °C. Each coverslip was incubated at 37 °C for 2 h in 0.5 ml NF-κB: PBS (1:75) antibody, washed with 1× PBS (plus 0.05% Tween 20) three times, and incubated with fluorescein (FITC)-conjugated goat anti-mouse antibody (1:100) 1 h. Cover slips were washed with 1× PBS (plus 0.05% Tween 20) three times and incubated with 2 μg/ml propidium iodide for double staining. The association of FITC-labeled ligands was viewed under a confocal microscope (model FV5 00IX70, Olympus, Tokyo, Japan). Samples were excited using an argon-ion laser (488 nm) to observe the green fluorescence of the fluorescein.

2.9. RNAi-mediated suppression of MKP-1

MKP-1 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) was used for MKP-1 RNAi knockdown. Transfection of siRNA in RAW 264.7 cells was performed using an oligofectamine reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). Transfected cells were applied to advanced assays and Western blot analysis.

2.10. Transfection and NF-kB luciferase assays

For these assays 5×10^5, RAW 264.7 macrophages were seeded into six-well (35-mm) plates. Cells were transfected on the following day by the Lipofectamine method with 0.5 mg of pGL2-ELAM-Luc and pBK-CMV-LacZ (Invitrogen). After 24 h, the medium was aspirated and replaced with fresh DMEM containing 10% fetal bovine serum. Cells were then stimulated with LPS for another 24 h before harvesting. Dipyridamole was added to the cells 30 min before LPS stimulation to assess the inhibitory effect of dipyridamole. Luciferase activity was determined with a luciferase assay system (Promega) and was normalized on the basis of LacZ expression. The level of induction of luciferase activity was determined as a ratio in comparison to control cells.

2.11. Statistical analysis

All data are expressed as the mean ± S.E.M. Statistical analysis was performed using one-way analysis of variance and Student's *t*-test. A difference between groups of $P<0.05$ was considered statistically significant.

3. Results

3.1. Dipyridamole inhibits LPS-induced iNOS, COX-2, IL-6 and MCP-1 expression in RAW 264.7 cells

To evaluate the effects of dipyridamole on the inflammatory responses in macrophages, we monitored LPS-induced iNOS, COX-2, IL-6 and MCP-1 expressions, and nitrite production in RAW 264.7 cells. When cells were treated with 1 μg/ml of LPS, the expression levels of iNOS and COX-2 significantly increased (data not shown). Treatment of cells with dipyridamole prior to the addition of LPS inhibited LPS-induced iNOS and COX-2 expressions in RAW 264.7 cells (Fig. 1A). Dipyridamole also inhibited LPS-stimulated nitrite accumulation, and the maximum inhibition was seen with 3 μM of dipyridamole (Fig. 1B). Similar to the results for iNOS and COX-2, LPS-induced IL-6 and MCP-1 expressions in RAW 264.7 were also significantly suppressed by dipyridamole treatment at as little as 1 μM (Fig. 1C, D). The MTT cell viability assay revealed that dipyridamole caused no cytotoxicity at concentrations lower than 10 μM (data not shown).

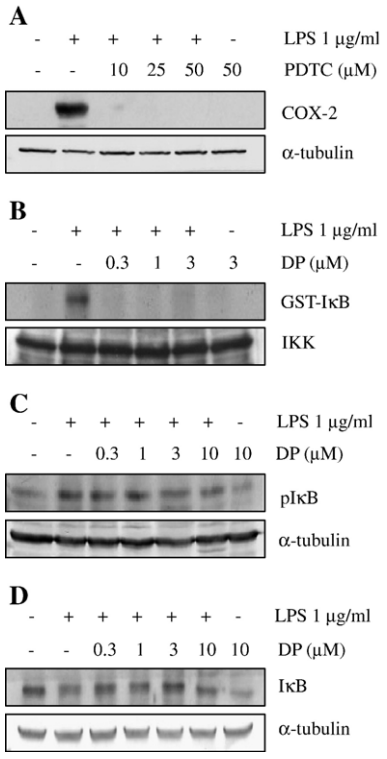

Fig. 2. Dipyridamole inhibited the NF-κB-dependent signaling pathway in RAW 264.7 macrophages. In (A), cells were pretreated with the NF-κB inhibitor, PDTC (10, 25, or 50 μM), for 30 min before incubation with LPS (1 μg/ml) for 24 h. Cell lysates were subjected to Western blot analysis using COX-2-specific antibodies. The equal loading in each lane was demonstrated by the similar intensities of α-tubulin. In (B), cells were incubated with different concentrations (0.3, 1, or 3 μM) of dipyridamole for 30 min before the addition of LPS (1 μg/ml) for 30 min. Cell lysates were immunoprecipitated with an anti-IKK-β monoclonal antibody, and IKK kinase activity was determined by the extent of phosphorylation on bacterially expressed GST-IkBα. In (C) and (D), cell lysates were subjected to Western blot analysis using anti-phospho-IκB antibodies (C) or anti-IκB antibodies (D). Results are representative of three independent experiments.

3.2. Dipyridamole inhibited LPS-induced NF-κB activation in RAW 264.7 cells

NF-κB is an important mediator of the LPS-induced inflammatory response. Treatment of cells with LPS resulted in IKK phosphorylation, IκB phosphorylation, and IκB degradation (data not shown). As shown in Fig. 2A, pretreatment of cells with pyrrolidone dithiocarbamate (PDTC), an NF-κB inhibitor, suppressed LPS-induced COX-2 expression in RAW 264.7 cells in a concentration-dependent manner. To elucidate whether the NF-κB-mediated pathway is influenced by dipyridamole, cells were treated with 1 μg/ml of LPS and different concentrations of dipyridamole, and then IKK-β activity, IκB-α phosphorylation, and IκB-α degradation were examined. Treatment of cells with dipyridamole before LPS stimulation inhibited the LPS-induced increases in IKK-β activity (Fig. 2B), IκB-α phosphorylation (Fig. 2C), IκB-α degradation (Fig. 2D). Incubation of RAW 264.7 cells with LPS (1 μg/ml) stimulated p65 translocation from the cytosol to the nucleus (data not shown), and dipyridamole treatment inhibited LPS-stimulated NF-κB translocation in a dose-dependent manner (Fig. 3A). Similar inhibition of nuclear entry of p65 by dipyridamole was further demonstrated by immunofluorescent staining of RAW 264.7 cells using an anti-p65 antibody and examination under confocal microscopy. As shown in Fig. 3B, p65 was primarily localized in the cytosol in the resting condition. LPS stimulated p65 entry into nuclei, and this effect was inhibited by pretreatment of cells with dipyridamole (10 μM). To confirm whether dipyridamole also inhibits LPS-stimulated NF-κB binding to its response element, an EMSA was used. As shown in Fig. 3C, dipyridamole inhibited the NF-κB-specific DNA binding activity. In agreement, pretreatment of cells with dipyridamole (1–10 μM) for 30 min inhibited LPS-induced NF-κB-luciferase activity (Fig. 3D).

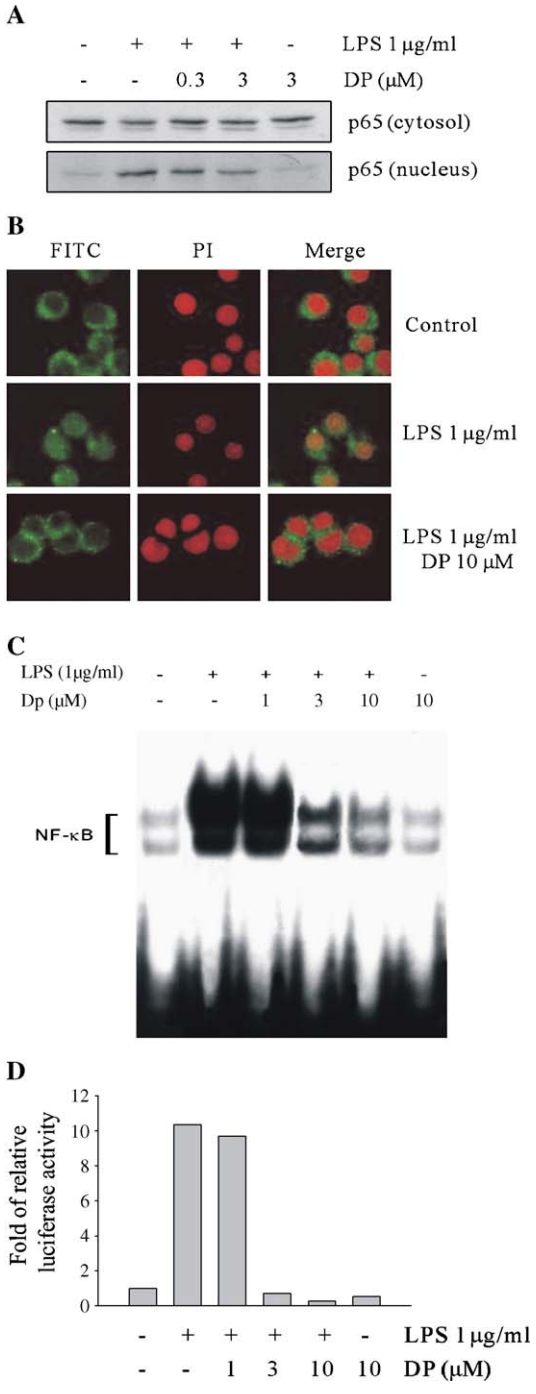

Fig. 3. Dipyridamole inhibited LPS-stimulated NF-κB translocation to nuclei, DNA binding, and NF-κB-responsive luciferase reporter gene expression. In (A), the p65 NF-κB contents in the nuclear and cytosolic fractions were determined by Western blot analysis using p65 NF-κB-specific antibodies. In (B), subcellular localization of p65 NF-κB was examined using immunofluorescence staining and analyzed under confocal microscopy. In (C), RAW 264.7 cells were pretreated with or without dipyridamole for 30 min before incubation with 1 μg/ml LPS for 24 h. Nuclear protein extracts of treated cells were prepared and applied to the EMSA. In (D), RAW 264.7 cells were transfected with plasmids containing the thymidine kinase promoter-driven luciferase gene with 6X of the NF-κB-responsive enhancer elements at the upstream regulatory region. Cells were incubated in the presence of different concentrations of dipyridamole for 30 min before stimulation with LPS (1 μg/ml) for 30 min. Luciferase activity was determined with a luciferase assay system (Promega) and was normalized on the basis of LacZ expression. The level of induction of luciferase activity was determined as multiples of luciferase activity in comparison with control cells. Results are representative of three independent experiments.

3.3. Inhibition of the NF-κB signaling pathway by dipyridamole can be attributed to inactivation of upstream p38 MAPK in RAW 264.7 macrophages

Given that COX-2 gene expression can be regulated by NF-κB and ATF-2 (the downstream effector of p38 MAPK), NF-κB and p38 MAPK are important for mediating COX-2 expression by LPS stimulation in RAW 264.7 macrophages. As shown in Fig. 4A, pretreatment of cells with a p38 MAPK-specific inhibitor (SB 203580), but not a MEK-specific inhibitor (PD 98059), suppressed LPS-induced COX-2 expression. We next examined whether p38 MAPK is the upstream regulator of IKK-β. An IKK-β immunoprecipitate-kinase assay was performed in the presence or absence of SB 203580. Pretreatment of cells for 30 min with SB 203580 (0.3 or 3 μM) inhibited the LPS-induced increase in IKK-β activity (Fig. 4B), suggesting that LPS may activate NF-κB through an upstream p38 MAPK signal pathway. To further

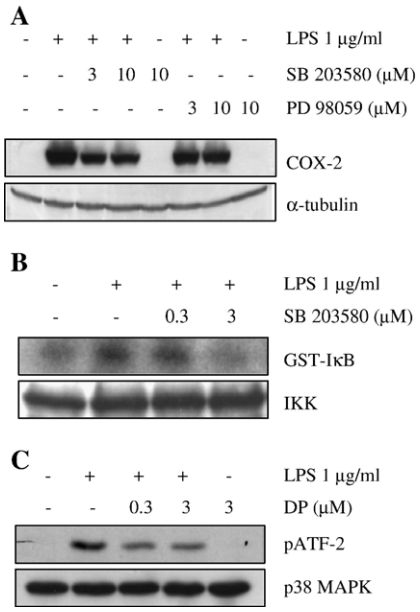

Fig. 4. Inhibition of the NF-κB signaling pathway by dipyridamole can be attributed to inactivation of upstream p38 MAPK in RAW 264.7 macrophages. In (A), cells were pretreated with the p38 MAPK-specific inhibitor, SB 203580 (3 or 10 μM), or the MEK-specific inhibitor, PD 98059 (3 or 10 μM), for 30 min before incubation with LPS (1 μg/ml) for 24 h. Cell lysates were subjected to Western blot analysis using COX-2-specific antibodies. In (B), cells were pretreated with SB 203580 (0.3 or 3 μM) for 30 min before incubation with LPS (1 μg/ml) for 30 min. Cell lysates were immunoprecipitated with an anti-IKK monoclonal antibody, and IKK kinase activity was determined by the extent of phosphorylation on bacterially expressed GST-IkBα. In (C), cells were incubated with different concentrations of dipyridamole (0.3 or 3 μM) for 30 min before incubation with LPS (1 μg/ml) for 30 min. Cell lysates were immunoprecipitated with anti-p38 MAPK antibodies, and p38 MAPK activity was determined by the extent of phosphorylation on ATF-2 by immunoblotting with phospho-ATF-2-specific antibodies. Equal loading in each lane was demonstrated by the similar intensities of α-tubulin (A), IKK-β (B), and p38 MAPK (C). Results are representative of three independent experiments.

confirm whether p38 MAPK is inhibited by dipyridamole, a p38 MAPK kinase assay was performed in the presence or absence of dipyridamole. As shown in Fig. 4C, pretreatment of cells with dipyridamole (0.3 or 3 μM) inhibited LPS-stimulated p38 MAPK activity. These results suggest that dipyridamole may inhibit p38 MAPK and subsequently suppress LPS-induced COX-2 expression in RAW 264.7 macrophages.

3.4. Dipyridamole inhibited COX-2 expression through MKP-1 activation in RAW 264.7 cells

It has been shown that glucocorticoids inhibit p38 MAPK activity via MKP-1. We next investigated the role of MKP-1 in dipyridamole-induced inhibition of p38 MAPK activity in RAW 264.7 cells. Immunoprecipitation with anti MKP-1 antibodies followed by Western blot analysis using phosphotyrosine-specific antibodies revealed a dose-dependent increase in phosphorylated MKP-1 after dipyridamole stimulation(Fig. 5A). MKP-1 phosphorylation became evident at 20 min, which was followed by a decrease in phosphorylation of p38 MAPK at 60 min (Fig. 5B). Dipyridamole is an inhibitor of phosphodiesterase, which increases intracellular levels of cyclic AMP and cyclic GMP. The roles of these cyclic nucleotides in activation of MKP-1 by dipyridamole were monitored with 8-Br cGMP or 8-Br cAMP treatment. As shown in Fig. 5C, none of these agents increased MKP-1 phosphorylation in RAW 264.7 cells, suggesting that cyclic nucleotides are not involved in the increase in phosphorylation of MKP-1 by dipyridamole. To further determine the crucial role of MKP-1 in the inhibitory effect of dipyridamole, triptolide (an MKP-1-specific inhibitor) and MKP-1 siRNA were used to block MKP-1 activity in RAW 264.7 cells. As shown in Fig. 6A, the inhibition of LPS-induced COX-2 expression by dipyridamole was reversed by pretreatment of RAW 264.7 cells with triptolide, an MKP-1 inhibitor. The MKP-1 knockdown experiment revealed that transfection of MKP-1 siRNA (Santa Cruz Biotechnology) (50 nM), but not mock controls, decreased MKP-1 protein expression and increased COX-2 protein expression. The inhibition of LPS-induced COX-2 expression by dipyridamole was reversed by transfection of MKP-1 siRNA (Fig. 6B). These

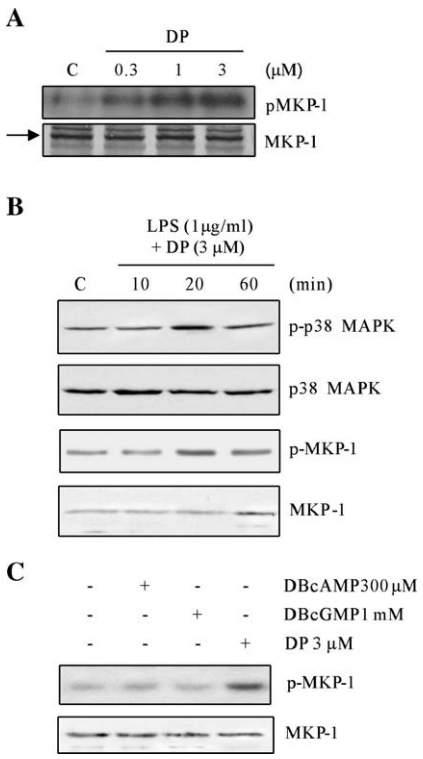

Fig. 5. Dipyridamole increased phosphorylation of MKP-1 in RAW 264.7 macrophages. In (A), cells were incubated without or with different concentrations of dipyridamole (0.3, 1, or 3 μM), cell lysates were immunoprecipitated with an anti-MKP-1 antibody, and MKP-1 phosphorylation was determined by Western blot analysis using phosphotyrosine-specific antibodies. In (B), cells were co-incubated with 1 μg/ml LPS and 3 μM dipyridamole for different time periods (10, 20, and 60 min). Cell lysates were immunoprecipitated with an anti-p38 MAPK antibody or anti-MKP-1 antibody, and p38 MAPK or MKP-1 phosphorylation was determined by Western blot analysis using phospho-p38 MAPK-(for p-p38 MAPK) or phosphotyrosine (for p-MKP-1)-specific antibodies. In (C), cells were incubated with dibutyl cAMP (300 μM), dibutyl cGMP (1 mM), or dipyridamole (3 μM). Cell lysates were immunoprecipitated with an anti-MKP-1 antibody, and MKP-1 phosphorylation was determined by Western blot analysis using phosphotyrosine-specific antibodies. Results are representative of three independent experiments.

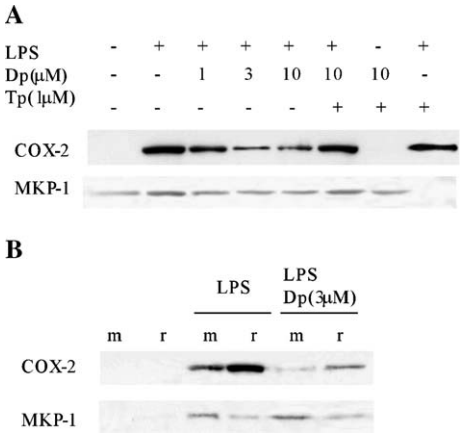

Fig. 6. Dipyridamole inhibited COX-2 expression through MKP-1 activation in RAW 264.7 cells. In (A), RAW 264.7 cells were incubated in the presence or absence of dipyridamole or triptolide for 30 min after overnight starvation. After pretreatment, cells were treated with 1 μg/ml LPS for 24 h. COX-2 and MKP-1 were detected by Western blot analysis respectively using anti-COX-2 or anti-MKP-1 antibodies. In (B), RAW 264.7 cells were either transfected with control siRNA as mock controls (m) or 50 nM of MKP-1 siRNA (r). MKP-1 expression was determined using anti-MKP-1 antibodies. Cells were pretreated with dipyridamole for 30 min before treatment with 1 μg/ml LPS for 6 h. Results are representative of three independent experiments.

data confirmed that MKP-1 plays a pivotal role in the inhibition of LPS-induced COX-2 expression by dipyridamole.

4. Discussion

Dipyridamole is a non-selective PDE inhibitor, which has been shown to improve proteinuria in membranous glomerulonephritis, mesangial IgA glomerulonephritis, and focal and segmental glomerulonephritis (Harmankaya et al., 2001). However, the molecular basis of its beneficial effect on renal disease is still not clear. LPS is a bacterial endotoxin which induces the expression of a number of proteins associated with inflammation. LPS-induced COX-2 expression is a key mediator of inflammatory responses (Chen and Wang, 1999; Dean et al., 1999). In the present study, we provide evidence that dipyridamole inhibits iNOS, COX-2, MCP-1, and IL-6 expressions in LPS-stimulated RAW 264.7 macrophages. We demonstrate that dipyridamole activates MKP-1, which leads to inhibition of p38 MAPK activation, IKK-β, and the NF-κB signaling pathway and subsequently suppresses LPS-induced COX-2 expression in RAW 264.7 macrophages.

Glucocorticoids are typical anti-inflammatory agents. The anti-inflammatory effects of glucocorticoids involve direct interactions of the glucocorticoid receptor with the transcription factor, NF-κB (Adcock and Caramori, 2001), and subsequent inhibition of pro-inflammatory gene expression. NF-κB can be activated in response to a broad range of stimuli and conditions, including interleukin-1 (IL-1) and tumor necrosis factor alpha (Bowie and O'Neill, 2000). LPS activation of NF-κB may increase the release of cytokines, and subsequently increase the transcription of COX-2 in macrophages and many other cell types (Huttunen et al., 1999; Li et al., 2000; Mohamed et al., 1999). LPS-induced COX-2 expression is a consequence of IKK activation, IκB phosphorylation and degradation, and activation of NF-κB-dependent transcription as shown by others and the data herein: 1) pretreatment of cells with PDTC inhibited LPS-mediated COX-2 induction; 2) LPS treatment phosphorylated and degraded IκB in the cytosol; 3) treatment of RAW 264.7 macrophages with LPS increased p65 translocation from the cytosol to the nucleus; and 4) treatment of RAW 264.7 macrophages with LPS enhanced NF-κB-specific DNA binding and luciferase activity. In addition, we found that dipyridamole inhibited IKK activation, IκB phosphorylation and degradation, NF-κB translocation, DNA binding, and activation of NF-κB-dependent transcription in RAW 264.7 macrophages. These data are consistent with a recent report that dipyridamole blocked MCP-1 and MMP-9 generated by LPS-treated monocytes via inhibiting NF-κB activation (Weyrich et al., 2005). Thus, dipyridamole may inhibit LPS-induced inflammatory responses, which may contribute to its beneficial effects of controlling the progression of a variety of diabetic complications.

The three mitogen-activated protein kinases, p44/42, JNK/SAPK, and p38 MAPK, regulate proinflammatory gene expression in response to proinflammatory stimuli (Chang and Karin, 2001). A previous study showed that in RAW 264.7 cells, p38 MAPK but not p44/42 MAPK is required for LPS stimulation of NF-κB DNA binding and subsequent induction of COX-2 in RAW 264.7 macrophages (Chen and Wang, 1999). In agreement with this, we showed that LPS-induced COX-2 expression was inhibited by SB 203580 but not PD 98059. The involvement of a p38 MAPK-specific pathway was confirmed by LPS stimulation

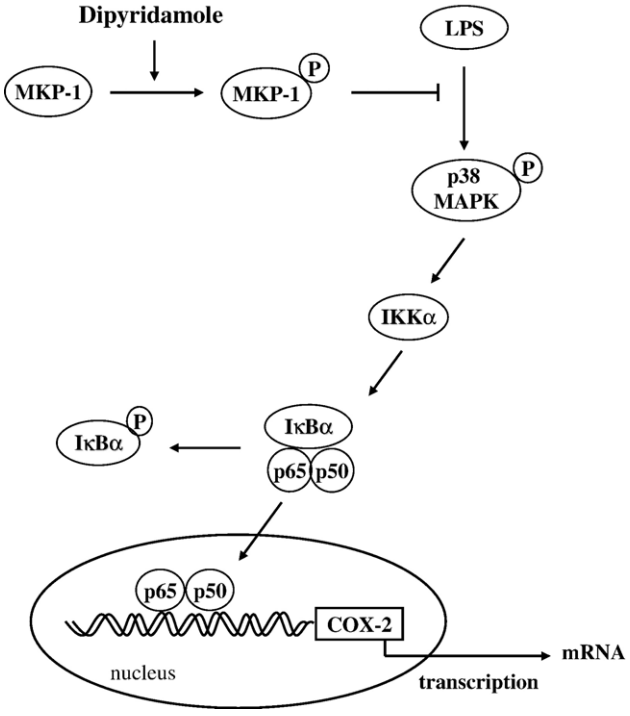

Fig. 7. Schematic diagram illustrating the signaling pathways involved in dipyridamole's inhibition of COX-2 expression through MKP-1 activation in RAW 264.7 cells. Dipyridamole activates MKP-1, which dephosphorylates and inactivates LPS-stimulated p38 MAPK activation. Inhibition of p38 MAPK inactivates IKK-β and the NF-κB signaling cascade, which subsequently results in suppression of LPS-induced COX-2 expression in RAW 264.7 macrophages.

of p38 MAPK in RAW 264.7 cells. LPS activation of p38 MAPK is linked to NF-κB activation, because pretreatment of cells with SB 203580 inhibited the LPS-stimated IKK-β activation. Dipyridamole blocks the LPS-induced activation of p38 MAPK and NF-κB, and the subsequent induction of COX-2, suggesting that p38 MAPK is regulated by dipyridamole. These data are consistent with those of selenium on LPS-induced activation of p38 MAPK and NF-κB induction. Selenium specifically blocks the LPS-induced activation of p38 MAPK and NF-κB, and these effects are independent of JNK and extracellular signal-regulated kinases (Kim et al., 2004).

Dephosphorylation of both threonine and tyrosine residues in MAPKs is regulated by the dual-specificity phosphatases or MAPK phosphatases (Camps et al., 2000). Overexpression or phosphorylation of MKP-1 is able to dephosphorylate and inhibit p38 MAPK (Lasa et al., 2002). We hypothesized that inhibition of p38 MAPK by dipyridamole involves activation of MKP-1's function. Indeed, we demonstrated that dipyridamole activates MKP-1, which in turn inactivates these proinflammatory signaling pathways. These observations are consistent with MKP-1-mediated inhibition of p38 MAPK by glucocorticoids (Adcock and Caramori, 2001; Lasa et al., 2002). However, it has to be noted that inhibition of p38 MAPK activity by glucocorticoids requires the glucocorticoid receptor-mediated gene expression of MKP-1 (Chen et al., 2002), whereas dipyridamole phosphorylates and activates MKP-1 without altering the protein level. Inhibition of MKP-1 by triptolide or MKP-1 gene knockdown by siRNA provides strong evidence of the involvement of MKP-1 in inhibition of LPS-induced COX-2 expression by dipyridamole, as these treatments restore the LPS-induced response. Because incubation of cells with neither 8-Br cGMP nor 8-Br cAMP altered the phosphorylation pattern of MKP-1, and this effect is independent of cyclic nucleotides, our results are consistent with those of Matsumori et al. (2000), who demonstrated that treatment of LPS-activated J774.1 macrophages with 8-Br cAMP enhanced, rather than inhibited, nitrite accumulation. The mechanisms by which dipyridamole activates MKP-1 remain unclear. Recently, dipyridamole was shown to attenuate NF-κB translocation and block MMP-9 and MCP-1 generated by LPS-treated monocytes. The inhibitory effects of dipyridamole were reversed by the addition of exogenous adenosine deaminase, which removes extracellular adenosine (Weyrich et al., 2005). These results indicate that inhibition of adenosine uptake by dipyridamole is attributed to its anti-inflammatory effects. Whether adenosine plays a role in induction of MKP-1 expression warrants further investigation.

In conclusion, our results suggest that dipyridamole may exert its anti-inflammatory effects through a mechanism shown schematically in Fig. 7. Dipyridamole inhibits LPS-induced inflammatory mediator expression in RAW 264.7 macrophages through p38 MAPK-, IKK-α-, and NF-κB-dependent mechanisms. The rapid activation of MKP-1 by dipyridamole may provide a novel mechanism for suppression of proinflammatory gene expression. Nevertheless, the possibility exists that other signaling pathways other than those highlighted in the present study may also contribute to the inhibition of LPS in RAW 264.7 macrophages.

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