Increased cyclooxygenase-2 expression and prostaglandin E₂ production in pressurized renal medullary interstitial cells

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Submitted 28 August 2009; accepted in final form 30 June 2010

Cyclooxygenase (COX) is involved in the oxytocin-induced COX-2 expression and prostaglandin E₂ production in pressurized renal medullary interstitial cells. COX exists in two isoforms: COX-1 and COX-2. COX-1 is believed to be expressed in most tissues and thought to be responsible for the production of prostanoids involved in regulating normal house-keeping cellular processes. COX-2 is undetectable in most tissue under normal physiological conditions. However, it can rapidly and transiently be induced by local osmotic, inflammatory, and physical stimuli in addition to its homeostatic role (25). In the kidney, COX-2 is constitutively expressed in macula densa and thick ascending limb (5, 10) in cortex and in inner medulla; COX-2 is found in interstitial cells (33).

In vivo studies have shown that COX-2 is induced in inner medulla in response to both 24 h of bilateral ureteral (BUO) and UUO (7, 22), and immunohistochemical analysis demonstrated marked labeling of COX-2 in interstitial cells in the base of the inner medulla in the obstructed kidney (22). Previous studies using rabbit RMICs have demonstrated that hypertonic stress induces COX-2 expression, and this is critical for enabling the cells to survive lethal changes in environmental tonicity, which is essential to the regulation of urinary concentrating ability (14). To investigate whether hypertonic stress could play a role in the induction of COX-2 in renal medullary interstitial cells (RMICs) in response to 24-h BUO, we have previously determined the inner medulla osmolality (22.) As osmolality was decreased, it is unlikely that the BUO-induced COX-2 expression in the RMICs is caused by changes in osmolality. The mechanism responsible for the COX-2 induction in the medullary interstitial cells remains incompletely understood.

Several in vitro studies have demonstrated increased COX-2 expression in numerous cell culture models, including renal podocytes, urothelial cells, vascular endothelial cells, and osteoblasts, which have been subjected to mechanical stretch (11, 15, 17, 20). These data led us to hypothesize that elevated pressure increases COX-2 expression and activity in RMICs isolated from rats. Using a novel apparatus described by Broadbelt et al. (4) in which pressure can be applied directly to cultured cells, we investigated whether pressure stimulates COX-2 expression and activity in RMICs as well as signaling pathways involved in pressure-induced COX-2 expression. Furthermore, we also examined whether mechanical and biochemical stimuli have similar effects on COX-2 protein abundance and the production of PGE₂ in RMICs. In addition, COX-2 expression was characterized in vivo, and these studies were compared with the in vitro experiments.

METHODS

Cell culture. RMICs were obtained from Dr. C. Maric (University of Mississippi Medical Center, Jackson, MS). The cells were obtained...
from fresh renal medullary tissue of Sprague-Dawley rats (80–90 g), using a modified version of the method described by Fontoura et al. (12). These cells show cytoplasmic lipid droplets, vacuolated cytoplasm, and elongated cellular outline, features that are characteristic of papillary interstitial cells (19). Cells were grown in RPMI 1640 supplemented with 10% FBS, 4 mM l-glutamine and 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 was changed at 48-h intervals, and cells were passaged at confluency. Homogeneous cell populations were generally reached by passage 10 and cells between passages 10 and 20 were used in the experiments. Cultures were at 80–90% confluency at the start of the experiments, and media was replaced with serum-free medium 24 h before the experiment. Actinomycin D and p38 MAPK inhibitor SB202190 were employed in serum-free medium, with 1-h incubation prior to stimulation experiments.

**Pressure apparatus.** We used a recently developed system to study the effects of pressure on cells in vitro (4). Cells were placed in a T-75 flask equipped with a rubber stopper containing inlet and outlet valves. The entire system was placed in a CO₂ incubator to maintain constant temperature, atmosphere, and humidity. A motorized pump allows for the continuous exchange of the incubator atmosphere with the flask using the inlet and outlet valves. This novel system can apply pressures of 20 to 200 mmHg, which are regulated by the outlet valve. Pressures of 0 mmHg (control) or 60 mmHg were applied to the RMICs for varying times.

**Experimental animals.** All procedures conformed with the Danish national guidelines for the care and handling of animals and to the published guidelines from the National Institute of Health. The animal protocols were approved by the board of the Institute of Clinical Medicine, University of Aarhus according to the licenses for use of experimental animals issued by the Danish Ministry of Justice. Studies were performed in male Munich-Wistar rats initially weighing 220 g (Møllegaard Breeding Centre, Eiby, Denmark). The rats had free access to a standard rodent diet (Altromin Lager, Germany) and tap water. During the experiments, rats were kept in individual metabolic cages, with a 12:12-h light-dark cycle, a temperature of 21°C, a humidity of 55%, and a light intensity of 110 lux. Rats were allocated to the protocols indicated below. Age- and sex-matched controls were prepared and were observed in parallel with each UUO group in the following protocols.

**Protocol 1:** UUO was induced for 6 h. The kidneys were prepared for semiquantitative immunoblotting and quantitative PCR (QPCR; n = 6). Sham-operated controls were prepared in parallel (n = 6), and the kidneys were prepared for semiquantitative immunoblotting and PCR (n = 6). Protocol 2: UUO was induced for 12 h. The kidneys were prepared for semiquantitative immunoblotting and PCR (n = 6). Sham-operated controls were prepared in parallel (n = 6), and the kidneys were prepared for semiquantitative immunoblotting and PCR (n = 6).

**RNA extraction and cDNA synthesis.** Total RNA was extracted from RMICs using the Trizol-chloroform extraction procedure. RNA extraction from inner medulla tissue was performed according to the protocol of Qiagen’s RNeasy Mini Kit. cDNA synthesis was performed with StrataScript First-Strand synthesis system (Stratagene, AH Diagnostics, Aarhus, Denmark) in accordance to the manufacturer’s instructions.

**QPCR.** QPCR was performed on RMICs and inner medulla tissue. We used 100 ng cDNA as a template for PCR amplification using Brilliant SYBR Green QPCR Master Mix according to the manufacturer’s instruction (Stratagene, AH Diagnostics). Serial dilution (1 ng to 1 fg/μl) of cDNA was used as template for generation of a standard curve. Nested primers were used to amplify standards and kidney cDNA samples: COX-1: sense, 5′-GGA ATT CCA CCT TTA TCGT; antisense, 5′-GAC ACC GTC GTA CAC CAG CAT A-3′ (GenBank accession no. NM_017043, 121 bp). COX-2: sense, 5′-CCT TGA AGA CGG ACT TGC TCA C-3′; antisense, 5′-TCT TCT TGC TCT GGT CAA TGG A-3′ (GenBank accession no. U03389, 131 bp). TATA box-binding protein: sense, 5′-GAC TCC TGT CTC CCC TAC CC-3′; antisense, 5′-CTC AGT GCA GAG GAG GGA AC-3′ (GenBank accession no. NM_001004198, 162 bp). β-actin: sense, 5′-CTG ACA GGA TGC AGA AGG-3′; antisense, 5′-GAG TAT CGT TGC TCA GGA G-3′ (GenBank accession no. NM_031144).

**Immunocytochemistry.** For COX-2 immunocytochemistry, RMICs were fixed with 4% formaldehyde for 10 min. Cells were then permeabilized with 0.1% Triton X-100, blocked in PBS containing 10% normal goat serum and TBS-TX for 30 min at room temperature. Afterward the cells were incubated with primary antibody, 2% goat serum and TBS-TX for 1 h. After being washed with PBS-T, the cells were incubated with a Pierce BCA protein assay kit (Roche Diagnostics, Vedbaek, Denmark) using enhanced chemiluminescence system (Amersham Pharmacia Biotech). Immunoblotting controls were performed using a polyclonal antibody against COX-2 in RMICs.

**Immunoblotting.** RMICs and inner medulla tissue were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at room temperature. Afterward the sections were washed in 0.1 M phosphate buffer, pH 7.4, and then in 0.1 M phosphate buffer, pH 7.4, containing 0.1% Tween 20 for 30 min. Sections were then washed in 0.1 M phosphate buffer, pH 7.4, containing 0.1% Tween 20, 0.1% Triton X-100, and 5% nonfat dry milk in PBS-T (0.1 M NaH₂PO₄, 0.1 M NaCl, 0.1% Triton X-100) for 60 min. The sections were then incubated with primary antibody, 2% goat serum and TBS-TX for 1 h. After being washed with PBS-T, the cells were incubated with secondary antibody goat FITC anti-rabbit (1:200; Molecular Probes, Eugene, OR) and DAPI (1:200; Molecular Probes) in TBS-TX. After being washed, sections were mounted with a coverslip in slow-fade Medium (Dako).

**Primary antibodies.** For semiquantitative immunoblotting and immunocytochemistry, we used the following primary antibodies: 1) COX-1 (cat. no. 160109; Cayman Chemical, Ann Arbor, MI), an affinity-purified rabbit polyclonal antibody against COX-1; 2) COX-2...
(cat. no. 160126, Cayman Chemical), an affinity-purified rabbit polyclonal antibody against COX-2; 3) inducible nitric oxide synthase (iNOS; NOS2, cat. no. SC-650; Santa Cruz Biotechnology, Santa Cruz, CA), an affinity-purified rabbit polyclonal antibody against iNOS; 4) p38 MAP kinase (cat. no. 9212; Cell Signaling, Danvers, MA), an affinity-purified rabbit polyclonal antibody against p38 MAP kinase; 5) phospho-p38 MAP kinase (cat. no. 4511; Cell Signaling), an affinity-purified rabbit polyclonal antibody against phospho-p38 MAP kinase; 6) NF-κB (cat. no. 3034; Cell Signaling), an affinity-purified rabbit polyclonal antibody against NF-κB; 7) β-actin (cat. no. 3597–100; BioVision, Mountain View, CA), an affinity-purified rabbit polyclonal antibody against β-actin.

Measurement of PGE2 production. PGE2 in culture media was measured by ELISA (Cayman Chemical; AH Diagnostics, Aarhus, Denmark) according to the manufacturer’s instructions. RMICs were subjected to pressure or IL-1β treatment, and another group of cells was exposed to pressure or IL-1β administration in the presence of a COX-2-specific inhibitor, 10 μM parecoxib (Dynastat; Pfizer, Ballerup, Denmark).

Cell viability. Cell death was assessed using the Trypan blue exclusion method. RMICs were exposed to 60 mmHg pressure for 6 h. Cells were counted using a hemacytometer with the addition of trypan blue. Only cells that excluded the dye were counted as viable cells, whereas those that stained blue were counted as dead. As a positive control for cell death, hydrogen peroxide was used.

A methylthiazol tetrazolium (MTT) assay was used to measure cell viability by seeding cells in a 96-well plate with 100 μl medium and 20 μl of CellTiter 96 Reagent. Cells were pressurized and incubated in the incubator for 6 h. At the end of the allotted time, plates were placed individually in a spectrophotometer for reading at 490 nm.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. Apoptosis was assessed using The ApopTag Peroxidase In Situ Apoptosis Detection Kit S7100 (Intergen, Purchase, NY). Briefly, monolayers at ambient pressure or subjected to 60 mmHg for 6 h were fixed to chamber slides with 4% paraformaldehyde before the addition of terminal deoxynucleotidyl transferase enzyme and diaminobenzidine solution. Following PBS wash, endogenous peroxidases were quenched with 3% H2O2/methanol for 30 min. Nonspecific binding was blocked using 5% BSA for 30 min. Equilibrium buffer was applied to the slides for 10 s and removed, and terminal deoxynucleotidyl transferase enzyme was then applied for 1 h at room temperature. Enzyme was removed and stop/wash buffer was applied for 10 min. Antidigoxigenin peroxidase was applied to slides for 30 min. Cells were developed with diaminobenzidine and counterstained with hematoxylin.

Statistics. Values are presented as means ± SE. Statistical comparisons between experimental groups were made by a standard unpaired t-test. P values < 0.05 were considered significant.

RESULTS

Effect of serum, IL-1β, and osmotic stress on COX-2 expression. Previous studies have documented that IL-1β is a potent inducer of COX-2 expression and PGE2 production in numerous cell lines including endothelial cells, macrophages, monocytes, fibroblast, and osteoblastic cells (2, 3, 8). To test whether IL-1β may induce COX-2 expression in RMICs, cells were incubated with a different concentration of IL-1β in a dose- and time-dependent manner. COX-2 protein abundance increased in a dose-dependent manner (Fig. IA) and time-dependent manner (Fig. IB).

The effect of serum on IL-1β-induced COX-2 expression at different time points was examined. In the presence of serum, 5 ng/ml of IL-1β was not able to induce COX-2 protein abundance (Fig. IC). However, cells that were serum starved for 24 h showed a marked induction of COX-2 occurring in the presence of IL-1β. However, at 48 and 72 h it was not possible to increase COX-2 protein levels in response to IL-1β administration (Fig. IC). Furthermore, RMICs serum starvation for 48 and 72 h also promoted changes in morphology, and cells started to detach (data not shown). Further experiments were carried out after a 24-h period of serum starvation.

To localize COX-2 in RMICs we performed immunocytochemistry. COX-2 immunoreactivity was present within the cytoplasm in RMICs (Fig. ID).

Effect of pressure on COX-2 expression. In animal models of UUO, a marked elevation of ureteral pressure occurs, increasing to 35 to 60 mmHg (6, 9, 23). We therefore investigated whether pressure at 60 mmHg increases COX-1 and COX-2 expression in renal interstitial cells (RMICs) subjected to treatment with IL-1β and FBS. All Western blots were reacted with anti-COX-2 antibody and revealed a 72-kDa band. A total of 20 μg protein was used for the COX-2 assay. All results are representative of 4 similar experiments. A: COX-2 protein abundance in RMICs in response to different concentrations of IL-1β (0.5, 1, 5, 10, 20 ng/ml) for 6 h. Increased COX-2 protein was observed at 5 ng/ml of IL-1β and stays elevated at 10 and 20 ng/ml as well. B: COX-2 protein abundance in RMICs as result of time-dependent IL-1β stimulation (5 ng/ml) for 0, 2, 4, 6, 8, 12, and 24 h. C: COX-2 protein abundance in RMICs grown in medium with and without FBS as well as subjected to administration of 5 ng/ml of IL-1β for 6 h. RMICs cultured in serum-free medium for 24 h prior to IL-1β treatment for 6 h showed increased COX-2 protein level, whereas cells subjected to medium with 10% FBS or serum-free media for 48 h did not show any change in COX-2. D: COX-2 immunocytochemistry in RMICs cultures. COX-2 immunoreactivity (green) is present in the cytoplasm of RMICs. As a nuclear marker DAPI (blue) was used. Scale bar = 50 μM.

Fig. 1. Cyclooxygenase type 2 (COX-2) protein level in renal medullary interstitial cells (RMICs) subjected to treatment with IL-1β and FBS. All Western blots were reacted with anti-COX-2 antibody and revealed a 72-kDa band. A total of 20 μg protein was used for the COX-2 assay. All results are representative of 4 similar experiments. A: COX-2 protein abundance in RMICs in response to different concentrations of IL-1β (0.5, 1, 5, 10, 20 ng/ml) for 6 h. Increased COX-2 protein was observed at 5 ng/ml of IL-1β and stays elevated at 10 and 20 ng/ml as well. B: COX-2 protein abundance in RMICs as result of time-dependent IL-1β stimulation (5 ng/ml) for 0, 2, 4, 6, 8, 12, and 24 h. C: COX-2 protein abundance in RMICs grown in medium with and without FBS as well as subjected to administration of 5 ng/ml of IL-1β for 6 h. RMICs cultured in serum-free medium for 24 h prior to IL-1β treatment for 6 h showed increased COX-2 protein level, whereas cells subjected to medium with 10% FBS or serum-free media for 48 h did not show any change in COX-2. D: COX-2 immunocytochemistry in RMICs cultures. COX-2 immunoreactivity (green) is present in the cytoplasm of RMICs. As a nuclear marker DAPI (blue) was used. Scale bar = 50 μM.
mRNA and protein levels in a time-dependent manner (n = 4). RMIC cells were serum starved for 24 h prior to each experiment. QPCR showed that COX-2 mRNA expression normalized to β-actin was increased in response to 60 mmHg for 4 h and stayed elevated during 6 h as well (Fig. 2A). COX-2 protein abundance normalized to β-actin increased in a time-dependent manner when subjected to an elevated pressure of 60 mmHg for 2, 4, and 6 h, but levels returned toward control after 8 h of pressure. Densitometric analysis revealed that COX-2 protein abundance was highest compared with control at 6 h when pressure of 60 mmHg was applied (Fig. 2B). We examined the COX-2 protein abundance in RMICs exposed to increased pressure of 30, 60, 90, or 120 mmHg. A marked increase in COX-2 protein abundance was observed when cells were stimulated with pressure at 60 and 90 mmHg for 6 h (Fig. 2C). To validate the effectiveness of pressure, we also examined iNOS protein level; immunoblot analysis demonstrated induction of iNOS in RMICs subjected to pressure of 60 mmHg (Fig. 2D). In contrast, COX-1 mRNA and protein levels normalized to β-actin did not change in response to pressure of 60 mmHg at any time point (Fig. 3, A and B).

**Effect of pressure and IL-1β stimulation on COX activity.** The increase in COX-2 protein abundance was accompanied by a corresponding increase in COX enzyme activity. As shown in Fig. 4A, there was a significant increase in PGE2 levels in the culture media after 6 h pressure for 60 mmHg (329 ± 36 pg/ml in control vs. 710 ± 98 pg/ml in pressurized cells; n = 6 for each group). This increase in PGE2 production in response to 60 mmHg pressure for 6 h was prevented in the presence of a selective COX-2 inhibitor parecoxib at 10 μM (329 ± 39 pg/ml in control vs. 202 ± 8 pg/ml in pressurized RMICs + parecoxib; n = 6 for each group).

RMICs were treated with 5 ng/ml of IL-1β for 6 and 24 h, and PGE2 production was evaluated. Figure 4B demonstrates that PGE2 production was significantly increased in response to IL-1β administration for 6 h (241 ± 53 pg/ml in control vs. 567 ± 10 pg/ml in IL-1β stimulated RMICs, n = 4) and 24 h (395 ± 19 pg/ml in control vs. 1,510 ± 79 pg/ml in IL-1β stimulated RMICs, n = 4). Parecoxib (10 μM) inhibited the IL-1β-induced PGE2 production (6 h: 216 ± 47 pg/ml and 24 h: 285 ± 63 pg/ml; n = 4).

**Signaling pathways involved in stimulation of pressurized COX-2 induction.** We next wanted to investigate potential signaling pathways involved in the observed induction of COX-2 in pressurized RMICs. Therefore, a first-step experiment was performed to examine whether COX-2 is regulated at the transcriptional level. By adding the transcription inhibitor actinomycin D, the pressure-induced COX-2 protein expression was significantly decreased (Fig. 5A). It has previously been shown that hypertonic stress activates an NF-κB/COX-2-linked survival mechanism in rabbit RMICs (14). Furthermore, it has been demonstrated that COX-2 expression occurs via p38 MAPK signaling cascade in renal podocytes and bladder smooth muscle cells subjected to mechanical forces (20, 26). We therefore examined these two pathways by using the selective p38 MAPK inhibitor SB202190 and siRNA knockdown of NF-κB.

Immunoblotting analysis demonstrated an increased p38 phosphorylation in RMICs subjected to 60 mmHg for 6 h (Fig. 5B). The pressurized COX-2 induction was p38 MAPK-dependent as the p38 MAPK inhibitor blocked this effect in a
dose-dependent manner (Fig. 5C). Transfection of RMICs with siRNA directed against NF-κB reduced NF-κB level by 80% (Fig. 5D). Immunoblotting of COX-2 demonstrated that NF-κB siRNA attenuated the pressurized COX-2 induction; however, data were not significant due to variations among the experiments (Fig. 5E).

Effect of pressure on cell death, viability, and apoptosis. To evaluate whether RMICs subjected to 60 mmHg pressure for 6 h had any effect on cell death, viability, and apoptosis, we used trypan blue exclusion, MTT assay, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. Cell death at ambient pressure or in cells exposed to elevated pressure of 60 mmHg for 6 h was < 10% (Fig. 6A). Compared with RMICs at ambient pressure, there was no difference in the number of apoptotic cells when cells were subjected to pressure of 60 mmHg for 6 h (Fig. 6B). Cell viability in RMICs was examined by MTT assay. As shown in Fig. 6C pressure at 60 mmHg for 6 h did not affect cell viability. As a positive control, hydrogen peroxide was added to the RMIC cells to verify that the cells can be killed (Fig. 6D).

Increased inner medullary COX-2 expression in response to 6- and 12-h UUO. It has previously been demonstrated that the COX-2 protein level in inner medulla is increased in the obstructed kidney in response to 24-h UUO (7). We therefore investigated whether COX-1 and COX-2 mRNA and protein level are regulated in response to 6- and 12-h UUO. COX-2 mRNA was increased in inner medulla in the obstructed kidney compared with both sham-operated and unobstructed kidney after both 6- and 12-h UUO (Fig. 7A). COX-2 protein abundance was increased in inner medulla in the obstructed kidney compared with both sham-operated and unobstructed kidney in response to 12-h UUO. However, at 6-h UUO, the COX-2 protein level was only significantly increased in the obstructed kidney compared with the unobstructed kidney in inner medulla (Fig. 7B). The abundance of COX-1 mRNA and protein was unchanged in both 6- and 12-h UUO rats (Fig. 7, A and B).

Fig. 4. PGE2 production in response to 60 mmHg pressure and IL-1β treatment in RMICs with and without the COX-2-specific inhibitor parecoxib. A: PGE2 production was increased in RMICs in response to 60 mmHg pressure for 6 h, and administration of a specific COX-2 inhibitor parecoxib attenuated this increase. Each bar represents the mean ± SE of 5 experiments. *P < 0.05 compared with control; #P < 0.05 parecoxib-treated pressurized RMICs compared with untreated pressurized RMICs.

Fig. 3. COX-1 mRNA and protein level in RMICs in response to pressure. A: QPCR for COX-1 and β-actin in RMICs through pressure stimulation. QPCR was performed using 100 ng cDNA and illustrated unchanged COX-1 mRNA abundance normalized to β-actin. B: immunoblot was reacted with anti-COX-1 antibody and revealed a 71-kDa band. A total of 10 μg protein was used for the COX-1 assay. Densitometric analysis demonstrated unchanged COX-1 protein level normalized to β-actin in RMICs subjected to 60 mmHg for 6 h. Each column represents the mean ± SE.
DISCUSSION

The main findings of the present study are COX-2 protein induction in RMICs stimulated with the cytokine IL-1β and induction of COX-2 mRNA and protein abundance in pressurized RMICs. Our results suggest that p38 MAPK and NF-κB are involved in the increased expression of COX-2 as a result of pressure. In addition, our data demonstrated increased production of PGE2 in RMICs in response to pressure as well as IL-1β stimulation. This enhanced PGE2 production was attenuated by administration of a COX-2-specific inhibitor. COX-1 mRNA and protein level were unchanged in pressurized RMICs. Furthermore, COX-2 is upregulated in rats subjected to both 6-h and 12-h UUO, demonstrating that pressure plays a role in the induction of COX-2 and production of PGE2 in RMICs. Importantly, the study indicates that COX-2 protein expression likely is induced by similar mechanisms both in vitro and in vivo.

In vivo, RMICs are subjected to inflammatory and mechanical stress as a result of ureteral obstruction. IL-1β is known to be a potent inducer of COX-2 expression and PGE2 production in numerous cell lines including endothelial cells, macrophages, monocytes, fibroblast, and osteoblastic cells (2, 3, 8). This is consistent with our findings in rat RMICs showing increased COX-2 expression and activity in RMICs treated with IL-1β as an inflammatory stress factor.

Serum is a potent inducer of COX-2 gene expression in several types of cells, including mouse fibroblast and osteoblasts as well as rat vascular smooth muscle cells and human oral gingival epithelial cells (21, 31, 32). We wanted to exclude serum from the media, to ensure that its presence did not confound our results. Our data demonstrated that IL-1β-induced COX-2 after 24 h of serum starvation. If the cells were not serum starved or starved for more than 24 h it was not possible to increase COX-2 levels in response to IL-1β administration. In previous studies in rabbit RMICs, the 10% serum medium was replaced with 0.5% serum for 24 to 48 h prior to experimental analysis (13). Furthermore, we also observed changed morphology in RMICs; and cells start to detach when they have been serum starved for 48 and 72 h, suggesting that these cells were not able to survive without medium containing FBS.

Using a pressure apparatus developed by Broadbelt et al. (4) the present study demonstrated an increased COX-2 mRNA expression in RMICs subjected to 60 mmHg pressure for 4 h and enhanced COX-2 protein level when RMICs were pressurized for 6 h. COX-1 mRNA and protein level was unchanged. We also demonstrated that COX-2 expression by pressure was functionally significant by documenting the increase in PGE2 excretion in response to 60 mmHg pressure. The fact that this increased PGE2 production was almost
completely suppressed by administration of a specific COX-2 inhibitor provides further evidence that COX-2 activation, rather than COX-1, is mainly responsible for enhanced PGE2 production in pressurized RMICs.

This system is placed in the CO2 incubator to maintain constant temperature atmosphere as well as humidity and elevates pressure in the culture chamber with the use of a pump that pumps and circulates incubator atmosphere (95% air/5% CO2). The amount of pressure can easily be changed by decreasing or increasing the opening of the outlet valve. In vivo, RMICs are subjected to pressure as a result of ureteral obstruction, and this cell culture pressure apparatus may be critical for studies aimed at identifying key mechanotransduction events in cells by mimicking the situation observed in the obstructed kidney. Pressure up to 48–60 mmHg have been documented in the obstructed ureter in UUO (6, 9, 28). Furthermore, we have recorded an intrapelvic pressure at 60 mmHg after 1-h occlusion in rats (23) and Persson et al. (27) demonstrated several years ago that 2 h of UUO increase interstitial hydraulic pressure.

Fig. 6. Effect of 60 mmHg pressure on cell death, viability, and apoptosis in RMICs. A: % dead RMICs in response to 60 mmHg pressure for 6 h. B: apoptotic RMICs (brown stain) were determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay, and no change was detected between control and pressurized cells. C: cells viability was determined by methylthiazolite tetrazolium (MTT) assay, and no change was detected between control and pressurized RMICs. D: RMICs were treated with hydrogen peroxide and showed increased cells death by 5 mM of hydrogen peroxide. White bars represented alive RMICs and black bars represented dead RMICs. *P < 0.05 compared with dead cells. Data are expressed as means ± SE of 4 experiments.

Fig. 7. Expression of COX-2 and COX-1 mRNA and protein in inner medulla tissue from sham-operated rats and rats subjected to unilateral ureteral obstruction (UUO) for 6 and 12 h. A: representative QPCR for COX-2, COX-1, and TATA box-binding protein. QPCR was performed using 100 ng cDNA. COX-2 mRNA expression was increased in response to 6- and 12-h UUO in inner medulla. COX-1 mRNA level was unchanged. Each column represents the mean ± SE. *P < 0.05 Obstr. compared with sham rats; #P < 0.05 Obstr. compared with non-obstr. B: immunoblot was reacted with anti-COX-2 antibody and anti-COX-1 antibody. A total of 20 µg protein was used for the COX assay. Densitometric analyses of all the samples from sham-operated and obstructed kidneys of rats with 6- and 12-h UUO revealed that there was an increase of COX-2 protein level in the UUO group compared with sham-operated rats. COX-1 protein level was unchanged. *P < 0.05 Obstr. compared with sham rats; #P < 0.05 Obstr. Compared with non-obstr (n = 6).
It has previously been demonstrated that COX-2, but not COX-1, can be induced in inner medulla in response to 24-h UUO (7) as well as 12- and 24-h BUO (22). Furthermore, we have in response to both 12-h and 24-h BUO demonstrated marked labeling of COX-2 in medullary interstitial cells in the base of the inner medulla (22). Our in vivo study of UUO, demonstrated a significant increase in COX-2 mRNA and protein level at 6 and 12 h in the obstructed kidney. This is in contrast with our in vitro study, whereby COX-2 mRNA and protein induction occur within 4 h and 6 h, respectively, of applying 60 mmHg pressure. COX-2 protein expression remains elevated through 12 h in vivo but is reduced from its maximal value at this time point in the pressurized cell model. This effect is probably due to isolation of renal interstitial cells as well as rapid and precise pressure in this cell culture pressure apparatus. In vivo ureteral pressures that trigger distension and cell stretch develop more gradually than in the direct application of pressure that is achievable in the cell model system. Furthermore, numerous other factors present in systemic circulation or released in the paracrine tissue may be affecting COX-2 protein abundance in vivo. Jerde et al. (16) also demonstrated that COX-2 induction occurs faster in an porcine urothelial cell model subjected directly to mechanical stretch compared with ureteral obstruction in vivo.

Our data clearly demonstrated that COX-2 is regulated at the transcriptional and that p38 MAPK is involved in the increased induction of COX-2 as a result of pressure. Furthermore, data showed that NF-κB seems to be involved in the COX-2 induction in response to pressure. However, data were not significant due to variations among the experiments, which might be explained by the lack of a complete NF-κB knockdown. Several in vitro studies have demonstrated that mechanical stretch activates COX-2 expression in bladder smooth muscle and vascular endothelial cells as well as renal podocytes, urothelial cells, and osteoblasts (11, 15, 17, 20). Both in urothelial cells (17) and podocytes (20) increased COX-2 protein level was observed after stretching for 6 h in agreement with our data showing significant induction of COX-2 protein abundance in RMICs pressurized for 6 h. Many cellular signal transduction cascades of COX-2 induction have been identified, including those involving mitogen-activated protein kinases, PKA, PKC, NF-κB, extracellular matrix proteins, and glycogen synthase kinase-3β. However, most of these studies involved hormone, mitogen-induced, or hypertonic conditions, and data investigating mechanosensing pathways of COX-2 induction remain sparse. Several consensus elements for NF-κB have been indentified as regulatory sequences in the promoter region of COX-2, and previous studies demonstrated that hypertonic stress activates an NF-κB-COX-2-linked survival mechanism in rabbit RMICs (14) and that this COX-2-dependent pathway can be antagonized by glycogen synthase kinase-3β (29). Broadbelt et al. (4) also showed that NF-κB is involved in the increased expression of iNOS in renal epithelial cells subjected to 60 mmHg pressure. We found that siRNA knockdown of NF-κB leads to a decreased in the pressure-induced COX-2 expression, suggesting that NF-κB could play a role in the regulation of COX-2 in pressurized RMICs. Furthermore, in renal podocytes and bladder smooth muscle cells subjected to mechanical forces, it has been demonstrated that COX-2 expression occurs via the p38 MAP kinase signaling cascade (20, 26). The findings of the present study show increased phosphorylation of p38 as a result of pressure and the p38 inhibitor SB202190 reduced the pressure-induced COX-2 expression, confirming that the p38 MAPK pathway is involved in the induction of COX-2 in pressurized RMICs. Moreover, previous studies have shown that mechanical stretch upregulates COX-2 expression in mesangial cells via PKC (1) and a recent study showed that stretch induction of COX-2 in urothelial cells is calcium- and PKCζ-dependent (17). However, the triggering cascades of these molecules are yet to be verified in pressurized cells.

Ju et al. (18) have shown that hydrostatic pressure can induce apoptotic cell death in cultured retinal ganglion cells (RGCs) when they have been exposed to 30 mmHg for 3 days in a pressurized incubator. In addition, another study demonstrated pressure-induced apoptosis of RGCs exposed to 70 mmHg of pressure for 48 h. However, they did not observe any change in survival when RGCs were pressurized in 6 h (30). Furthermore, Broadbelt et al. (4) demonstrated, using the same kind of pressure apparatus, that the application of 20–120 mmHg had no effect on cell death or apoptosis, while proliferation was significantly increased at 24 and 36 h following 60 mmHg in kidney epithelial cells. In this study, we did not observe any apoptosis or cell death when RMICs were subjected to 60 mmHg for 6 h. Previous reports have suggested that COX-2 plays an important role in RMICs survival when cells are subjected to hyperosmotic stress or treatment with a specific COX-2 inhibitor (13). The addition of the selective COX-2 inhibitor SC-58236 induces RMIC cell death at concentration of 30 μM or higher for 48 h (13). Furthermore, RMICs treated with a submicromolar concentration (1–5 μM) of SC-58236 and exposure to hypertonic media for 12 h markedly reduces cell survival (14). Future studies will be conducted to determine whether COX-2 also plays an important role in cell survival in pressurized RMICs.

**Perspectives and Significance**

The present study is an effort to elucidate whether the induction of COX-2 in response to ureteral obstruction in RMICs may be mediated by the increased pressure in the kidney tissue. Our data indicate that pressure causes an induction of COX-2 mRNA and protein level and also activation of PGE2 production in RMICs. This pressure-induced COX-2 expression involves signaling through p38 MAPK. COX-2 could play a role in RMICs survival in response to ureteral obstruction, and elucidation of the effects of pressure at the molecular level may lead to pharmacologic targets of intervention for pressure-related diseases.

**ACKNOWLEDGMENTS**

The authors thank Line V. Nielsen, Gitte Skou, and Gitte Kall for expert technical assistance.

**GRANTS**

The Water and Salt Research Centre at the University of Aarhus is established and supported by the Danish National Research Foundation (Danmarks Grundforskningsfond). Support for this study was provided by Lundbeck Foundation, The Novo Nordisk Foundation, The Danish Medical Research Council, The University of Aarhus, and the intramural budget of the National Heart, Lung, and Blood Institute, National Institutes of Health, the Katherine and Vigo Skovgaard Foundation and the A. P. Møller Foundation for the Advancement of Medical Science.
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