CaR-mediated COX-2 expression in primary cultured mTAL cells

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Wang, Dairong, Shao-Jian An, Wen-Hui Wang, John C. McGiff, and Nicholas R. Ferreri. CaR-mediated COX-2 expression in primary cultured mTAL cells. Am J Physiol Renal Physiol 281: F658–F664, 2001.—Primary cultures of medullary thick ascending limb (mTAL) cells retain the capacity to express calcium-sensing receptor (CaR) mRNA and protein. Increases in cyclooxygenase-2 (COX-2) mRNA accumulation, protein expression, and PGE2 synthesis were observed in a dose- and time-dependent manner after exposure of these cells to extracellular calcium (Ca2+). Moreover, transfection of mTAL cells with a CaR overexpression vector significantly enhanced COX-2 expression and PGE2 production in response to calcium compared with cells transfected with an empty vector. Challenge with the CaR-selective agonist poly-L-arginine (PLA) also increased COX-2 mRNA accumulation, protein expression, and PGE2 synthesis. Furthermore, Ca2+- and PLA-mediated PGE2 production was abolished in the presence of NS-398 or nimesulide, two different COX-2-selective inhibitors. These data suggest that intracellular signaling mechanisms initiated via activation of CaR contribute to COX-2-dependent PGE2 production in rat primary cultured mTAL and to assess the role of CaR in this response.

METHODS

Animals. Male Sprague-Dawley rats (Charles River Lab, Wilmington, MA), weighing 100–110 g, were maintained on

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EXPRESSION OF CYCLOOXYGENASE-2 (COX-2) APPEARS to be differentially regulated in the kidney. For instance, low salt increases COX-2 expression in the cortex, whereas high salt increases expression in the medulla (36). These differences are consistent with the ability of renal prostaglandins to affect vascular or tubular events in the cortex and medulla, respectively. The medullary thick ascending limb (mTAL), which is impermeable to water but actively reabsorbs salt from tubular fluid, helps to establish the osmolarity gradient along the loop of Henle. The Na+ pump located on the basolateral membrane of the mTAL provides the energy for this process. Na+, K+, and Cl− are reabsorbed from the tubular fluid via the Na+-K+-2Cl− cotransporter on the apical membrane, and K+ is recycled via apical K+ channels back to the tubular fluid. PGE2, the major prostaglandin produced in the kidney, has been reported to inhibit the Na+-K+-2Cl− cotrans-
standard rat chow (Ralston-Purina, Chicago, IL) and given tap water ad libitum.

Reagents. Tissue culture media, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and COX-2 primers (sense: TACAAGCTGGAAAGGAC, antisense: CAGTATGGAGGAAACACGAG) were obtained from Life Technologies (Grand Island, NY). Reagent-grade chemicals and collagenase (type IA) were from Sigma (St. Louis, MO). COX-2 antisera were from Cayman (Ann Arbor, MI). NS-398 and nimesulide were from Biomol (Ann Arbor, MI). Polyvinylidene difluoride (PVDF) membranes were obtained from Amersham (Arlington Heights, IL).

Isolation of mTAL cells. mTAL cells (~95% purity) were isolated and characterized as previously described (9, 17).

Briefly, male Sprague-Dawley rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (0.65 mg/100 g body wt). The kidneys were perfused with sterile 0.9% saline, via retrograde perfusion of the aorta and cut along the corticopapillary axis. The inner stripe of the outer medulla was excised, minced with a sterile blade, and incubated for 10 min at 37°C in a 0.1% collagenase (3 solution) gassed with 95% O2. The suspension was sedimented on ice, bated for 10 min at 37°C in a 0.1% collagenase solution, then at room temperature for 10 min to allow extension of the suspension of tubules was collected. The collagenase digestion was repeated three times with the remaining undigested tissue. The combined tubule suspensions were spun, resuspended in HBSS, and filtered through 52-μm nylon mesh (Fisher Scientific, Springfield, NJ). The filtrated solution was discarded, and the tubules retained on the mesh were resuspended in HBSS. Then, the solution was centrifuged at 500 rpm for 5 min, the supernatant was aspirated away, and the cells were cultured in DMEM-Ham’s F-12 medium (1:1), 10% fetal bovine serum (FBS), epidermal growth factor (20 ng/ml), 1% glucose, streptomycin-penicillin (100 U/ml), and Fungizone (1 μg/ml; Gemini, Woodland, CA). After 3 days, monolayers of cells were 80–90% confluent. The cells were kept quiescent in RPMI containing 0.42 mM Ca2+ and 0.5% FBS for 18–24 h before use.

Isolation of total RNA/RT-PCR analysis. Total RNA was isolated by lysing cells or outer medullary tissue in TriZol for 18–24 h before use. 

Gene transfection. mTAL cells were cultured with calcium chloride (1–2 mM) or poly-l-arginine (PLA; 10–100 μg/ml) in media containing 0.5% serum for varying times, after which the cell-free supernatants were assayed for PGE2 by ELISA (Neogen, Lexington, KY). Briefly, 50 μl of the sample and 50 μl of horseradish peroxidase (HRP)-conjugated PGE2 were added to wells of a 96-well plate that had previously been coated with anti-PGE2 antibody for 1 h. After incubation, substrate for HRP was added to each well for 30 min, and the reaction was terminated by the addition of 50 μl/well 1 N HCl. Quantitation was achieved by measuring absorbance at 450 nm.

Statistical analysis. The responses were compared by unpaired Student’s t-test or by one-way ANOVA when multiple comparisons were made. Data are presented as means ± SE; *P < 0.05 was considered statistically significant.

RESULTS

Expression of CaR in cultured mTAL cells. Expression of CaR has been reported for TAL tissues. However, the ability of primary cultures of mTAL cells to express CaR has not been studied. Thus CaR mRNA accumulation was assessed in mTAL cells after 3 days of culture. The inner stripe of the outer medulla (OM) was used as a positive control because this region was used to prepare tubule suspensions from which primary cultures of mTAL cells were then established. RT-PCR analysis of an equal amount of total RNA
isolated from OM and primary cultures of mTAL cells revealed a 400-bp fragment predicted by primers designed to detect the presence of CaR mRNA (Fig. 1). The identity of the 400-bp fragment was confirmed by DNA sequencing analysis, which demonstrated that the sequence was identical to that reported for rat CaR (data not shown). CaR protein was detected by Western blot analysis of equal amounts of protein samples indicating that cultured mTAL cells retained the ability to express this receptor (Fig. 1).

Ca$_{2+}$ and PLA increase COX-2 protein expression. The effects of Ca$_{2+}$ or PLA (a CaR-selective agonist) on COX-2 protein expression in cultured mTAL cells were determined. Exposure of mTAL cells to increasing concentrations of Ca$_{2+}$ or PLA increased expression of COX-2 protein in a dose-dependent manner (Figs. 2 and 3). Significant increases in COX-2 protein expression were observed after a challenge for 9 h with 1.5, 1.7, and 2.0 mM CaCl$_2$; a fourfold enhancement of COX-2 protein levels was observed after a challenge with 2 mM CaCl$_2$ (Fig. 2). The enhanced COX-2 protein expression levels were maintained after a challenge with CaCl$_2$ for up to 22 h (data not shown). Previous studies have demonstrated that the EC$_{50}$ for activation of CaR by PLA is $\sim$40 nM (3). Our data indicate that at this dose, PLA increased COX-2 protein expression by twofold; additional increases were observed when cells were exposed to 100 nM PLA for 9 h (Fig. 3).

Ca$_{2+}$ and PLA increase COX-2 mRNA accumulation. RT-PCR data revealed that both Ca$_{2+}$ (1 mM) and PLA (40 nM) increased COX-2 mRNA levels in mTAL by about 40% (Figs. 4 and 5). The kinetics of the response to Ca$_{2+}$ and PLA were similar as significant increases in mRNA accumulation were observed after exposure of cells for 3 or 6 h. These data suggest that the increase in COX-2 protein expression in response to either CaCl$_2$ or PLA may be related to an increase in the transcription of the mRNA for COX-2. Moreover, the relatively smaller increases in COX-2 mRNA levels compared with COX-2 protein expression and PGE$_2$ production (see below) suggests that a posttranscriptional regulatory mechanism(s) and/or modification of COX-2 enzyme activity may be involved.

Ca$_{2+}$ increases PGE$_2$ production by mTAL cells. To confirm that Ca$_{2+}$, and not the Cl$^-$ ion, stimulates PGE$_2$ synthesis in mTAL cells, PGE$_2$ levels in supernatants were measured by ELISA after treatment with 1.7 mM CaCl$_2$ or 3.4 mM NaCl for 1, 6, and 22 h (Fig. 6). We found that CaCl$_2$ strongly enhanced PGE$_2$ production compared with NaCl (Fig. 6). The effects of Ca$_{2+}$ or PLA (a CaR-selective agonist) on COX-2 mRNA accumulation were determined by RT-PCR using primers specific for COX-2 and CaR. The primers used, sense 5-CCCTTACCTGTCCCTGAAG and antisense 5-GGCAACAAAACCTCAGTGCC-3, were designed to span intron-exon boundaries to eliminate the possibility that PCR amplification of genomic DNA would lead to false positives.

Fig. 1. Calcium-sensing receptor (CaR) expression in the medullary thick ascending limb (mTAL). The presence of mRNA (A) and protein (B) for CaR was determined using either total RNA or tissue lysates, respectively, from mTAL cells cultured for 3 days (mTAL), outer medullary tissue (OM), or heart. +, with RT-PCR; −, PCR without RT; Control, RT-PCR of a 500-bp RNA (a positive control for RT-PCR). The primers used, sense 5-CCCTTACCTGTCCCTGAAG and antisense 5-GGCAACAAAACCTCAGTGCC, were designed to span intron-exon boundaries to eliminate the possibility that PCR amplification of genomic DNA would lead to false positives.

Fig. 2. Extracellular calcium (Ca$_{2+}$) increased mTAL cyclooxygenase-2 (COX-2) protein expression. Primary cultures of mTAL were equilibrated in RPMI-1640 containing 0.42 mM Ca$_{2+}$ and 0.5% FCS for 18 h then exposed for 9 h to CaCl$_2$. Control indicates that cells were incubated in media containing 0.42 mM Ca$_{2+}$; this amount should be added to the amounts used to challenge the cells to obtain the total Ca$_{2+}$ concentration present. Top: cell lysates (30 μg) were separated on a 10% SDS-PAGE gel, and COX-2 protein expression was assessed by Western blot analysis. Molecular mass of COX-2 (72/74 kDa) is indicated. Bottom: phosphorimaging and analysis with Imagequant software were used to determine relative intensities of bands. COX-2 level in control is assigned a value of 1. *P < 0.05 (n = 4).

Fig. 3. Poly-l-arginine (PLA) increased mTAL COX-2 protein expression. Primary cultures of mTAL were exposed for 9 h to PLA. Top: cell lysates were separated on a 10% SDS-PAGE gel, and COX-2 protein expression was assessed by Western blot analysis. Molecular mass of COX-2 (72/74 kDa) is indicated. Bottom: phosphorimaging and analysis with Imagequant software were used to determine relative intensities of bands. COX-2 level in control is assigned a value of 1. *P < 0.05 (n = 4).
duction in primary cultured mTAL cells. In contrast, PGE$_2$ levels were similar to those observed for unstimulated cells after exposure to NaCl (with the same Cl$^-$ concentration as the CaCl$_2$ treatment) (Fig. 6). These data demonstrate that the enhanced PGE$_2$ production by CaCl$_2$ is caused by the Ca$^{2+}$ ion but not by Cl$^-$. 

Effects of COX-2-selective inhibitors on Ca$^{2+}$- and PLA-mediated PGE$_2$ production. NS-398 and nimesulide are selective inhibitors of COX-2. The IC$_{50}$ of NS-398 is 1 μM for COX-2, and COX-1 enzyme activity is not affected at concentrations up to 100 μM (11). Thus these inhibitors were used to determine if the PGE$_2$ produced by mTAL cells in response to Ca$^{2+}$ was derived from COX-2. Primary cultured mTAL cells were preincubated in the absence or presence of 1 μM NS-398 or nimesulide for 15 min then challenged with 1 mM Ca$^{2+}$ for 9 h. Both NS-398 and nimesulide inhibited PGE$_2$ production after a challenge with 1 mM Ca$^{2+}$, suggesting that COX-2 contributed significantly to PGE$_2$ production in response to Ca$^{2+}$ (Fig. 7A). Cells were treated with or without COX-2 inhibitors for 15 min, and then challenged with PLA (10–100 nM) for 9 h. PGE$_2$ production increased in a dose-dependent manner after exposure to PLA. Both COX-2 inhibitors blocked the increase in PGE$_2$ production after a challenge with PLA (Fig. 7B). These data indicate that the CaR-selective agonist PLA exerts effects on PGE$_2$ production that are similar to those observed with Ca$^{2+}$.

Overexpression of CaR in mTAL cells. We recently showed that addition of Ca$^{2+}$ increased intracellular calcium (Ca$^{2+}$) in cultured mTAL cells (34). Because this response is a feature of CaR activation, and because cultured mTAL cells express this receptor, gene transfection studies were performed to determine if the effects of Ca$^{2+}$ could be enhanced by overexpression of CaR. Cells were transfected with plasmids expressing CaR (3 μg/ml) or the corresponding empty plasmid vector without the CaR gene. Expression of CaR protein was increased in cells transfected with CaR overexpression vector compared with mTAL cells transfected with an empty vector (Fig. 8, left). Transfected cells were quiesced, treated with 1.2 mM CaCl$_2$ for 9 h, and COX-2 expression and PGE$_2$ synthesis were determined. Cells transfected with empty vector or the CaR expressing vector produced similar amounts of PGE$_2$ in the absence of Ca$^{2+}$ (Fig. 8). Moreover, the levels of PGE$_2$ in these cells were similar to those observed in untransfected cells (data not shown). However, COX-2
expression and PGE₂ synthesis were significantly greater in cells transfected with CaR overexpression vector compared with cells transfected with empty vector after being challenged with Ca²⁺ (Fig. 8). These data suggest that the enhanced COX-2-derived PGE₂ production in response to calcium may be mediated via activation of CaR expressed on mTAL cells.

**DISCUSSION**

We demonstrated that mTAL cells in primary culture express CaR. COX-2 mRNA accumulation and protein expression were enhanced after being challenged with Ca²⁺ or the CaR-agonist, PLA. Production of PGE₂ increased in response to either Ca²⁺ or PLA and was dependent on activation of COX-2, as the COX-2-selective inhibitors NS-398 or nimesulide completely blocked the response. Overexpression of CaR in mTAL cells resulted in greater COX-2 expression and PGE₂ production in response to Ca²⁺, compared with cells transfected with empty vector.

The COX-1 and COX-2 proteins are encoded by separate genes located on different chromosomes. Multiple signaling pathways have been linked to stimulation of COX-2 gene expression including the protein kinase A pathway, the PKC pathway, viral transformation, and tyrosine kinase (28). We previously demonstrated that PMA and tumor necrosis factor, direct and indirect activators of PKC activity, respectively, enhance COX-2 protein expression and PGE₂ production in cul-

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**Fig. 7.** COX-2 inhibitors block Ca²⁺- or PLA-dependent PGE₂ production. mTAL cells were pretreated with 1 μM NS-398 or nimesulide for 15 min and then treated with 1 mM CaCl₂ (A) or 10–100 nM PLA (B) for 9 h. *P < 0.05.

**Fig. 8.** Effects of CaR overexpression in mTAL. Cells were transfected with 3 μg/ml of either pcDNA3 control or pcDNA3-CaR plasmid vector and then incubated for 9 h in the absence or presence of 1.2 mM CaCl₂. Expression of CaR and COX-2 were determined by Western blot analysis; PGE₂ levels were determined by ELISA (*n = 3*).
CA2+ has been shown to increase COX-2 gene expression in the mTAL via CaR activation, which involves activation of PKC in some cell types.

The free calcium concentration ranges from 1.0 to 1.2 mM in serum and is tightly regulated by several mechanisms including the CaR (1, 14). Mutations in this receptor have been shown to cause disorders of calcium homeostasis such as familial hypocalciuric hypercalcaemia. Heterozygous and homozygous CaR knockout mice exhibit mild and severe alterations in Ca2+ homeostasis, respectively, confirming the importance of CaR in Ca2+ homeostasis (14). COX-2 mRNA accumulation, protein expression, and PGE2 production in mTAL cells were stimulated after increasing the Ca2+ concentration from 0.45 to 1.45 mM. NS-398 and nimesulide, at concentrations that do not inhibit COX-1 enzyme activity, abolished the increased PGE2 production, suggesting that PGE2 was derived from COX-2. The CaR agonist, PLA (40–100 nM), was similarly affected by NS-398 and nimesulide. Moreover, mTAL cells transfected with a CaR overexpression vector expressed higher levels of COX-2 and produced significantly more PGE2 in response to 1.2 mM CaCl2 compared with cells transfected with a plasmid vector without CaR. These data suggest that small changes in the concentration of Ca2+ increase COX-2-derived PGE2 production via CaR activation.

Ca2+ has been reported to inhibit K+ recycling in TAL (35) and to disrupt both NaCl and divalent cation reabsorption by the TAL (27). Thus modulation of TAL NaCl and divalent cation reabsorption by CaR activation provides a mechanism to regulate both monovalent and divalent mineral ion homeostasis (1). For instance, raising the serum ionized Ca2+ level by 25% increased the urinary excretion of Na+ by 150% (8, 23). CaR modulation of TAL function also may be linked to alterations in the urinary concentrating capacity of the kidney via alterations in medullary tonicity. Thus hypercalcemia patients have diminished urinary concentrating ability. Hypercalcemia stimulated the expression of intrarenal phospholipase A2 and COX-2 in rats (18), and endogenous PGE2 mediated the inhibition of rat TAL Cl− reabsorption in chronic hypercalcemia (22). Thus the extracellular calcium concentration gradient along the loop of Henle (30) may provide the necessary concentration range to alter local COX-2 protein expression and PGE2 production in the mTAL.

Renal cortical COX-2 mRNA levels decreased 2.9-fold in rats on a high-salt diet and increased 3.3-fold in rats on a low-salt diet (13). In contrast, medullary COX-2 level was increased in rats on a high-salt diet (36). Divergent regulation of COX-2 in cortex and medulla by dietary salt suggests that prostaglandins in different kidney regions serve different functions, with medullary production playing a role in promoting the excretion of salt and water in volume overload, whereas cortical prostaglandins may protect glomerular circulation in volume depletion (13, 36). Recent studies have shown that adrenalectomy (ADX) caused higher COX-2 protein expression in rat TAL segments (32, 37). Previous studies showed that ADX caused inhibition of sodium reabsorption by 33% in the loop of Henle, an effect mediated by PGE2 in the mTAL and reversed by aldosterone (6, 15, 29, 37). Thus aldosterone and other steroid hormones produced by the adrenal gland may be one group of physiological factors that inhibit COX-2 protein expression in the mTAL in vivo to promote salt reabsorption. Immunohistochemical data showed that the mTAL appears to express COX-2 protein constitutively in a subpopulation of cells (33). Given the effects of PGE2 on mTAL ion transport (15, 16), Ca2+ may contribute to salt and water regulation in the kidney via regulation of COX-2 protein expression and PGE2 production in the mTAL. However, in vivo COX-2 protein expression may be low in mTAL due to the inhibitory effects of adrenal steroid hormones. Under conditions of experimentally induced hypercalcemia, COX-2 protein expression was high in OM tissue (18), perhaps reflecting the ability of Ca2+ along the TAL to overcome the inhibitory effects of adrenal hormones and other factors.

Ca2+-dependent COX-2-derived PGE2 production could contribute to the polyuria observed in hypercalcemia patients. Also, recent meta-analysis of randomized controlled trials revealed that consumption of more calcium caused a small but consistent drop in blood pressure (12, 19). The effect of calcium has been suggested to reside in promoting sodium excretion. Thus calcimimetics may offer a novel means of modulating salt and water reabsorption in conditions associated with volume expansion via enhancing local PGE2 production in mTAL by CaR activation. The use of COX-2-selective inhibitors may be associated with salt and water retention, and severe renal problems are observed in COX-2 knockout mice (7, 21). Establishing a link between CaR and COX-2 gene expression may help clarify the mechanism(s) that regulate local COX-2 gene expression and PGE2 production in the mTAL.
REFERENCES


