Celecoxib Induces Heme-Oxygenase Expression in Glomerular Mesangial Cells

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ABSTRACT: **Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used as analgesics. They inhibit cyclooxygenases (COX), preventing the formation of prostaglandins, including prostacyclin and thromboxane. A serious side effect of COX-1 and COX-2 inhibitors is renal damage. To investigate the molecular basis of the renal injury, we evaluated the expression of the stress marker, heme oxygenase–1 (HO-1), in celecoxib-stimulated mesangial cells. We report here that a COX-2 selective NSAID, celecoxib, induced a concentration- and time-dependent increase of HO-1 expression in glomerular mesangial cells. Celecoxib-induced HO-1 protein expression was inhibited by actinomycin D and cycloheximide, suggesting that *de novo* transcription and translation are required in this process. *N*-acetylcysteine, a free radical scavenger, strongly decreased HO-1 expression, suggesting the involvement of reactive oxygen species (ROS). Celecoxib-induced HO-1 expression was attenuated by pretreatment of the cells with SP 600125 (a specific JNK inhibitor), but not SB 203580 (a specific p38 MAPK inhibitor), or PD 98059 (a specific MEK inhibitor). Consistently, celecoxib activated c-Jun N-terminal kinase (JNK) as demonstrated by kinase assays and by increasing phosphorylation of this kinase. *N*-acetylcysteine reduced the stimulatory effect of celecoxib on stress kinase activities, suggesting an involvement of JNK in HO-1 expression. On the other hand, LY 294002, a phosphatidylinositol 3-kinase (PI-3K)–specific inhibitor, prevented the enhancement of HO-1 expression. This effect was correlated with inhibition of the phosphorylation of the PDK-1 downstream substrate Akt/protein kinase B (PKB). In conclusion, our data suggest that celecoxib-induced HO-1 expression in glomerular mesangial cells may be mediated by ROS via the JNK–PI-3K cascade.**

KEYWORDS: **celecoxib; glomerular mesangial cells; heme oxygenase**

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used in the treatment of rheumatisms and other chronic inflammatory diseases.[1] Most NSAIDs in clinical use are cyclooxygenase (COX) inhibitors. COX catalyzes the synthesis of eicosanoids from arachidonic acid.[2] There are two isoforms of COX: a constitutively expressed COX-1 and an inducible COX-2.[3] Both isoforms are expressed in the adult mammalian kidney. Prostaglandins modulate renal microvascular hemodynamics, renin release, and tubular salt and water reabsorption.[4] Depletion of prostaglandins in kidney may result in renal damages reflected by a reduction in glomerular filtration rate, renal blood flow, and diminished sodium and potassium excretion.[5] Inhibition of COX activity in the kidney by NSAIDs has relatively mild consequences in healthy individuals but in vulnerable subjects can lead to serious adverse events in kidney due to depletion of prostaglandins. The decline in renal function is especially pronounced in the elderly and in patients with pre-existing renal disease.[6]

Heme oxygenase-1 (HO-1) catalyzes the rate-limiting step in heme degradation, resulting in the formation of iron, carbon monoxide, and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase. Recent attention has focused on the biological effects of the reaction product(s), which exert important antioxidant, anti-inflammatory, and cytoprotective functions. There are three heme oxygenase isoforms: an inducible isoform, HO-1, and two constitutively expressed isoforms, HO-2 and HO-3. Induction of HO-1 occurs as an adaptive and beneficial response to several injury signaling processes and has been implicated in many clinically relevant disease states including acute renal injury. Increased HO-1 expression protects kidneys from oxidative injuries,[7] rhabdomyolysis,[8] cisplatin nephrotoxicity,[9] acute renal failure,[10] and ischemic/reperfusion-mediated tissue injury.[11]

The cellular signaling mechanisms that mediate HO-1 expression in glomerular mesangial cells remain unclear. It has been shown that HO-1 gene expression is mediated through activation of the JNK and p38 MAPK pathways in primary cultures of rat hepatocytes.[12] In the present study, we examined the effects of a selective COX-2 inhibitor, celecoxib (Celebrex), on the induction of HO-1 expression in glomerular mesangial cells. This study also investigated the mechanisms by which celecoxib induced HO-1 expression. The data clearly show that the treatment of glomerular mesangial cells with celecoxib results in increased free radical generation, which triggers a signal transduction cascade involving JNK and PI-3K activation. These stress signals ultimately induce HO-1 expression in glomerular mesangial cells.

MATERIALS AND METHODS

PD 98059, SB 203580, and SP 600125 were purchased from Calbiochem (San Diego, CA). Dubecco's modified Eagle medium (DMEM), fetal calf serum (FCS), penicillin, and streptomycin were purchased from Life Technologies (Gaithersburg, MD). Affinity-purified polyclonal antibodies to c-Jun phosphorylated ATF-2 and phosphorylated c-Jun, phosphorylated Akt (Thr^{473}), and phosphorylated p38 MAPK were obtained from Transduction Laboratory (Lexington, KY). Antibodies specific for HO-1 and β-actin were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). Horseradish peroxidase–conjugated anti–rabbit IgG antibody was purchased from

BioRad (Hercules, CA). 5-Bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium substrate was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim (Mannheim, Germany). The p38 MAPK assay kit was purchased from New England Biolabs (Beverly, MA). Celecoxib is a gift from Pharmacia Co. (Northumberland, England). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture and Preparation of Cell Lysates

Glomerular mesangial cells from an SV40 transgenic mouse (ATCC CRL-1927) were cultured in DMEM supplemented with 13.1 mM $NaHCO_3$, 13 mM glucose, 2 mM glutamine, 10% of heat inactive fetal calf serum (FCS), and 10% horse serum and penicillin (100 U/mL)/streptomycin (100 mg/mL). Cells were attached to a petri dish after a 24-h incubation. Cells were plated at a concentration of 1×10^5 cells/mL and used for experiments when they reached 80% confluency. All reagents were added directly to the culture at a volume of 100 µL/10 M medium. Cultures were maintained in a humidified incubator under 5% CO_2 at 37°C. After reaching 80% confluence, cells were treated with various concentrations of celecoxib for indicated time intervals and incubated in a humidified incubator under 5% CO_2 at 37°C. In some experiments, cells were pretreated with specific inhibitors as indicated followed by celecoxib and incubated for more 16 h. After incubation, cells were harvested, chilled on ice, and washed three times with ice-cold phosphate-buffered saline (PBS). Cells were lysed by adding lysis buffer containing 10 mM Tris HCl (pH 7.5), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM sodium orthovanadate, 1 mM DTT, 0.1% mercaptoethanol, 0.5% Triton X-100, and the protease inhibitor cocktails (final concentrations: 0.2 mM PMSF, 0.1% aprotinin, 50 µg/mL leupeptin). Cells adhering to the plates were scraped off using a rubber policeman and stored at –70°C for further measurements.

Polyacrylamide Gel Electrophoresis and Western Blotting

Electrophoresis was ordinarily conducted at different percentages of sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS-PAGE). After electrophoresis, proteins on the gel were electrotransferred onto a polyvinyldifluoride (PVDF) membrane. After transfer, the PVDF paper was washed once with PBS and twice with PBS plus 0.1% Tween 20. The PVDF membrane then was blocked with blocking solution containing 3% bovine serum albumin in PBS containing 0.1% Tween 20 for 1 h at room temperature. The PVDF membrane was incubated with a solution containing primary antibodies in the blocking buffer for 2 h. Finally, the PVDF papers were incubated with enzyme-linked secondary antibodies for 1 h and then visualized by incubating with colorigenic substrate (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate; Sigma) or developed using a chemiluminescence kit (Amersham, Buckinghamshire, UK), and the immunoreactive bands were visualized by autoradiography.

Measurement of Intracellular Reactive Oxygen Species Generation

Intracellular reactive oxygen species (ROS) generation was assessed using 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Leiden, the

Netherlands) as described by Chandel *et al.*[13] In brief, mesangial cells were cultured in petri dishes and incubated with 10 μM DCFH-DA for 30 min. ROS in the cells caused oxidation of DCFH, which produces a fluorescent product. The intracellular fluorescence then was measured using flow cytometry.

Statistical Analysis

Results are expressed as means ± SEM from the number of independent experiments performed. One-way analysis of variance (ANOVA) was used to assess the difference of means among groups, and the Student's two-tailed *t*-test was used to determine the difference of means between two groups. A *P* value less than 0.05 was considered significant.

RESULTS

Celecoxib Induction of HO-1 Expression

Exposure of mesangial cells to celecoxib induced the expression of a 32-kDa band corresponding to HO-1 in a dose-dependent (FIG. 1A) and time-dependent (FIG. 1B) manner. HO-1 expression was maximum at approximately 10 μM of Celebrex. Celecoxib-induced HO-1 expression was apparent at 4 h and reached maximum at 16 h. The induction of HO-1 by celecoxib could be blocked with actinomycin D (1 μM; FIG. 1C) or cycloheximide (10 μg/mL) pretreatment (FIG. 1D), suggesting that celecoxib-induced HO-1 expression required *de novo* transcription and translation.

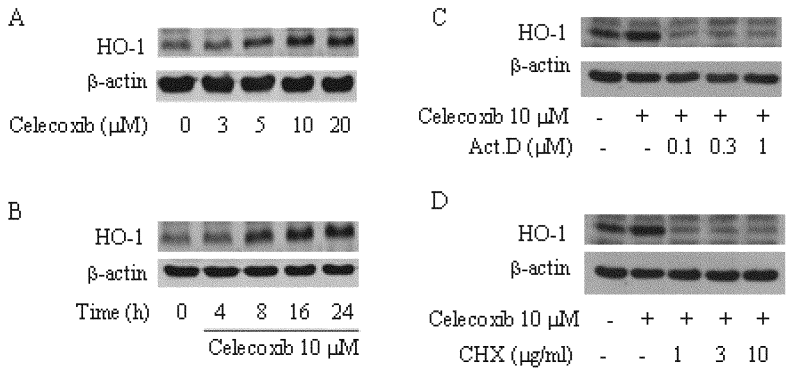

FIGURE 1. Celecoxib induced a time- and dose-dependent increase in HO-1 expression in mesangial cells. (**A**) Cells were incubated with various concentrations (3, 5, 10, 20 μM) of celecoxib at 37ºC for 24 h; (**B**) cells were incubated with celecoxib (10 μM) at 37°C for various time periods (4, 8, 16, 24 h). In (**C**) and (**D**), cells were pretreated with actinomycin D (0.1, 0.3, 1 μM) (**C**) and cycloheximide (1, 3, 10 μg/mL) (**D**) for 30 min before being incubated with celecoxib (10 μM) for 16 h. Cell lysates were electrophoresed and probed by Western blot with HO-1–specific antibodies. Equal loading in each lane was demonstrated by the similar intensities of β-actin.

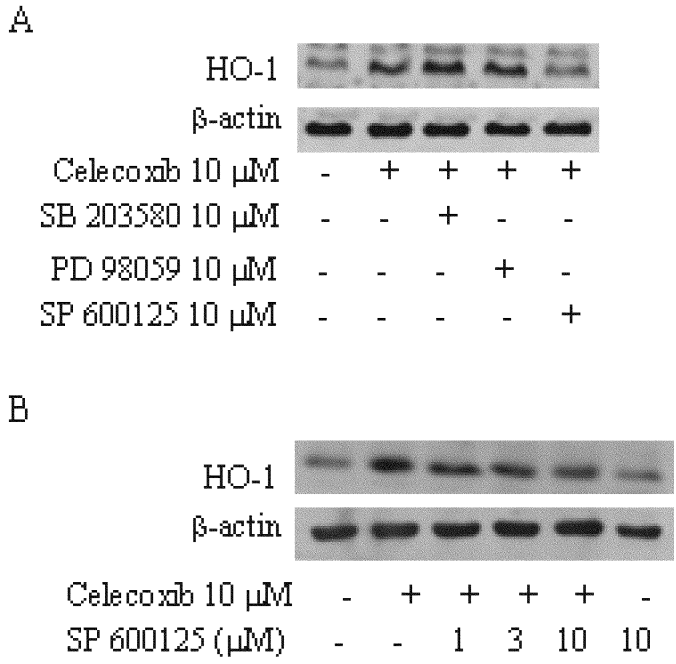

FIGURE 2. Roles of MAPKs in celecoxib-induced HO-1 expression in mesangial cells. (**A**) Cell were pretreated with various MAPK inhibitors, including SB 203580 (10 μM), PD 98059 (10 μM), and SP 600125 (10 μM) for 30 min before being incubated with celecoxib (10 μM) for 16 h. (**B**) Cells were pretreated with various concentrations SP 600125 (1, 3, 10 μM) for 30 min before being incubated with celecoxib (10 μM) for 16 h and lysed. Cell lysates were electrophoresed and probed by Western blot with HO-1–specific antibodies. Equal loading in each lane was demonstrated by the similar intensities of β-actin.

Involvement of the MAPK Signaling Pathway in Celecoxib-Induced HO-1 Expression

It has been demonstrated that mitogen-activated protein kinases (MAPKs) play important roles in celecoxib-induced HO-1 expression in macrophages.[12] To elucidate the mechanisms responsible for changes in HO-1 protein expression, we pretreated mesangial cells with MEK inhibitor (PD 98059), or JNK inhibitor (SP 600125), or p38 MAPK inhibitor (SB 203580), before incubation with celecoxib for 16 h. Pretreatment for 30 min with SP 600125 (10 μM) attenuated celecoxib-induced HO-1 expression. However, pretreatment with PD 98059 (10 μM) or SB 203580 (10 μM) did not alter celecoxib-induced HO-1 protein expression (FIG. 2). These findings suggest that JNK and p38 MAPK, but not MEK, are involved in the induction of HO-1 expression by celecoxib.

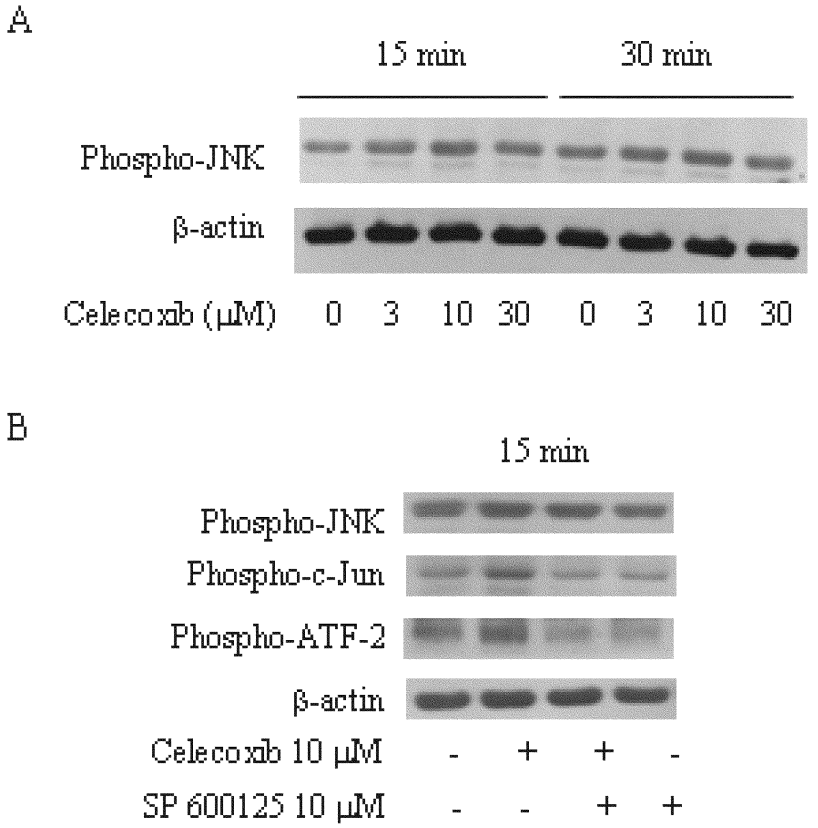

FIGURE 3. Celecoxib activates JNK in mesangial cells. (**A**) Cells were pretreated with various concentrations of celecoxib (3, 10, 30 μM) for 15 or 30 min and lysed. Cell lysates were electrophoresed and probed by Western blot with phospho-JNK–specific antibodies. (**B**) Cells were pretreated with 10 μM SP 600125 for 30 min before being incubated with 10 μM celecoxib for 15 min and lysed. Cell lysates were electrophoresed and probed by Western blot with phospho-JNK, phospho-c-Jun, and phospho-ATF-2–specific antibodies. Equal loading in each lane was demonstrated by the similar intensities of β-actin.

Activation of JNK by Celecoxib

To determine whether JNK was activated by celecoxib, we measured the activity of this kinase after celecoxib stimulation. As shown in FIGURE 3A, addition of celecoxib increased the phosphorylation of JNK; the maximum response was seen at 10 μM. Furthermore, celecoxib (10 μM) increased the phosphorylation of c-Jun and ATF-2; both are the downstream substrates of JNK. This effect of celecoxib could be blocked by pretreatment with SP 600125, a JNK inhibitor (FIG. 3B).

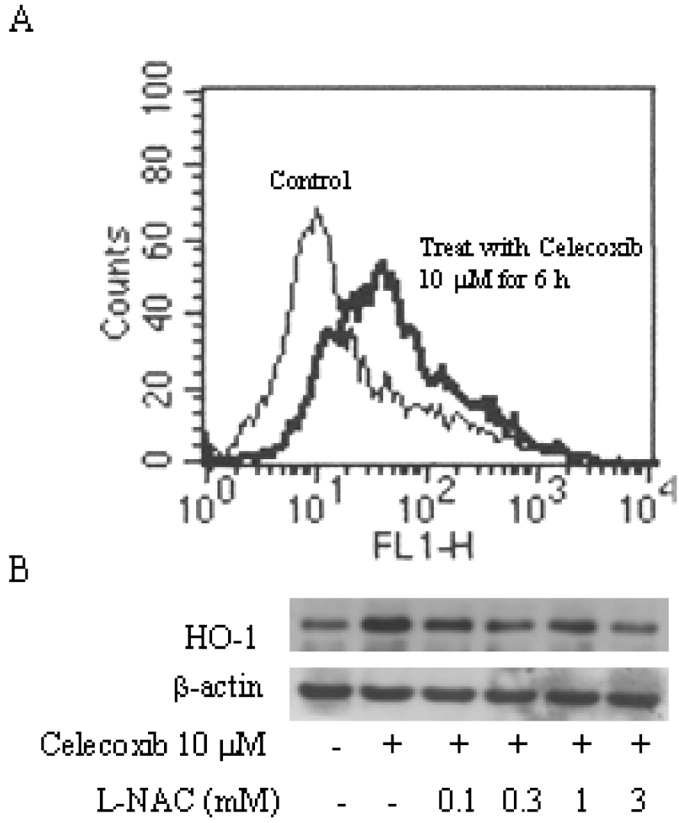

FIGURE 4. Roles of reactive oxygen species in celecoxib-induced HO-1 expression in mesangial cells. (**A**) Cells were incubated with DCFH-DA (10 μM) for 6 h in the presence of celecoxib (10 μM) as described in MATERIALS AND METHODS. (**B**) Cells were pretreated with various concentrations of L-NAC (0.1, 0.3, 1, 3 mM) before being incubated with celecoxib (10 μM) for 16 h and lysed. Cell lysates were electrophoresed and probed by Western blot with HO-1–specific antibodies. Equal loading in each lane was demonstrated by the similar intensities of β-actin.

Roles of ROS Generation in Celecoxib-Induced HO-1 Expression

The PI-3K pathway has been implicated in the stress-induced increase in ROS. We therefore examined whether celecoxib stimulated ROS generation using 2′,7′-dichlorofluorescein diacetate (DCFH-DA). The fluorescent probe can be oxidized by ROS and generates a fluorescent compound. As shown in FIGURE 4A, celecoxib increased ROS production. *l*-NAC (1 mM), an antioxidant, abolished celecoxib-induced HO-1 protein expression, suggesting that ROS generation caused by celecoxib is involved in this signaling process (FIG. 4B). Taken together, these data suggest that celecoxib may increase ROS generation, leading to activation of JNK, which, in turn, regulates HO-1 expression in mesangial cells.

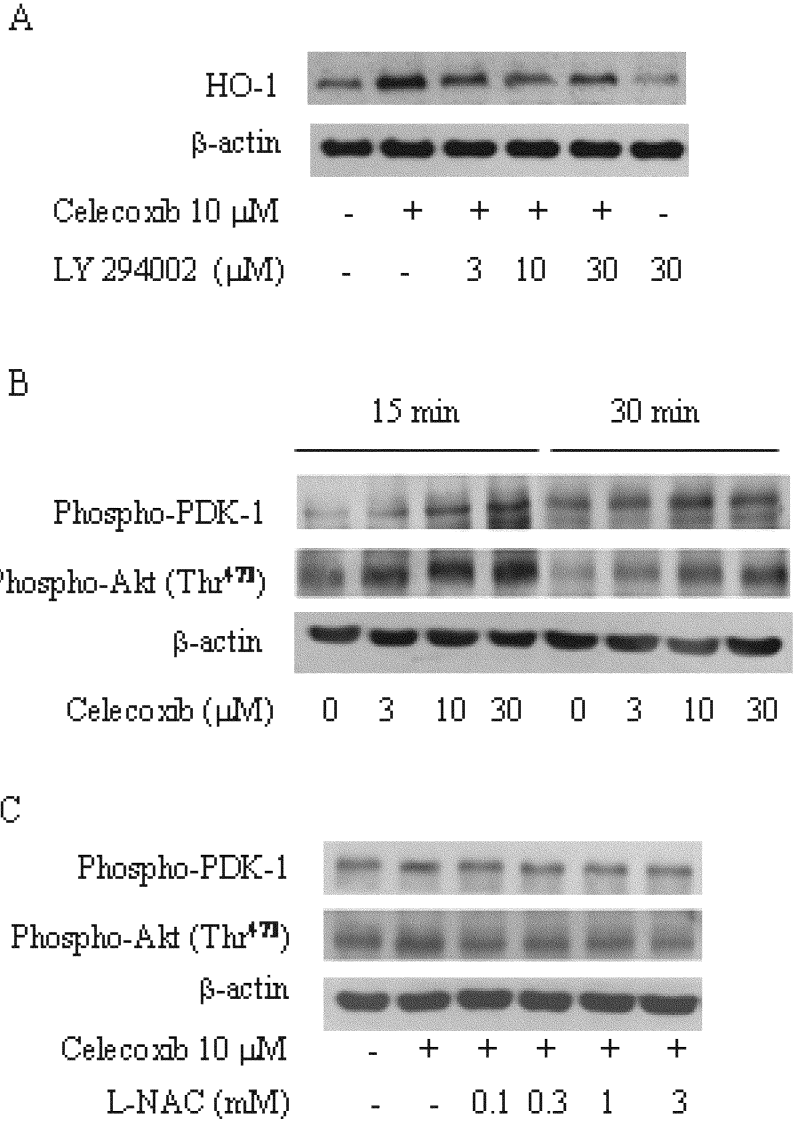

FIGURE 5. Roles of PI-3K signaling pathway in celecoxib-induced HO-1 expression in mesangial cells. (**A**) Cells were pretreated with various concentrations LY 294002 (3, 10, 30 μM) for 30 min before being incubated with celecoxib (10 μM) for 16 h. Cell lysates were electrophoresed and probed by Western blot with HO-1– and β-actin–specific antibodies. (**B**) Cells were treated with various concentrations of celecoxib (3, 10, 30 μM) for 15 or 30 min and lysed. Cell lysates were electrophoresed and probed by Western blot with phospho-PDK-1 and phospho-Akt (Thr^{473})–specific antibodies. (**C**) Cells were pretreated with various concentrations of L-NAC (0.1, 0.3, 1, 3 mM), and cell lysates were electrophoresed and probed by Western blot with phospho-PDK-1 and phospho-Akt (Thr^{473})–specific antibodies. Equal loading in each lane was demonstrated by the similar intensities of β-actin.

Roles of PI-3K in Celecoxib-Induced HO-1 Expression in Glomerular Mesangial Cells

The ROS-dependent HO-1 expression has been shown to be mediated through the phosphatidylinositol 3-kinase (PI-3K)/Akt pathway. We next investigated whether the PI-3K/Akt signaling pathway is involved in celecoxib-induced HO-1 expression in glomerular mesangial cells. As shown in FIGURE 5A, pretreatment with a specific inhibitor of PI-3K (LY 294002) reduced HO-1 expression. FIGURE 5B shows that celecoxib (10 μM) increased the phosphorylated form of its downstream effectors, Akt/PKB in mesangial cells, suggesting celecoxib can activate the PI-3K–PKB/Akt signaling pathway. *l*-NAC, an antioxidant, also inhibited celecoxib-stimulated PDK-1 and Akt phosphorylation (FIG. 5C).

DISCUSSION

In the present study, we demonstrate that celecoxib induced HO-1 expression in glomerular mesangial cells. HO-1 is a stress enzyme which can be induced under various experimental paradigms, including exposure to heavy metals, UV radiation, and other oxidative stresses. Given that NSAIDs have been implicated in causing renal damage, HO-1 expression may be considered as a marker of cellular stress. On the contrary, induction of HO-1 in macrophages has been shown to be essential for the anti-inflammatory effects of 15-deoxy-Delta 12,14-prostaglandin J2.[14] HO-1 induction may mediate its anti-inflammatory response through production of carbon monoxide, a product of heme catabolism catalyzed by heme oxygenase. Carbon monoxide, in turn, mediates the anti-inflammatory response by activation of the MAPK pathway.[15] The findings that celecoxib and another COX-2 inhibitor, SC58125, upregulate HO-1 induction in RAW 264.7 macrophages[16] suggest that these NSAIDs may exert their anti-inflammatory effect via induction of HO-1 expression.

Transcriptional regulation of HO-1 gene expression involves the activation of the JNK and p38 MAPK pathways in primary cultures of rat hepatocytes[12] and in mammary epithelial cells.[17] We demonstrated that inhibition of JNK/SAPK but not MEK or p38 MAPK by specific pharmacological inhibitors blocked celecoxib-induced HO-1 expression in glomerular mesangial cells. We further confirmed that celecoxib may activate JNK and increase the phosphorylation of its downstream substrates in mesangial cells. The finding that *N*-acetylcysteine, a free radical scavenger, substantially reduced HO-1 expression induced by celecoxib suggests ROS is involved in this process. We also provide direct evidence that celecoxib increased ROS generation, which is likely responsible for the signaling event leading to JNK activation. The stress signal ultimately induces HO-1 expression in glomerular mesangial cells.

Furthermore, we demonstrated that celecoxib activated the PI-3K pathway as demonstrated by increasing phosphorylation of its downstream effectors PKB/Akt. These results support the notion that celecoxib may exert its protective effect by activating a PI-3K–HO-1 cascade. However, our results differ from those found in cancer cell lines. In human prostate cancer cells[18] and in human colon cancer HT-29 cells,[19] celecoxib inhibits the PI-3K signaling pathway which leads to cancer cell apoptosis. In cultured mesangial cells, inhibition of PI-3K by a specific PI-3K inhibitor inhibited the phosphorylation of the downstream substrate PDK-1 and PKB/Akt and

HO-1 expression without inducing cell apoptosis. Note that the concentrations used in our study (10 μM) are slightly lower than those used in prostate cancer cells (25–50 μM) and in HT-29 cells (25–100 μM). Whether celecoxib induces mesangial cell apoptosis at higher concentrations remains to be determined.

In conclusion, this study demonstrates that celecoxib stimulates ROS generation, activating a signal transduction cascade involving JNK and PI-3K. These stress signals ultimately induce HO-1 expression in glomerular mesangial cells.

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