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Dipyridamole inhibits lipopolysaccharide-induced cyclooxygenase-2 and monocyte chemoattractant protein-1 via heme oxygenase-1-mediated reactive oxygen species reduction in rat mesangial cells

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A B S T R A C T

Dipyridamole contributes to its beneficial effects on inflammatory responses in many cell types. The anti-inflammatory mechanisms of dipyridamole on glomerular mesangial cells are mostly uncharacterized. In this study, we monitored the influence of dipyridamole on the expression levels of cyclooxygenase-2 (COX-2) and monocyte chemoattractant protein-1 (MCP-1) in rat mesangial cells stimulated with lipopolysaccharide. Dipyridamole was found to inhibit lipopolysaccharide-induced COX-2 and MCP-1 expression, and reduced lipopolysaccharide-induced reactive oxygen species generation in rat mesangial cells. This inhibitory effect of dipyridamole is independent on cyclic AMP and cyclic GMP increase. Tin protoporphyrin IX (SnPP), a heme oxygenase-1(HO-1) inhibitor, blocked the inhibitory effect of dipyridamole on lipopolysaccharide-induced COX-2 and MCP-1 expression. By applying specific inhibitors in rat mesangial cells, ERK1/2 and p38 MAPK signaling pathways were demonstrated to be involved in the lipopolysaccharide-induced inflammatory responses, and were inhibited by SnPP and N-acetylcysteine treatment. Additionally, dipyridamole was also found to upregulate HO-1 in rat mesangial cells. Therefore, our data suggest that dipyridamole inhibits the expression of COX-2 and MCP-1 in lipopolysaccharide-treated rat mesangial cells via HO-1-mediated reactive oxygen species reduction.

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1. Introduction

Glomerular mesangial cells play an important role in the pathogenesis of several renal diseases (Radeke and Resch, 1992; Veis, 1993). Mesangial cell proliferation is obvious in many types of glomerulonephritis, which are also characterized by the expression of various inflammatory cytokines and inducible enzymes, such as cyclooxygenase-2 (COX-2), and monocyte chemoattractant protein-1 (MCP-1). COX-2 is an inducible enzyme often found at sites of inflammation and results in the production of prostaglandin E2, which inhibits interleukin 1β-induced proliferation of rat mesangial cells (Stahl et al., 1990). COX-2 is strongly expressed in the glomeruli of clinical and experimental glomerulonephritis (Komers and Epstein, 2002). MCP-1 is a member of chemokines and mediates monocyte/macrophage infiltration. The glomerular expression of MCP-1 correlates with the degree of renal damage in inflammatory (Panzer et al., 2001) and non-inflammatory models (Taal et al., 2000) of glomerular injury.

Dipyridamole is a drug that is frequently used in nephrology clinics because it improves proteinuria in a variety of glomerulonephritis (Camara et al., 1991). At clinically relevant concentrations, dipyridamole inhibits profibrotic activities of renal fibroblasts (Hewitson et al., 2002) and peritoneal mesothelial cell proliferation (Hung et al., 2001) suggesting that dipyridamole may have therapeutic potential for treating fibrosis. Dipyridamole is a non-selective phosphodiesterase (PDE) inhibitor, which inhibits degradation of cyclic AMP (cAMP) and cyclic GMP (cGMP), thereby increasing cellular levels of cAMP and cGMP (Bravo, 1995). Dipyridamole, also an inhibitor of nucleoside transport (Hammond et al., 1985), can inhibit platelet function by blocking adenosine reuptake and degradation (Stafford et al., 2003). Increasing evidences have indicated that many specific inhibitors for PDEs possess anti-inflammatory effects (Cheng and Grande, 2007; Dastidar et al., 2007; Vijayakrishnan et al., 2007). In our previous study, we found that dipyridamole exerts its anti-inflammatory effect in RAW 264.7 cells, which involved the activation of mitogen-activated kinase phosphatase-1 (MKP-1) (Chen et al., 2006). However, this anti-inflammatory effect of dipyridamole was not dependent on the quantity of cAMP and cGMP in our finding. It is...
supposed that dipyridamole exerts its anti-inflammatory effect via other mechanisms besides PDE inhibition.

Dipyridamole has been found to inhibit rat mesangial cell proliferation and collagen synthesis (Tsai et al., 1995). The anti-inflammatory mechanisms of dipyridamole on glomerular mesangial cells are mostly uncharacterized. In this study, we monitored the influence of dipyridamole on the expression levels of COX-2 and MCP-1 in rat mesangial cells stimulated with lipopolysaccharide. Dipyridamole was found to decrease lipopolysaccharide-induced inflammatory factors and elevate the expression of heme oxygenase-1 (HO-1). HO catalyzes the conversion of heme to biliverdin, releasing equimolar amounts of carbon monoxide and iron. Subsequently, biliverdin is converted to bilirubin by biliverdin reductase (Montellano, 2000). HO-1 is induced in many cell types in response to inflammation and endotoxin exposure, and functions as cytoprotective mechanism against inflammatory responses and oxidative stress (Takahashi et al., 2004). Here, we demonstrated that HO-1-mediated reactive oxygen species reduction is essential for the anti-inflammatory function of dipyridamole in rat mesangial cells.

2. Materials and methods

2.1. Materials

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, and tissue culture reagents were from Life Technologies, Inc. (Grand Island, NY, USA). Antibodies used in this research were purchased from BD Laboratories (San Diego, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). All materials for SDS-PAGE were obtained from Bio-Rad (Hercules, CA, USA). The inhibitors for MAP kinases were purchased from Calbiochem (La Jolla, CA, USA). Lipopolysaccharides from E. coli, tin protoporphyrin IX (SnPP), N-acetylcysteine (NAC), apocynin, dibutyryl cGMP, and 8-Bromo-cAMP were purchased from Sigma-Aldrich Co. (ST. Louis, MO, USA).

2.2. Cell culture

Rat glomerulus mesangial cells were purchased from Food Industry Research and Development Institute (Taiwan) and cultured in DMEM culture medium supplemented with antibiotic/antifungal solution and 15% fetal bovine serum. The medium was changed to serum-free medium after cells were grown to confluence, and cells were incubated overnight before experiments.

2.3. Western blotting

Electrophoresis was ordinarily carried out on different percentages of SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, separated proteins on the gel were electrotransferred onto a polyvinylidifluoride (PVDF) membrane. After blocking and antibody incubation, the membrane was incubated with alkaline phosphatase-conjugated secondary antibody for 1 h, and visualized by chemiluminescence according to the manufacturer’s instructions (Pierce Chemical Co., Rockford, IL, USA).

2.4. Determination of monocyte chemoattractant protein-1 (MCP-1)

MCP-1 was quantified in the sample medium using ELISA kits according to instructions provided by the manufacturer (RayBiotech, Inc., GA, USA).

2.5. Assay of intracellular reactive oxygen species

Prior to the chemical treatment, rat mesangial cells were incubated in culture medium containing 30 μM 2′,7′-dichlorofluorescein (DCF; a fluorescent dye) for 30 min to establish a stable intracellular level of the probe. Subsequently, the cells were washed with PBS, removed from Petri dishes by scraping, and measured for DCF fluorescence intensity, which was used an index of intracellular levels of reactive oxygen species. DCF fluorescence was determined using a fluorescence spectrophotometer with excitation and emission wavelengths at 475 and 525 nm, respectively. The cell number in each sample was counted and utilized to normalize the fluorescence intensity of DCF.

2.6. Statistical analysis

All data are expressed as the mean ± S.E.M. Statistical analysis was performed using Student’s t-tests. A difference between groups of P < 0.05 was considered statistically significant.

3. Results

3.1. Dipyridamole inhibits lipopolysaccharide-induced COX-2 and MCP-1 expression in rat glomerular mesangial cells

The effect of lipopolysaccharide on the expression of COX-2 and MCP-1 was determined in rat mesangial cells. We determined COX-2 and MCP-1 1, 2, 4, 8, 12 and 24 h after lipopolysaccharide treatment by Western blotting and ELISA analysis, respectively. Lipopolysaccharide significantly induced COX-2 expression, which peaked 2 h after the treatment and lasted over the next time points (Fig. 1A). Lipopolysaccharide also induced MCP-1 secretion in rat mesangial cells over the treatment time (Fig. 1B). For monitoring the influence of dipyridamole on lipopolysaccharide-induced COX-2 and MCP-1, rat mesangial cells were incubated with dipyridamole for 30 min before the addition of lipopolysaccharide (1 μg/ml) for 2 h (for COX-2 detection) or 24 h (for MCP-1 detection). We found that lipopolysaccharide-induced COX-2 and MCP-1 expression were reduced by dipyridamole treatment, whereas dipyridamole alone didn’t influence the expression of COX-2 and MCP-1 in rat mesangial cells (Fig. 1C, D). The dosage of dipyridamole was 5 μM for the maximum inhibitory effect on lipopolysaccharide-induced COX-2. Additionally, the inhibitory effect of dipyridamole on lipopolysaccharide-induced MCP-1 reached a maximum within the dosage range of 1–5 μM.

3.2. The inhibitory effect of dipyridamole on lipopolysaccharide-induced MCP-1 is not dependent on cyclic AMP and cyclic GMP

Dipyridamole is an inhibitor of phosphodiesterase, which increases intracellular levels of cyclic AMP (cAMP) and cyclic GMP (cGMP). To evaluate the role of the cAMP and cGMP in the inhibitory effect of dipyridamole, we monitored lipopolysaccharide-induced MCP-1 secretion in rat mesangial cells with the treatment of dipyridamole with or without the stable analogue of cAMP, 8-Bromo-cAMP, or the cGMP analogue, dibutyryl cGMP. As shown in Fig. 2, 8-Bromo-cAMP and dibutyryl GMP didn’t influence the lipopolysaccharide-induced MCP-1 secretion. Actually, 8-Bromo-cAMP or dibutyryl GMP alone was capable of inducing MCP-1 secretion in rat mesangial cells. This induction was not inhibited by dipyridamole treatment, and caused the inhibition effect of dipyridamole on lipopolysaccharide-induced MCP-1 secretion unobvious. These results suggest that the inhibition effect of dipyridamole on lipopolysaccharide-induced MCP-1 secretion is independent on the increase of cAMP or cGMP in rat mesangial cells.

3.3. HO-1-mediated reactive oxygen species reduction is involved in the dipyridamole inhibitory effect on COX-2 and MCP-1 expression

Reactive oxygen species is known as an important mediator in lipopolysaccharide signaling pathways (Gloire et al., 2006). Using 2′,7′-dichlorofluorescein diacetate (DCF-DA) staining, we monitored the influence of dipyridamole on lipopolysaccharide-induced reactive oxygen species generation. Reactive oxygen species generation was
found in lipopolysaccharide-treated rat mesangial cells, and was significantly reduced by the reactive oxygen species scavenger N-acetylcysteine (NAC) and the specific NADPH oxidase inhibitor apocynin (Fig. 3A). This result shows that lipopolysaccharide induces reactive oxygen species in rat mesangial cells through NADPH oxidases. The lipopolysaccharide-induced increase in reactive oxygen species was significantly reduced approximately 70% by dipyridamole at 1 and 5 μM, and 50% at 10 μM (Fig. 3B). This reduction was blocked by tin protoporphyrin IX (SnPP), a well-documented HO-1 inhibitor. To further elucidate the connection between the dipyridamole function and HO-1-mediated reactive oxygen species reduction, we monitored lipopolysaccharide-induced COX-2 and MCP-1 expression with SnPP and the reactive oxygen species scavenger, N-acetylcysteine (NAC). The inhibitory effect of dipyridamole on lipopolysaccharide-induced COX-2 and MCP-1 was found to be blocked by SnPP
treatment (Fig. 4A, B). Besides, lipopolysaccharide-induced COX-2 and MCP-1 was also reduced by NAC treatment (Fig. 4A, B). These results imply that HO-1-mediated reactive oxygen species reduction is involved in the inhibitory effect of dipyridamole on the lipopolysaccharide-induced inflammatory response in rat mesangial cells.

HO-1 plays critical roles in antioxidant defense and anti-inflammation (Morse and Choi, 2002). In rat mesangial cells, the high concentration (1 and 5 μg/ml) of lipopolysaccharide reduced the expression of HO-1 although the low concentration (0.5 μg/ml) of lipopolysaccharide stimulated the HO-1 expression (Fig. 5A). By western blotting assay, we found dipyridamole alone was able to increase the HO-1 expression level in rat mesangial cells (Fig. 5B). This stimulation effect of dipyridamole reached a maximum at 5 μM, but was not significant at 10 μM. In the presence of 1 μg/ml lipopolysaccharide, dipyridamole also significantly elevated the protein level of HO-1 in rat mesangial cells (Fig. 5B). This result shows that the inhibitory effect of lipopolysaccharide on HO-1 expression is blocked by dipyridamole treatment.

3.4. Dipyridamole inhibits the lipopolysaccharide-induced phosphorylation of ERK1/2 and p38 via HO-1-mediated reactive oxygen species reduction

To identify the influence of dipyridamole on the signaling pathways of lipopolysaccharide-induced inflammatory responses, the involvement of ERK1/2, JNK1/2 and p38 MAPK was examined. To assess the role of these pathways, the effect of ERK1/2, JNK1/2 and p38 MAPK inhibitors on the lipopolysaccharide-induced COX-2 and MCP-1 expression were examined. Both PD98059 (the selective inhibitor of ERK1/2) and SB203580 (the specific inhibitor of p38) had a similar effect, decreasing the lipopolysaccharide-induced COX-2 expression (Fig. 6A) and MCP-1 secretion (Fig. 6B) without evidence of toxicity. There was no significant influence of SP600125 (the inhibitor of JNK1/2) on the lipopolysaccharide-induced COX-2 expression and MCP-1 secretion. These findings implicate both the ERK1/2 and p38 MAPK signaling pathways are involved in the lipopolysaccharide-induced inflammatory responses. The western blotting of phospho-MAP kinases revealed that lipopolysaccharide increased the phosphorylation level of ERK1/2 and p38 (Fig. 6C), but not JNK1/2 (data not shown). Both dipyridamole and NAC also inhibited the lipopolysaccharide-induced phosphorylation of ERK1/2 and p38 (Fig. 6C). The inhibitory effect of dipyridamole on the kinase phosphorylation was blocked by SnPP treatment. These results reveal that dipyridamole inhibits the lipopolysaccharide-induced phosphorylation of ERK1/2 and p38 via HO-1-mediated reactive oxygen species reduction in rat mesangial cells.
4. Discussion

In the present study, we demonstrated that the certain dosages of dipyridamole inhibit lipopolysaccharide-induced COX-2 and MCP-1 in rat mesangial cells (Fig. 1). This inhibitory effect of dipyridamole is not dependent on cAMP and cGMP increase (Fig. 2). Dipyridamole also reduced lipopolysaccharide-induced reactive oxygen species generation in rat mesangial cells (Fig. 3). Both HO-1 inhibitor SnPP and reactive oxygen species scavenger NAC blocked the inhibitory effect of dipyridamole on lipopolysaccharide-induced inflammatory mediators and reactive oxygen species generation (Fig. 4). Moreover, dipyridamole also elevated the HO-1 expression in rat mesangial cells (Fig. 5). HO-1-mediated reactive oxygen species reduction is supposed to be involved in the inhibitory effect of dipyridamole on the inflammatory response in rat mesangial cells. We also found that ERK1/2 and p38 MAPK signaling pathways are involved in the lipopolysaccharide-induced inflammatory responses, and are inhibited by SnPP and NAC (Fig. 6). Therefore, our data suggest that dipyridamole is able to induce HO-1 expression in rat mesangial cells to reduce lipopolysaccharide-induced reactive oxygen species generation, and subsequently suppresses lipopolysaccharide-induced ERK1/2 and p38 MAPK signaling pathways. By this mechanism, dipyridamole inhibits the expression of COX-2 and MCP-1 in lipopolysaccharide-treated rat mesangial cells. The antioxidant effect of dipyridamole via HO-1 induction may provide a major mechanism for suppression of pro-inflammatory gene expression.

Dipyridamole has been shown to improve proteinuria in membranous glomerulonephritis, mesangial IgA glomerulonephritis, and focal and segmental glomerulonephritis (Harmankaya et al., 2001). However, there is no report mentioned the different effects of dipyridamole in different dosages. In our data, the anti-inflammatory effect of dipyridamole in a relative high concentration (10 μM) was lower than that in a relative low concentration (1–5 μM) (Fig. 1). The HO-1 induction ability of dipyridamole was also reduced in a high concentration (Fig. 5). Dipyridamole is a non-selective PDE inhibitor and inhibits degradation of cAMP and cGMP (Beavo, 1995). The higher concentration of dipyridamole will increase more cyclic cAMP and cGMP in rat mesangial cells. Many reports have indicated that many specific inhibitors for PDEs increase cellular CAMP and cGMP to exert anti-inflammatory effects (Cheng and Grande, 2007; Dastidar et al., 2007; Vijayakrishnan et al., 2007). In our system, on the contrary, increasing cellular CAMP and cGMP by adding 8-Bromo-cAMP and dibutyryl cGMP induced MCP-1 expression in rat mesangial cells (Fig. 2). Ikead et al. have shown that IL-1 beta stimulates cAMP and cGMP accumulation in mesangial cells to modulate the glomerular response to inflammation (Ikedo et al., 1994). It is possible that the high cellular CAMP and cGMP levels caused by the high dosage of dipyridamole will induce more serious inflammatory response in rat mesangial cells, and therefore reduce the anti-inflammatory effect of dipyridamole.

Numerous studies showed that lipopolysaccharide induced the expression of HO-1 in many cell types (Camhi et al., 1995; Kitamura et al., 1998; Li et al., 2008). We found a low concentration (0.5 μg/ml) of lipopolysaccharide upregulated HO-1, but a high concentration (1 or 5 μg/ml) of lipopolysaccharide reduced the protein level of HO-1 in rat mesangial cells (Fig. 5A). This HO-1 expression pattern has not been found in other systems, may be unique to glomerular mesangial cells. Lipopolysaccharide has been reported to induce membrane-bound Fas ligand, a well-known death factor, in cultured human mesangial cells (Tsukinoki et al., 2004). A high concentration of lipopolysaccharide is supposed to cause mesangial cell injury via Fas ligand pathway, and then reduce HO-1 expression. This is a possible mechanism involved in the influence of lipopolysaccharide on HO-1 expression in mesangial cells. Nevertheless, the detailed mechanism needs to be clarified by further studies.

HO-1 is a cytoprotective protein whose expression is consistently associated with therapeutic benefits in a number of pathologic conditions such as atherosclerotic vascular disease and inflammation (Takahashi et al., 2004). Although the expression of HO-1 in most tissues is low, a large number of clinical and experimental pharmacologic compounds have been demonstrated to induce HO-1. This induction is suggested to be at least partially responsible for the
perceived therapeutic efficacy of these compounds. Our data reveal that dipyridamole is also an HO-1 inducer, and reduces lipopolysaccharide-induced reactive oxygen species generation and the phosphorylation of ERK1/2 and p38 MAPK in rat mesangial cells. This supports the notion that dipyridamole inhibits lipopolysaccharide-induced inflammatory responses, which may contribute to its beneficial effects on retarding the progression of a variety of renal diseases.

References


