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Hypertonic sodium choloride and mannitol induces COX-2 via different signaling pathways in mouse cortical collecting duct M-1 cells

WonChung Lim [a], JinSup Jung [b], YoungJoon Surh [c], Hiroyasu Inoue [d], YoungJoo Lee [a,*]

[a] College of Engineering, Institute of Biotechnology, Department of Bioscience and Biotechnology, Sejong University, Seoul, South Korea
[b] Department of Physiology, College of Medicine, Pusan National University, Busan, South Korea
[c] National Research Laboratory of Molecular Carcinogenesis and Chemoprevention, College of Pharmacy, Seoul National University, Seoul, South Korea
[d] Department of Food Science and Nutrition, Faculty of Human Life and Environment, Nara Women's University, Kita-Uoya-Nishimachi, Nara 630-8506, Japan

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Abstract

The kidney cortical collecting duct is an important site for the maintenance of sodium balance. Previous studies have shown that, in renal medullary cells, hypertonic stress induces expression of cyclooxygenase-2 (COX-2) via NF-κB activation, but little is known about COX-2 expression in response to hypertonicity in the cortical collecting duct. Therefore, we examined the mechanism of hypertonic induction of COX-2 in M-1 cells derived from mouse cortical collecting duct. Induction of COX-2 protein was detected within 6 h of treatment with hypertonic sodium chloride. The treatment also increased COX-2 mRNA accumulation in a cycloheximide-independent manner, suggesting that ongoing protein synthesis is not required for COX-2 induction. Using reporter plasmids containing 0.2-, 0.3-, and 1.5-kb fragments of the COX-2 promoter, we found that hypertonic induction of COX-2 was due to an increase in promoter activity. The COX-2-inductive effect of hypertonicity was inhibited by SB203580, indicating that the effect is mediated by p38 MAPK. Since p38 MAPK can activate NF-κB, we made point mutations in the NF-κB binding site within the COX-2 promoter. The mutations did not block the induction of COX-2 promoter activity by hypertonic sodium chloride, and hypertonic sodium chloride failed to activate NF-κB binding site-driven reporter gene constructs. In contrast, hypertonic mannitol activated NF-κB, indicating that hypertonic mannitol and hypertonic sodium chloride activate COX-2 by different mechanisms. Thus, induction of COX-2 expression in M-1 cells by hypertonic sodium chloride does not involve activation of NF-κB. Furthermore, the signal transduction pathways that respond to hypertonic stress vary for different osmolytes in cortical collecting duct cells.

Keywords: Kidney cortical collecting duct; Hypertonicity; Cyclooxygenase-2; NF-κB

Introduction

The kidney collecting duct is exposed to a wide range of extracellular osmolarity *in vivo*, and thus requires regulatory mechanisms that maintain cell volume and epithelial transport (Sun and Herbert, 1989; Natke, 1990). Prostaglandin E_2 (PGE_2), an important regulator of salt and water transport along the nephron (Breyer, 1998), is generated by cyclooxygenase (COX)-1 or COX-2. These COX enzymes are derived from different genes and differ significantly in their regulation (Smith et al., 1996). COX-1 is constitutively expressed in most cell types, where it plays a housekeeping role. COX-2 has been termed the inducible COX because it is rapidly upregulated in response to a variety of stimuli, including growth factors, cytokines, and phorbol esters (Jones et al., 1993; Kujubu et al., 1993; Dewitt and Meade, 1993; Evett et al., 1993). COX-2 activity and expression are required for osmolyte accumulation and adaptation of renal medullary interstitial cells to hypertonic stress (Moeckel et al., 2003; Hao et al., 2000). Recent studies have shown that COX-2 regulation in the kidney by salt is dependent on NF-κB (Hao et al., 2000) and occurs through transactivation of the EGF receptor (Zhao et al., 2003). In addition, the C/EBPβ pathway plays a dominant role in hypertonicity-induced expression of COX-2 via NF-κB (Chen et al., 2005). In studies using cultured cortical cells from the

* Corresponding author. Tel.: +82 2 3408 3640; fax: +82 2 3408 3334.
E-mail address: yjlee@sejong.ac.kr (Y. Lee).

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thick ascending limb of Henle, low salt also induced COX-2, via a p38- and NF-κB-dependent signaling pathway (Cheng et al., 2000; Cheng and Harris, 2002).

In the renal medulla osmolality may exceed 1 Osm/kg H_2O and cortical cells are also suggested to be under possible volume regulation (Sansom et al., 1990; Strange, 1991). Thus cells in kidney could be faced with extreme hypertonicity and would need to be adapt to high osmolality. M-1 cells, which are morphologically and functionally similar to collecting duct principal cells *in vivo*, express COX-1 and COX-2 enzymes that function in the production of PGE_2 (Korbmacher et al., 1993; Ferguson et al., 1999). Hypertonic sodium chloride is known to activate nonselective cation channels in M-1 cells (Volk et al., 1995), but the effects of hypertonic stress on COX-2 expression in M-1 cells, and the mechanism involved, are unknown. The aims of this study were to assess the COX-2 activation response to hypertonicity in cortical collecting duct cells of mouse kidney and to investigate the possible signaling pathway(s) involved, with special emphasis on the role of NF-κB.

Materials and methods

Material

Cycloheximide, SB203580, PD98059, U0126, urea, NaCl, mannitol, and Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), Trizol Reagent, and penicillin/streptomycin were purchased from GIBCO Invitrogen (Grand Island, NY, USA).

Cell culture, transfection, and luciferase assays

M-1 mouse cortical collecting duct cells were grown in DMEM/Ham's F12 nutrient mixture containing 10% FBS and penicillin/streptomycin in a humidified incubator at 37 °C. Plasmid COX-2-Luc, which contains the firefly luciferase reporter gene under control of the human COX-2 gene promoter (3.4 kb) was kindly provided by Dr. Huifang Cheng (Vanderbilt University School of Medicine, Tennessee, USA) (Cheng and Harris, 2002). Plasmid 3× (NF-κB)tk-Luc, which contains three copies of an NF-κB-responsive element and the firefly luciferase reporter gene, was kindly provided by Dr. Sam Okret (Karolinska University Hospital Huddinge, Huddinge, Sweden) (Bladh et al., 2005). Additional luciferase reporter plasmids containing various segments of the upstream COX-2 regulatory region (−1432/+59, −327/+59, −220/+59, −124/+59, and −52/+59) and mutant −327/+59 constructs KBM and CRM/ILM were kindly provided by Dr. Hiroyasu Inoue (Nara Women's University, Nara, Japan). KBM contains a mutated NF-κB binding site, and CRM/ILM contains mutated CRE binding protein (CREB) and NF-IL6 binding sites (Inoue et al., 1995).

M-1 cells were transiently transfected with COX-2-Luc, −1432/+59, −327/+59, −220/+59, −124/+59, −52/+59, KBM, CRM/ILM or 3× (−327/+59)tk-Luc by electroporation using a Gene Pulser II (Bio-Rad, Hercules, CA, USA). Cells were trypsinized, washed in cold PBS, and resuspended in PBS. A 400-μl aliquot of the cell suspension was then mixed with 20 μg of plasmid DNA, incubated for 5 min at room temperature, and pulsed at 1000 μF and 330 V. After 10 min at 37 °C, the suspension was diluted in medium and cultured for 24 h. M-1 cells were treated after being cultured for 24 h. The medium was removed, replaced with fresh medium, and high-salt medium (100 mM NaCl; 200 mOsm/kg H_2O) was added, resulting in a final osmolarity of 500 mOsm/kg H_2O. In some cases, cells were pretreated with specific inhibitors for 30 min before the high-salt treatment. The cells were then cultured for 24 h, harvested, and lysed with Luciferase Assay Lysis Buffer (Promega). The cell extract was mixed with luciferase assay reagent and analyzed using a Lumat LB 9507 luminometer (EG & G Berthold, Bad Widbad, Germany). Transfection experiments were performed in triplicate. The data presented are expressed as fold induction (observed experimental relative luciferase units (RLUs)/basal RLUs in absence of any stimulus).

Reverse transcription (RT)-PCR

Total RNA was extracted from cells using Trizol Reagent according to the manufacturer's instructions. RNA pellets were dissolved in diethylpyrocarbonate-treated water, and the yield of RNA was quantified by spectroscopy at 260 nm. Samples were aliquoted and stored at −80 °C until further processing. To synthesize first-strand cDNA, 3 μg of total RNA were incubated at 70 °C for 5 min with 0.5 μg of random hexamer and deionized water (up to 11 μl). The reverse transcription reaction was performed using 40 U of M-MLV reverse transcriptase (Promega, Madison, WI, USA) in 5× reaction buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT) containing 1 U RNase/μl and 1 mM mixed dNTPs at 37 °C for 60 min. Samples were heated at 70 °C for 10 min to terminate the reaction and then cooled at 4 °C.

The resulting cDNA was added to PCR reaction mixtures containing 10× PCR buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 25 U of rTaq polymerase (TakaRa, Shiga, Japan), 4 μl of 2.5 mM mixed dNTPs, and 10 pmol of each primer. The final volume was 50 μl. Samples were denatured at 94 °C for 5 min and then amplified using 23 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s on a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). Amplification of the β-actin gene was performed for 20 cycles and followed by a 5-min incubation at 72 °C. The primers used were (shown 5′ → 3′): COX-2 sense primer, ACACTCTATCACTGGCATCC; COX-2 antisense primer, GAAGGGACACCCTTTCACAT; β-actin sense primer, CCTGACCCTGAAGTACCCCA, β-actin antisense primer, CGTCATGCAGCTCATAGCTC; α-ENaC sense primer, TACGCGACAACAATCCCCAAGT; α-ENaC antisense primer ATGGAGGACATCCAGAGATTGGAG. The expected sizes of the COX-2, β-actin, and α-ENaC amplicons are 500, 550, 300, and 369 bp, respectively.

Western blot analysis

Cultured, treated M-1 cells were harvested, incubated in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 1%