ROS dependence of cyclooxygenase-2 induction in rats subjected to unilateral ureteral obstruction

Martin Østergaard, Michael Christensen, Line Nilsson, Inge Carlsen, Jørgen Frøkiaer, and Rikke Nørregaard

1Department of Clinical Medicine, Aarhus University Hospital, Aarhus University, Aarhus, Denmark; and 2Department of Clinical Physiology, Aarhus University Hospital, Aarhus University, Aarhus, Denmark

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Oxidative stress resulting from unilateral ureteral obstruction (UUO) may be aggravated by increased production of ROS. Previous studies have demonstrated increased cyclooxygenase (COX)-2 expression in renal medullary interstitial cells (RMICs) in response to UUO. We investigated, both in vivo and in vitro, the role of ROS in the induction of COX-2 in rats subjected to UUO and in RMICs exposed to oxidative and mechanical stress. Rats subjected to 3-day UUO were treated with two mechanistically distinct antioxidants, the NADPH oxidase inhibitor diphenyleneiodonium (DPI) and the complex I inhibitor rotenone (ROT), to interfere with ROS production. We found that UUO-mediated induction of COX-2 in the inner medulla was attenuated by both antioxidants. In addition, DPI and ROT reduced tubular damage and oxidative stress after UUO. Moreover, mechanical stretch induced COX-2 and oxidative stress in RMICs. Likewise, RMICs exposed to H2O2 as an inducer of oxidative stress showed increased COX-2 expression and activity, both of which were reduced by DPI and ROT. Similarly, ROS production, which was increased after exposure of RMICs to H2O2, was also reduced by DPI and ROT. Furthermore, oxidative stress-induced phosphorylation of ERK1/2 and p38 was blocked by both antioxidants, and inhibition of ERK1/2 and p38 attenuated the induction of COX-2 in RMICs. Notably, COX-2 inhibitors further exacerbated the oxidative stress level in H2O2-exposed RMICs. We conclude that oxidative stress as a consequence of UUO stimulates COX-2 expression through the activation of multiple MAPKs and that the induction of COX-2 may exert a cytoprotective function in RMICs.

ureteral obstruction; oxidative stress; cyclooxygenase-2; reactive oxygen species

OBSTRUCTIVE NEPHROPATHY is an important cause of renal insufficiency in both children and adults. Unilateral ureteral obstruction (UUO) is a well-established experimental rodent model that mimics the severe renal injury found in obstructive nephropathy (19).

The hydrostatic pressure increase as a result of obstruction causes massive tubular dilation, interstitial inflammatory infiltration, apoptotic tubular cell deletion, and progressive tubulointerstitial fibrosis (8). Importantly, oxidative stress plays an important role in the pathogenesis of UUO (16, 17). Several markers of oxidative stress, such as the oxidative stress response molecule heme oxygenase (HO)-1 and heat shock protein 27, are increased in UUO kidneys (16, 29). The general paradigm is that oxidative stress occurs when the production of ROS is greater than the ability of cells to detoxify the produced ROS; indeed, increased concentrations of ROS have been observed in the obstructed kidney (35). Although oxidative stress is involved in the UUO model, little is known about the source of the stress.

In vivo studies have demonstrated the induction of cyclooxygenase (COX)-2 in the inner medulla in response to both unilateral and bilateral ureteral obstruction (26–28), and immunohistochemical analysis showed marked labeling of COX-2 in renal medullary interstitial cells (RMICs) in the obstructed kidney (27). COXs, which are bifunctional enzymes that catalyze the conversion of arachidonic acid into PGs, can be divided into two isoforms, namely, COX-1 and COX-2 (5). COX-1 is constitutively expressed in most tissues and is thought to be responsible for the production of PGs involved in the regulation of normal “housekeeping” cellular processes, whereas COX-2 is undetectable in most tissues under normal physiological conditions (5). However, COX-2 can be rapidly and transiently induced by local osmotic, inflammatory, and mechanical stimuli, in addition to its homeostatic role (5).

Previous in vitro studies using collecting duct cells have demonstrated that COX-2 expression is increased by hyperoxic stress in a ROS- and MAPK-dependent signaling pathway (34). In addition, it has also been shown that hyperglycemia leads to increases in mitochondrial ROS production as well as COX-2 expression and activity in human mesangial cells (18). However, it remains unclear whether ROS production plays a role in the induction of COX-2 in RMICs of rats subjected to UUO. In this study, we hypothesized that ROS production might increase the expression and activity of COX-2 in rat RMICs in response to UUO and play an important role in the pathophysiology of obstructive nephropathy.

The present study was designed to elucidate the role of ROS in the expression and activity of COX-2 and to characterize the signal transduction pathway responsible for the regulatory mechanism in RMICs. To achieve this, we investigated, both in vivo and in vitro, the effects of two antioxidants that interfere with different ROS production sites (i.e., NADPH oxidase and mitochondria) on the regulation of COX-2 as well as oxidative stress in rats subjected to UUO for 3 days and in RMICs exposed to oxidative stress induced by H2O2 and mechanical stress produced by stretching the cells.

MATERIALS AND METHODS

Experimental Animals

Experiments were performed on male Munich-Wistar rats initially weighing 220 g. Animal protocols were approved by the board of the Institute of Clinical Medicine, Aarhus University, according to the
licenses for use of experimental animals issued by the Danish Ministry of Food, Agriculture and Fisheries. Animals had ad libitum access to rodent diet (Altromin, Lage, Germany) and tap water. Rats were kept in cages with a 12:12-h light-dark cycle, a temperature of 21 ± 2°C, and a humidity of 55 ± 5%.

Animals were treated with sevoflurane (Abbott Scandinavia, Solna, Sweden) under anesthesia and placed on a heating pad to maintain the rectal temperature at 37–38°C. Left ureter was exposed by making a midline abdominal incision were oculated with a silk ligature. The abdominal incision was closed. Age- and time-matched sham-operated control animals were prepared and observed in parallel with each UUO group in the following protocols.

Protocol 1. UUO was induced for 3 days. In group 1, rats were treated subcutaneously with diphenyleiodonium (DPI; 1.5 mg·kg⁻¹·day⁻¹, Sigma-Aldrich, Brøndby, Denmark) dissolved in isotonic glucose starting 1 day before the operation and throughout the experiment. DPI was administrated in the afternoon, which would minimize the time before rats were active and started eating, thereby minimizing the chance for hypoglycemia. The dose was chosen on the basis of work performed by Cooper et al. (9). At the end of the experiment, rats were euthanized for either immunoblot analysis (n = 6) or immunohistochemistry (n = 4). Sham-operated rats were prepared in parallel.

Protocol 2. UUO was induced for 3 days. In group 1, rats were fed rotenone (ROT; Sigma-Aldrich) mixed into their food (600 mg/kg food) starting 1 day before the operation and throughout the experiment. Given the important role of mitochondria in energy production, ROT is suspected to induce some degree of toxicity. The dose was chosen on the basis of work performed by Zang et al. (36) and the National Toxicology Program (25a), where rats tolerated ROT at doses of 600 mg/kg for up to 2 yr. At the end of the experiment, rats were euthanized for either immunoblot analysis (n = 6) or immunohistochemistry (n = 4). In group 2, rats were fed with normal rodent diet. Rats were euthanized for either immunoblot analysis (n = 6) or immunohistochemistry (n = 4). Sham-operated rats were prepared in parallel.

Blood Sampling and Kidney Removal

After rats were reanesthetized with sevoflurane, the midline incision was reopened, and the aortic bifurcation was dissected free. Blood samples (5–7 ml) were collected. Rapidly thereafter, the kidneys were removed, and the inner medulla was dissected. Blood samples were analyzed to determine the levels of plasma creatinine and urea by a Roche Cobas 6000 analyzer (Roche Diagnostic).

Cell Culture

RMICs (a gift from Dr. C. Maric, University of Mississippi Medical Center) were obtained from fresh renal medullary tissues of Sprague-Dawley rats (80–90 g) using a modified version of the method previously described by Fontoura et al. (11). These cells show features that are characteristic of papillary interstitial cells, including elongated cellular outlines, vacuolated cytoplasm, and cytoplasmic lipid droplets (22). Cells were grown in RPMI-1640 supplemented with 10% FBS, 4 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml). Cells were incubated at 37°C in a 5% CO₂–95% air humidified atmosphere. Culture media were changed every 48 h. Cells were passaged at confluence. By passage 10, homogeneous cell populations were generally reached, and cells between passages 10 and 20 were used in the experiments. Cultures were 80–90% confluent at the start of experiments, and media were replaced with serum-free media at 24 h before the experiments. The ERK1/2 inhibitor PD-98059, the p38 MAPK inhibitor SB-202190 (Sigma-Aldrich), and the COX-2 inhibitor SC-236 (Cayman Chemical, Ann Arbor, MI) were added into the serum-free medium and incubated for 1 h before the stimulation experiments.

Flexcell Apparatus

The effect of stretch on RMICs was studied in vitro using the Flexcell FX-5000T system (Dunn Labotechnik, Asbach, Germany), which applies stretch to adherent cell types. RMICs were cultured on collagen-coated BioFlex plates (six-well plates, Dunn Labotechnik) and exposed to uniform static stretch for 2, 6, and 12 h. To determine the optimal condition, we applied different amounts of static stretch to RMICs and increase the attached cell surface area by 10%, 15%, and 20%. As a control, nonstretched cells were used. The complete system was placed in a CO₂ incubator to maintain the temperature, humidity, and atmosphere during the stretch experiment. In the optimal condition, stretch of 0% (control) and 20% was applied to RMICs for 2 h.

Membrane Fractionation for Immunoblot Analysis

RMICs were collected and lysed using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Vedbaek, Denmark). Cell suspensions were centrifuged at 14,000 g at room temperature for 10 min. Tissues were prepared for immunoblot analysis by homogenization for 30 s at 1,250 rpm in dissection buffer (0.3 M sucrose, 25 mM imidazole, and 1 mM EDTA, pH 7.2) containing Complete mini protease inhibitor cocktail tablets (Roche) followed by centrifugation at 4,000 g for 15 min at 4°C. Gel samples were prepared from supernatants mixed with Laemmli sample buffer containing 2% SDS. The Pierce BCA Protein Assay Kit (Roche) was used to determine the total protein concentration of homogenates.

Electrophoresis and Immunoblot Analysis

Gel samples were run on 12% polyacrylamide minigels (Bio-Rad Mini Protein II, Bio-Rad, Copenhagen, Denmark). For each gel intended for Western blot analysis, an identical gel was run before blotting and subjected to Coomassie staining to ensure identical protein loading. β-Actin was used as a loading control for normalization.

Protein samples run on 12% polyacrylamide minigels were transferred to nitrocellulose membranes (Hybond ECL RPN 3032D, GE Healthcare Europe, Brøndby, Denmark). Blots were blocked in 5% skim milk dissolved in PBS with Tween 20 (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, and 0.1% Tween 20, adjusted to pH 7.4). After washes with PBS-Tween 20, blots were incubated with primary antibodies overnight at 4°C. Antigen-antibody complexes were visualized with horseradish peroxidase-conjugated secondary antibodies (PO448 or PO447, 1:3,000, DAKO, Glostrup, Denmark) using the ECL system (GE Healthcare Europe).

Primary Antibodies

For semiquantitative immunoblot analysis and immunohistochemistry, the following previously characterized monoclonal and polyclonal antibodies were used: COX-1 (Cayman Chemical), COX-2 (Abcam, Cambridge, UK), HO-1 (ENZO Life Sciences, Farmingdale, NY), β-actin (Sigma-Aldrich, St. Louis, MO), and p38, phospho-p38, ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, Bcl-2, and Bax (Cell Signal Technology, Danvers, MA).

Histology and Immunohistochemistry

Kidneys from UUO rats and sham-operated control rats were fixed by retrograde perfusion via the abdominal aorta with 4% paraformaldehyde in 0.1 M PBS buffer. Next, organs were fixed for an additional hour and washed three times (10 min) with 0.01 M PBS buffer. Fixed kidneys were then dehydrated, embedded in paraffin, and cut into 2-µm sections on a rotary microtome (Leica Microsystems, Herlev, Denmark).
Paraffin-embedded sections were stained with hematoxylin and eosin to assess the grade of tubular damage. Under high magnification (×200), 10 nonoverlapping fields from each section of the renal cortex were photographed. The tubular luminal area of each section was measured using image-analysis software (ImageJ, National Institutes of Health). A grid containing sampling points was superimposed on each photograph. Points falling on glomerular structures and large vessels were excluded from the total count. The tubular dilatation score was determined by the number of points overlying dilated tubular spaces and then converted to a percentage. All analyses were performed blind.

For immunoperoxidase labeling, sections were deparaffinized, rehydrated, and processed for immunolabeling using previously characterized antibodies as described elsewhere (27).

**Fig. 1.** Effects of diphenyleneiodonium (DPI) and rotenone (ROT) on tubular damage, oxidative stress, and apoptosis in rats subjected to 3-day unilateral ureteral obstruction (UUO). A: representative micrographs of hematoxylin and eosin-stained kidney specimens (top) and the quantitative analysis of tubular damage in various groups (bottom). B: apoptosis analyzed by TUNEL assay (top) and the quantitative analysis of apoptotic nuclei in various groups (bottom). C and D: Western blot analysis of the ratio of proapoptotic Bax to antiapoptotic Bcl-2 (C) as well as the oxidative stress marker heme oxygenase (HO)-1 (D) in inner medullary homogenates. Data are means ± SE of 6 rats/group. *P < 0.05, sham-operated (sham) rats vs. untreated UUO rats; #P < 0.05, vehicle-treated UUO rats vs. DPI- or ROT-treated UUO rats.
Paraffin-embedded sections were permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate in PBS and stained for apoptosis using an in situ cell detection kit (POD, Roche) followed by a counterstain in hematoxylin. Under magnification (×40), five to seven nonoverlapping sections were imaged from each inner medulla. The amount of TUNEL-positive cells was counted in Adobe Photoshop CS5 and normalized to the number of images per inner medulla and expressed as number of TUNEL-positive cells per section.

Table 1. Changes in body weight, kidney weight, and levels of plasma creatinine and urea after different treatments

<table>
<thead>
<tr>
<th></th>
<th>Sham Operation</th>
<th>3-Day UUO</th>
<th>3-Day UUO + Diphenyleneiodonium</th>
<th>3-Day UUO + Rotenone</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>257 ± 4</td>
<td>247 ± 3</td>
<td>248 ± 6</td>
<td>202 ± 3†</td>
</tr>
<tr>
<td>Kidney weight, mg/g rat</td>
<td>3.7 ± 0.06</td>
<td>7.03 ± 0.23*</td>
<td>7.21 ± 0.29</td>
<td>6.53 ± 0.32</td>
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<tr>
<td>Plasma creatinine, mg/dl</td>
<td>0.19 ± 0.01</td>
<td>0.32 ± 0.01*</td>
<td>0.35 ± 0.03</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>Plasma urea, mmol/l</td>
<td>6.30 ± 0.31</td>
<td>7.91 ± 0.32</td>
<td>9.44 ± 0.86</td>
<td>7.51 ± 0.57</td>
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</tbody>
</table>

Values are means ± SE of 6 rats/group. UUO, unilateral ureteral obstruction. *P < 0.05, 3-day UUO rats vs. sham-operated rats; †P < 0.05, untreated 3-day UUO rats vs. treated 3-day UUO rats.

TUNEL Assay

Paraffin-embedded sections were permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate in PBS and stained for apoptosis using an in situ cell detection kit (POD, Roche) followed by a counterstain in hematoxylin. Under magnification (×40), five to seven nonoverlapping sections were imaged from each inner medulla. The amount of TUNEL-positive cells was counted in Adobe Photoshop CS5 and normalized to the number of images per inner medulla and expressed as number of TUNEL-positive cells per section.
Measurements of ROS

Intracellular ROS generation in RMICs was quantified using 2′,7′-dichlorodihydrofluorescein diacetate (Sigma-Aldrich). Briefly, 1 day before the experiment, cells were seeded in 96-well plates and incubated for 6 h with H$_2$O$_2$ in the presence or absence of inhibitors of ROS production. Next, cells were washed twice with HBSS without phenol red and then incubated with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate in HBSS for 30 min at 37°C. Finally, 2,7-dichlorofluorescein fluorescence was measured with excitation at 485 nm and emission at 520 nm.

PGE$_2$ Concentration

Levels of PGE$_2$ in culture media were measured using the PGE$_2$ EIA kit (monoclonal, Cayman Chemical), according to the manufacturer’s protocol. Briefly, RMICs were subjected to H$_2$O$_2$ treatment for 6 h with or without inhibitors of ROS production followed by PGE$_2$ measurements.

Statistical Analysis

Data are expressed as means ± SE. Statistical comparisons were analyzed by an unpaired Student’s $t$-test when two groups were compared.
compared or by one-way ANOVA followed by a post hoc unpaired Student’s t-test with the Bonferroni correction when several groups were compared. *P values of <0.05 were taken as significant.

RESULTS
Effects of DPI and ROT on Tissue Damage and Oxidative Stress in Rats Subjected to 3-Day UUO

To examine the effects of two potential antioxidants that affect ROS production on tubular damage, oxidative stress, and apoptosis after UUO injury, we administered the NADPH oxidase inhibitor DPI and the mitochondrial respiratory chain complex I inhibitor ROT to rats subjected to 3-day UUO. To analyze apoptosis, both TUNEL staining and protein expression levels of proapoptotic Bax and antiapoptotic Bcl-2 were measured, whereas the oxidative stress marker HO-1 was used as an indicator of the oxidative stress level. As shown in Fig. 1A, obstructed kidneys had massive tubular dilatation and injury compared with kidneys from sham-operated control rats.

Fig. 4. Effects of DPI and ROT on the activation of the MAPK pathway in RMICs exposed to oxidative stress. A–D: RMICs preincubated with either 10 μM DPI (A and B) or 2.5 μM ROT (C and D) were challenged with H₂O₂ (75 μM) for 6 h. Total and phosphorylated (p) ERK1/2, p38, and JNK expression levels were determined by Western blot analysis. Values are means ± SE of 4 replicates. *P < 0.05 vs. H₂O₂ alone; #P < 0.05 vs. H₂O₂ with either DPI or ROT.
ROS and COX-2 in Ureteral Obstruction

DPI and ROT Prevent the Induction of COX-2 in Rats Subjected to 3-Day UUO

To examine whether ROS production plays a role in the induction of COX-2 in response to ureteral obstruction, the expression and localization of COX-2 in the inner medulla of DPI- and ROT-treated UUO rats were investigated. The protein abundance of COX-2 was significantly increased in the inner medulla of rats subjected to 3-day UUO compared with sham-operated rats, and this induction was attenuated by DPI or ROT treatment (Fig. 2, A and B). COX-1 expression was decreased in UUO rats compared with sham-operated control rats; however, DPI and ROT did not change COX-1 levels in UUO rats (Fig. 2, A and C). Immunohistochemical analysis also showed strong COX-2 labeling in RMICs in the inner medulla of obstructed kidneys compared with control kidneys (Fig. 2, D and E). In contrast, weaker labeling was observed in DPI- or ROT-treated UUO rats (Fig. 2, F and G) compared with untreated UUO rats (Fig. 2E). In the cortex, there was no change in COX-2 protein levels among all four groups (data not shown). These results suggest that ROS play a role in the induction of COX-2 in RMICs of rats subjected to 3-day UUO.

DPI and ROT Reduce the Induction of COX-2 in RMICs Exposed to Oxidative Stress

Since ROS might play a role in the regulation of COX-2 expression in RMICs of rats subjected to 3-day UUO in vivo, we further investigated the signaling pathway responsible for the induction of COX-2 in RMICs exposed to 75 µM H₂O₂ to mimic oxidative stress in vitro. COX-2 mRNA and protein abundance in RMICs were found to be increased in a time-dependent manner upon H₂O₂ exposure (Fig. 3, A and B). In contrast, preincubation of RMICs with either 2.5–10 µM ROT or 10–25 µM DPI for 6 h led to a dose-dependent inhibition of COX-2 induction (Fig. 3C). Especially, treatment with DPI (10 µM) and ROT (2.5 µM) for 6 h effectively reduced H₂O₂-induced COX-2 protein abundance and PGE₂ concentrations in RMICs (Fig. 3, D and E). Taken together, these in vitro observations are consistent with the in vivo results and support an important role for ROS in the increased COX-2 expression and activity in RMICs.

Fig. 5. Effects of MAPK pathway inhibitors on COX-2 and HO-1 expression in RMICs exposed to oxidative stress. A and B: RMICs preincubated with either the ERK1/2 inhibitor PD-98059 (30 µM; A) or the p38 inhibitor SB-202190 (20 µM; B) for 1 h were challenged with H₂O₂ (75 µM) for 6 h. COX-2 and HO-1 expression were determined by Western blot analysis. Values are means ± SE of 4 replicates. *P < 0.05 vs. H₂O₂ alone; #P < 0.05 vs. H₂O₂ with either ERK1/2 or p38 inhibitors.
Roles of DPI and ROT in MAPK Pathway Activation in RMICs Exposed to Oxidative Stress

The mechanism linking ROS and COX-2 was further examined. Our previous study (6) demonstrated that the MAPK pathway plays a role in the regulation of COX-2 expression in RMICs subjected to mechanical stress; therefore, we investigated whether ROS may act via MAPK. To test this, we determined the effects of the antioxidants DPI and ROT on the activation of ERK1/2, p38, and JNK in RMICs exposed to H$_2$O$_2$. The activation of ERK1/2, p38, and JNK was determined by immunoblot analysis using phosphorylation-specific antibodies, as previously described. The results showed that exposure of RMICs to H$_2$O$_2$ increased the phosphorylation of both ERK1/2 and p38 compared with untreated cells (Fig. 4, A–D). The activation of both ERK1/2 and p38 by oxidative stress was attenuated by DPI and ROT, even though the regulation of p38 did not completely reach a significant

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**Fig. 6.** Effects of DPI and ROT on ROS production, oxidative stress, and apoptosis in RMICs exposed to oxidative stress. A: the 2',7'-dichlorodihydrofluorescein diacetate method was used to determine intracellular ROS production in RMICs preincubated with DPI (10 μM) or ROT (2.5 μM) and challenged with H$_2$O$_2$ (75 μM) for 6 h. B and C: Western blot analysis of HO-1 expression (B) as well as proapoptotic Bax and antiapoptotic Bcl-2 expression (C) in RMICs preincubated with DPI (10 μM) or ROT (2.5 μM) and challenged with H$_2$O$_2$ (75 μM) for 6 h. Values are means ± SE of 4 replicates. *P < 0.05 vs. H$_2$O$_2$ alone; #P < 0.05 vs. H$_2$O$_2$ with either DPI or ROT.
level ($P = 0.06$ for DPI and $P = 0.07$ for ROT; Fig. 4, A–D). Notably, the abundance of total ERK1/2 and p38 as well as JNK and the active phosphorylated form of JNK was unchanged (Fig. 4, A–D).

To examine whether ROS-activated ERK1/2 and p38 pathways play a role in the induction of COX-2 in RMICs exposed to oxidative stress, RMICs were treated with H$_2$O$_2$ as well as inhibitors of ERK1/2 and p38 activation followed by an analysis of COX-2 and HO-1 expression. The immunoblot results showed that oxidative stress-induced COX-2 and HO-1 were attenuated by inhibition of the ERK1/2 and p38 pathway (Fig. 5, A and B). Taken together, these findings suggest a relationship among ROS, MAPK, and COX-2 in RMICs exposed to oxidative stress.

**Effects of DPI and ROT on ROS Production and Apoptosis in RMICs Exposed to Oxidative Stress**

Exposure of cells to H$_2$O$_2$ can induce both oxidative stress and apoptosis, depending on the cell type and H$_2$O$_2$ concentrations (30). H$_2$O$_2$-induced oxidative stress was examined by measuring ROS production and the oxidative stress marker HO-1. The effects of the antioxidants DPI and ROT were also investigated. As shown in Fig. 6A, treatment of RMICs with H$_2$O$_2$ (75 μM) for 6 h increased ROS production, which was significantly attenuated by the administration of both DPI and ROT. Similarly, the induction of HO-1 in RMICs upon H$_2$O$_2$ treatment was also attenuated by ROT and DPI (Fig. 6B). We found no significant increase in the Bax/Bcl-2 ratio between H$_2$O$_2$-exposed cells and control cells, nor did we observe any effect of either DPI or ROT (Fig. 6C). These findings suggest that the antioxidants DPI and ROT enhance the resistance of RMICs to oxidative stress.

**Effects of COX-2 Inhibition on HO-1 Expression in RMICs Exposed to Oxidative Stress**

It has previously been demonstrated that the ability of RMICs to tolerate H$_2$O$_2$ is dependent on COX-2 activity, since COX-2 inhibition with SC-58236 reduces cell viability and increases apoptosis (15). To test whether COX-2 activity plays a role in the regulation of oxidative stress in RMICs, we treated RMICs with the selective COX-2 inhibitor SC-236 (5 μg) and measured the expression of HO-1. The results demonstrated that COX-2 inhibition increases H$_2$O$_2$-induced HO-1 expression (Fig. 7), indicating reduced cell resistance to oxidative stress.

**Effects of DPI and ROT in the Regulation of Oxidative Stress and the MAPK Pathway in RMICs Exposed to Mechanical Stress**

Tubule hydrostatic pressure increases in response to ureteral obstruction. To evaluate whether mechanical stress plays a role in the regulation of oxidative stress and the MAPK pathway in RMICs, we exposed cells to stretch and treated them with the antioxidants ROT and DPI. The results demonstrated that stretch for 2 h increases COX-2 and HO-1 expression in RMICs, indicating that mechanical stress plays a role in the regulation of COX-2 and oxidative stress. The administration of DPI attenuated the stretch-induced HO-1 expression (Fig. 8, A and B).

The MAPK pathway was studied by analyzing the activation of ERK1/2, p38, and JNK in RMICs exposed to stretch. Our data demonstrated increased ERK1/2 and p38 activity after 2 h of stretch, whereas the activation of JNK was unchanged (Fig. 8, A and B). The administration of ROT abolished the stretch-induced activation of both ERK1/2 and p38. In contrast, DPI treatment further increased the activation of ERK1/2 in RMICs exposed to stretch (Fig. 8, A and B). These findings suggest that mitochondrial respiration chain complex I and NADPH oxidase play a role for the upstream regulation of the MAPK signaling pathway in RMICs exposed to stretch.

**DISCUSSION**

In this study, we investigated, both in vivo and in vitro, whether ROS play a role in the regulation of COX-2 in RMICs in response to UUO. Our main findings demonstrated ROS as an upstream regulatory mediator of COX-2 expression and activity in RMICs. To interfere with ROS production, we used two mechanistically distinct antioxidants, namely, the NADPH oxidase inhibitor DPI and the complex I inhibitor ROT. Our results show that UUO-induced tubular dilatation and oxidative stress were attenuated by the administration of DPI and ROT in obstructed kidneys. The induction of COX-2 in the inner medulla was also effectively inhibited by both antioxidants in rats subjected to 3-day UUO and in RMICs exposed to oxidative stress. Using an in vitro model that induces mechanical stress to mimic the in vivo situation in response to UUO, we demonstrated that stretch stimulates COX-2 and HO-1 expression as well as ERK1/2 and p38 activation in RMICs. In addition, oxidative stress-induced phosphorylation of ERK1/2 and p38 was partly attenuated by antioxidant treatment, whereas inhibition of ERK1/2 and p38 blocked the induction of COX-2 in RMICs. Notably, COX-2 inhibitors also exacerbated the oxidative stress level in H$_2$O$_2$-exposed RMICs. These results suggest that oxidative stress stimulates COX-2 expression through activation of the MAPK pathway and that the induction of COX-2 may exert a cytoprotective function in RMICs.

The mitochondrial respiration chain and NADPH oxidase have been considered as key sources of ROS production. Here, our data show that COX-2 expression and activity were induced by ROS derived from both NADPH oxidase and com-
plex 1 in the mitochondrial respiration chain in RMICs in the inner medulla of rats subjected to 3-day UUO as well as in cultured RMICs exposed to oxidative stress. These findings are consistent with previous in vitro studies demonstrating that mitochondria and NADPH oxidase increased ROS production and COX-2 expression in a variety of cells, such as human mesangial cells incubated with glucose (18) and collecting duct cells exposed to hypertonic treatment (34). In the present in vivo study, the link between oxidative stress and COX-2 induction in an experimental model of UUO was examined. We assume that increased UUO-associated oxidative stress may be an integral component of the mechanism involved in the induction of COX-2 in RMICs, even though we cannot exclude other manifestations of the obstruction state as contributing factors to the induction of COX-2. Nevertheless, a previous in vivo study (25) has demonstrated a similar effects of DPI in the attenuation of COX-2 induction during ischemia-reperfusion damage in rat stomachs. In addition, Li et al. (21) showed that chronic administration of the antioxidant tempol prevented increased renal expression of COX-2 in streptozocin-induced diabetic rats, indicating that oxidative stress resulted in the induction of COX-2 in diabetes. We and others (6, 15, 24, 27) have observed increased COX-2 expression in the renal inner medulla in response to ureteral obstruction. There is increasing evidence supporting a cytoprotective role of COX-2 in the renal medulla and increased COX-2 expression as a prerequisite for RMIC survival from hypertonic stress (13, 14, 33). Based on these studies, one might speculate that oxidative stress-induced COX-2 expression in response to UUO contributes to protection against oxidant injury. However, COX-2 has also been linked to renal damage in UUO models, in which the COX-2 inhibitor etodolac reduced renal tubular damage and apoptosis (23). Therefore, prevention of COX-2 induction with antioxidants can similarly be expected to ameliorate renal damage related to UUO. These findings reveal a counterbalanced intrarenal handling of COX-2, that is, the detrimental versus beneficial effects of COX-2 may depend on the pathophysiological condition, again highlighting the importance of the enzyme in renal physiology.

Fig. 8. Effects of DPI and ROT on COX-2 expression, oxidative stress, and activation of the MAPK pathway in RMICs exposed to mechanical stress. A and B: RMICs were preincubated with DPI (10 μM) or ROT (2.5 μM) for 0.5 h followed by stretch of 20% for 2 h. COX-2 and HO-1 as well as total and phosphorylated ERK1/2, p38, and JNK expression levels were determined by Western blot analysis. Values are means ± SE of 6 replicates. *P < 0.05 vs. stretch alone; #P < 0.05 vs. stretch with either DPI or ROT.
To explore the possible underlying mechanisms that link ROS and COX-2 in RMICs, the MAPK cascade was examined. MAPK pathways mediate the stimulatory effects of different extracellular stimuli on COX-2 expression in a stimulus- and cell type-specific manner (6, 34), and studies have also demonstrated that oxidative and mechanical stress can stimulate MAPK pathways in various cell types (1, 3, 10, 31). Here, we studied different classes of MAPKs, including ERK1/2, p38, and JNK, and our data showed that the antioxidants DPI and ROT partly attenuated oxidative stress-induced phosphorylation of both ERK1/2 and p38 in RMICs. These results indicate that ROS play a role in the activation of MAPK cascades, particularly the ERK1/2 and p38 pathway, in RMICs. In addition, we found that inhibition of ERK1/2 and p38 activation suppressed COX-2 expression in RMICs in response to oxidative stress, suggesting that ERK1/2 and p38 may function as downstream effectors of ROS to transduce signals for the induction of COX-2. This observation is consistent with a previous study (34) that identified a distinct role of the ROS/MAPK/COX-2 pathway in the osmotic response in collecting duct cells.

Growing evidence has demonstrated that MAPK cascades, especially ERK1/2 and p38, play roles in cytoprotection and oxidative stress in different cell types (3, 10, 20). Our present study shows that inhibition of ERK1/2 and p38 attenuated the increased expression of the oxidative stress marker HO-1, supporting that the MAPK pathway may protect against oxidant injury in RMICs. A previous study (32) has also demonstrated that COX-2 is critical for the capability of medullary epithelial cells to survive under hypertonic stress. Consistently, our present results show that the COX-2 inhibitor SC-236 increased oxidative stress-induced HO-1 expression in RMICs, indicating that COX-2 mediates protection against oxidant injury. Furthermore, it has been previously demonstrated that the ability of RMICs to tolerate H2O2 is dependent on COX-2 activity, since COX-2 inhibition with SC-58236 reduces cell viability and increases apoptosis (15). Taking together, our observations in RMICs suggest that mitochondria and NAPDH oxidase may contribute to H2O2-induced ROS production, which transduces the signal to MAPKs and leads to COX-2 activation and protection against oxidant injury. The NAPDH oxidase subunits involved in the oxidative response in RMICs were not directly addressed in the present study. However, both p22phox and p47phox have been shown to be involved in the development of oxidative stress in a number of animal models of diseases with renal involvement, and these subunits are also expressed in the renal medulla (12) and might play a role in the oxidative response in RMICs as well.

The tubule pressure increases in response to UUO, leading to renal tubular distention and cell deformation. To mimic this in vitro, we exposed RMICs to mechanical stress and demonstrated that stretch induces COX-2 and HO-1 expression as well as ERK1/2 and p38 activation in RMICs, indicating that stretch play a role in the regulation of COX-2, oxidative stress, and the MAPK signaling pathway. As a part of the investigation into the signaling pathways linking mechanical stretch to ERK1/2 and p38 activation in RMICs, we explore the possibility that signaling mediated by mitochondrial respiration chain complex I- or NAPDH oxidase-derived ROS production may be involved. Previous studies (4, 7) have demonstrated that ROS are associated with mechanical stress-induced phosphorylation of ERK1/2 and p38 in endothelial cells and vascular smooth muscle cells. We found that stretch induced the activation of ERK1/2 and p38 signaling involved complex I stimulation. The NADPH oxidase inhibitor DPI increased the phosphorylation of ERK1/2 in RMICs exposed to stretch, whereas p38 phosphorylation was not changed. These data support the concept of different regulation pathways for individual MAPKs.

In summary, this study shows, both in vivo and in vitro, that ROS/oxidative stress might play a role in the induction of COX-2 in RMICs of rats subjected to ureteral obstruction. The activation of MAPK pathways might be involved in signal transduction, leading to increased COX-2 expression in RMICs. Overall, we conclude that oxidative stress as a consequence of ureteral obstruction might stimulate COX-2 expression through activation of the MAPK cascade pathway and that the induction of COX-2 may exert a cytoprotective function in RMICs.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.O., M.C., L.N., and I.C. performed experiments; M.O., M.C., L.N., I.C., and R.N. analyzed data; M.O., M.C., L.N., I.C., and R.N. interpreted results of experiments; M.O., M.C., L.N., I.C., and R.N. prepared figures; M.O., M.C., L.N., I.C., J.F., and R.N. drafted manuscript; M.O., M.C., L.N., I.C., J.F., and R.N. edited and revised manuscript; M.O., M.C., L.N., I.C., J.F., and R.N. approved final version of manuscript; L.N., J.F., and R.N. conception and design of research.

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ROS AND COX-2 IN URETERAL OBSTRUCTION


