Analysis of Angiotensin II Mediated COX-2 Downregulation in Angiotensin II- or Aldosterone-Infused Hypertensive Rat

Won Chung LIM,^a Jeong Bae PARK,^b and Young Joo LEE^{*,a}

^a College of Bioscience, Institute of Biotechnology, Department of Bioscience and Biotechnology, Sejong University; Kwang-Jin-Gu, Seoul, 143–747 Korea; and ^b Division of Cardiology Medicine, Samsung Cheil Hospital, Sungkyunkwan University, School of Medicine; Seoul, 100–380 Korea. Received March 7, 2003; accepted May 9, 2003

The renin-angiotensin cascade plays an important role in blood pressure control and sodium homeostasis. This study investigated whether cyclooxygenase-2 expression is regulated in the kidney, in an angiotensin II- and aldosterone-induced hypertension model. For this purpose, we treated male Sprague-Dawley rats ($n=8$ per group) with angiotensin II (9 mg/h, subcutaneously) for 14 d and aldosterone (0.75 mg/h, subcutaneously) for 42 d. Systolic blood pressure was significantly increased by angiotensin II ($p<0.001$) and by aldosterone ($p<0.001$). We found that angiotensin II downregulated cyclooxygenase-2 protein in the kidney cortex, whereas aldosterone showed no effect. These results indicate that angiotensin II may act directly to inhibit kidney cortex cyclooxygenase-2 protein expression, rather than acting *via* stimulation of aldosterone.

Key words angiotensin II; aldosterone; cyclooxygenase-2; kidney

Cardiovascular disease will be the greatest healthcare burden of the twenty-first century.¹⁾ Hypertension, a major risk-factor for development of cardiovascular disease, is an ongoing and substantial public health problem, affecting 25% of the adult population in industrialized societies.²⁾ The renin-angiotensin cascade plays an important role in blood pressure regulation and sodium homeostasis of the body.³⁾ Angiotensin II directly affects the overall renal transepithelial transport of salt and water.⁴⁾ Angiotensin II exerts its vasoconstrictor and sodium-retaining actions *via* the angiotensin type 1 (AT₁) receptor, thus directly participating in the pathogenesis of cardiovascular and renal diseases.⁵⁾ Autoradiographic ligand binding studies showed the expression of AT₁ receptors at glomeruli, proximal convoluted tubules, and the inner stripe of the outer medulla of the kidney.⁶⁾ Among many factors affecting blood pressure variation, the kidney functions in the long-term regulation of sodium balance, blood volume, and blood pressure *via* pressure diuresis and pressure natriuresis.^{7,8)} Although the effects of angiotensin II on the physiology of the kidney have been extensively examined, the molecular mechanisms underlying the role of angiotensin II in the development of hypertension is not yet clear.

Prostaglandins regulate vascular tone, and salt and water homeostasis in the mammalian kidney; they are likewise involved in the mediation and/or modulation of hormonal action.⁹⁾ Prostaglandins have also been implicated in the mediation of increased renin production by the affected kidney in renovascular hypertension.^{10,11)} Cyclooxygenase (prostaglandin H synthase; COX) is a key regulatory enzyme in the biosynthesis of prostaglandins from arachidonic acid.¹²⁾ It is found as two distinct isoforms, COX-1 and COX-2, of which COX-2 is the inducible form.¹³⁾ Within the renal cortex, COX-2 is almost exclusively expressed in cells of the late thick ascending limb of the loop of Henle, including the macula densa cells.^{14–16)} COX-2 is thus an important candidate gene in human essential hypertension. Several studies have shown that angiotensin II inhibits COX-2 expression.^{9,17)} However, whether this is under direct control of angiotensin II or occurs *via* increased aldosterone production, stimulated by angiotensin II, is not yet fully understood.

Using angiotensin II- or aldosterone-infused rats as a model system, we investigated the effects of angiotensin II on renal cortical COX-2 expression and the role of aldosterone, which is stimulated by angiotensin II, as a mediator of angiotensin II regulation of COX-2. Our results show that angiotensin II dramatically downregulates renal cortical COX-2 protein levels, whereas aldosterone has no effect on COX-2 expression. Our findings suggest that angiotensin II inhibits renal cortex expression of COX-2 directly, rather than *via* an aldosterone-mediated mechanism.

MATERIALS AND METHODS

Animal Models The study was conducted according to the recommendations of the Animal Care and Use Committee of the Samsung Biomedical Research Institute. Male Sprague-Dawley rats weighing approximately 230 g were used in the experiments. The aldosterone-infused group and sham-infused group were uninephrectomized. Rats, under anesthesia with ketamine 50 mg/kg and xylazine 5 mg/kg given intramuscularly, received subcutaneous implants of model 2002 mini-osmotic pumps (Alza Corporation, Palo Alto, CA, U.S.A.), which infused either 9 mg/h angiotensin II (Sigma) or 0.75 mg/h d-aldosterone (Sigma), both dissolved in 0.9% saline. Untreated rats served as controls for angiotensin II-infused rats; sham-operated rats served as controls for aldosterone-infused rats. All rats were offered 1% saline to drink. Systolic blood pressure (SBP) was measured weekly by the tail-cuff method. Rats were sacrificed at the end of the experiments. The kidney and heart were carefully removed, cleaned of excess fat and adventitia, and placed in PSS composed of 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH₂PO₄, 1.17 mM MgSO₄·7H₂O, 14.9 mM NaHCO₃, 5.5 mM dextrose, 0.26 mM EDTA, and 1.6 mM CaCl₂. Left kidneys were quickly removed, cut into longitudinal halves, then macroscopically subdivided into cortex and medulla. These were immediately frozen in liquid nitrogen and stored at –80 °C until extraction of protein.

Western Blot Analysis Western blot analysis was used to examine the expression of the epithelial sodium channel alpha subunit (α ENaC), COX-2, and c-fos protein in rat kid-

* To whom correspondence should be addressed. e-mail: yjlee@sejong.ac.kr

ney cortex. Protein extracted from rat kidney was homogenized by serial passage through 18- and 22-gauge needles in radioimmune precipitation buffer (containing 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 1% SDS, with protease inhibitor cocktail (Sigma)) on ice for 1 h and then centrifuged for 20 min at 13000g. Supernatant was collected and protein concentrations were determined using the Bradford method (Bio-Rad). Fifty micrograms of kidney protein were dissolved in sample buffer and boiled for 5 min prior to loading onto a 8% acrylamide gel. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane, blocked with 5% non-fat dry milk in Tris-buffered saline (TBS), and incubated with rabbit polyclonal antibody to rat α ENaC (Affinity Bioreagents), c-fos (Santa Cruz), or COX-2 (Cayman) overnight at a 1 : 500 dilution. After washing with TBS, blots were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Zymed Laboratories, Inc.) at a 1 : 5000 dilution and visualized using enhanced chemiluminescence ECL kits (Amersham). Quantitative analysis was performed by computer-assisted densitometry.

Statistical Analysis Densitometry values were expressed as the mean $\pm$ S.D. Significant differences between means were estimated using the Student's *t*-test. Differences were considered significant at $p < 0.05$.

RESULTS

Systolic Blood Pressure, Body Weight, and Heart Weight SBP was significantly increased by both angiotensin II and aldosterone. At 2 weeks after infusion with angiotensin II, the SBP was 209 ± 21 mmHg, compared with 140 ± 8 mmHg in control rats ($p < 0.001$ versus control) (Table 2). At 2 weeks after infusion with aldosterone, the SBP was 199 ± 9 mmHg compared with 133 ± 9 mmHg in control rats ($p < 0.001$ versus control) (Table 1). Body weight was similar in all groups (Tables 1, 2). Relative heart weight (normalized for body weight) was increased in angiotensin II- and aldosterone-infused rats (Tables 1, 2). These results indicate that chronic administration of either angiotensin II or aldosterone induced cardiac hypertrophy.

Effects of Chronic Angiotensin II on α ENaC, COX-2, and c-FOS Protein Levels in the Kidney Cortex We analyzed the changes induced by angiotensin II on COX-2 protein levels in the kidney cortex of rats, using Western blot analysis. We used the left kidney for all experiments performed in this study to control for differences in expression between left and right kidneys. The level of expression of COX-2 decreased approximately 2.5-fold in the kidney cortices of rats infused with angiotensin II as compared with that of controls (Fig. 1A). As a control for the model system, we examined expression of two other proteins known to be regulated by angiotensin II: α ENaC and c-FOS. α ENaC protein levels were increased in the renal cortex when compared with levels observed in control rats, which correlates with previous results (Fig. 1A).¹⁸ In our model system, angiotensin II decreased renal cortical c-FOS protein expression about two-fold as compared with that of controls.

Effects of Chronic Aldosterone on COX-2, α ENaC, and c-FOS Protein Levels in the Kidney Cortex To investigate whether aldosterone regulates COX-2 expression in the

Table 1. Physiological Parameters from Aldosterone-Infused Hypertensive Rats

Parameter	BW (g)	SBP (mmHg)	HW/BW $\times 10^{-3}$	LV/BW $\times 10^{-3}$
Sham ($n=8$)	396 ± 11	148 ± 9	2.86 ± 0.16	2.30 ± 0.12
Aldo ($n=8$)	369 ± 16	$199 \pm 9^*$	$3.68 \pm 0.30^{**}$	$3.03 \pm 0.24^{**}$

Each value is the mean $\pm$ S.D., $n=8$. Aldo indicates aldosterone; BW, body weight; SBP, systolic blood pressure; HW, heart weight; LV, left ventricle. * $p < 0.001$ vs. sham-infused rats; ** $p < 0.01$ vs. sham-infused rats.

Table 2. Physiological Parameters from Angiotensin II-Infused Hypertensive Rats

Parameter	BW (g)	SBP (mmHg)	HW/BW $\times 10^{-3}$	LV/BW $\times 10^{-3}$
Control ($n=8$)	319 ± 16	140 ± 8	3.06 ± 0.14	2.42 ± 0.14
Ang II ($n=8$)	313 ± 14	$209 \pm 21^*$	$3.47 \pm 0.44^\dagger$	$2.75 \pm 0.39^{**}$

Each value is the mean $\pm$ S.D., $n=8$. Ang II indicates angiotensin II; BW, body weight; SBP, systolic blood pressure; HW, heart weight; LV, left ventricle. * $p < 0.001$ vs. control; ** $p < 0.01$ vs. control; $^\dagger p < 0.05$ vs. control.

Fig. 1. Western Blot Analysis of COX-2, α ENaC, and c-fos Protein in Angiotensin II (A) or Aldosterone (B) Infused Rat Renal Cortex; (C) Proposed Signaling Pathways for COX-2 Expression

Parallel Coomassie-stained gels (data not shown) demonstrated equality of loading among samples. Quantitative analysis was performed by computer-assisted densitometry. Each value is the mean $\pm$ S.D., $n=8$. Con indicates control; ALC, sham-infused rats. * Significantly different from control or ALC ($p < 0.005$).

kidney cortex, we determined levels of COX-2 protein in rat kidney cortex using Western blot analysis. Aldosterone infusion did not alter the abundance of either c-FOS or COX-2. These results indicate that the effect of aldosterone on c-FOS

and COX-2 expression is different from that of angiotensin II. As a positive control, we examined levels of α ENaC protein expression. Aldosterone is the main hormone controlling ENaC activity at the cell surface,¹⁹⁾ and several groups have reported that aldosterone regulates ENaC expression at both translational and posttranslational levels.^{20–22)} In addition, a recent study reported that aldosterone stimulation of α ENaC transcription occurs *via* mineralocorticoid receptor-responsive elements in the 5'-flanking region of the α ENaC gene.²³⁾ α ENaC protein levels were elevated significantly in kidney cortex from aldosterone-infused rats as compared with sham-infused rats (a 13-fold increase) (Fig. 1B), which is consistent with previous findings. However, as noted, aldosterone itself does not regulate levels of COX-2 expression.

DISCUSSION

In this study we examined the pathways by which angiotensin II regulates COX-2 expression. In particular, we attempted to differentiate the direct effects of angiotensin II on COX-2, independent of angiotensin II-induced aldosterone stimulation (Fig. 1C). We used angiotensin II-infused and aldosterone-infused rats as a model system for this comparison. The results demonstrate that chronic angiotensin II infusion downregulates levels of COX-2 expression in renal cortex, whereas chronic aldosterone infusion has no effect on COX-2 expression levels. Our data indicate that angiotensin II is capable of inhibiting COX-2 by a direct mechanism, rather than indirectly, by stimulation of aldosterone. AT₁ receptor knockout studies on mice showed that regulations of sodium transporters and channel proteins are independent of altered aldosterone concentrations²⁴⁾ as is proposed in this reports on COX-2 regulation. One possible explanation for our proposed mechanism is that angiotensin II may have a direct effect on COX-2 regulation in kidney cortex. In order to confirm our data, we examined two other known angiotensin II regulated genes, α ENaC and c-FOS. A previous study has shown that angiotensin II directly stimulates ENaC activity in the cortical collecting ducts *via* AT₁ receptors.¹⁸⁾ ENaC plays an important role in sodium homeostasis and blood pressure.²⁵⁾ Angiotensin II stimulates expression of immediate early response genes such as c-fos at early time points, but downregulates c-fos expression when administered chronically.²⁶⁾ C-fos heterodimerizes with proteins in the Jun family, forming the transcription factor AP-1.²⁷⁾ Some reports suggest that AP-1 is involved in mediating COX-2 expression.²⁸⁾ It has been suggested that COX inhibitors, which block the catalysis of arachidonic acid to prostaglandin, may signal through AP-1.²⁹⁾ Further studies, utilizing inhibitors of the mineralocorticoid receptor and AT₁ receptors, will clarify the signaling pathways involved in the observed downregulation of COX-2 expression by angiotensin II.

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REFERENCES

- 1) Yusuf S., Reddy S., Ounpuu S., Anand S., *Circulation*, **104**, 2746–2753 (2001).
- 2) Burt V. L., Whelton P., Roccella E. J., Brown C., Cutler J. A., Higgins M., Horan M. J., Labarthe D., *Hypertension*, **25**, 305–313 (1995).
- 3) Muller M. W., Todorov V., Kramer B. K., Kurtz A., *Pflugers Arch.*, **444**, 499–505 (2002).
- 4) Harris P. J., Hiranyachattada S., Antoine A. M., Walker L., Reilly A. M., Eitle E., *Clin. Exp. Pharmacol. Physiol.*, **3**, S112–S118 (1996).
- 5) Kim S., Iwao H., *Pharmacol. Rev.*, **52**, 11–34 (2000).
- 6) Harrison-Bernard L. M., Navar L. G., Ho M. M., Vinson G. P., el-Dahr S. S., *Am. J. Physiol.*, **273**, F170–F177 (1997).
- 7) Guyton A. C., Coleman T. G., Cowley A. W., Jr., Liard J. F., Norman R. A., Jr., Manning R. D., Jr., *Ann. Biomed. Eng.*, **1**, 254–281 (1972).
- 8) Liard J. F., Cowley A. W., Jr., McCaa R. E., McCaa C. S., Guyton A. C., *Circ. Res.*, **34**, 549–560 (1974).
- 9) Cheng H. F., Wang J. L., Zhang M. Z., Miyazaki Y., Ichikawa I., McKanna J. A., Harris R. C., *J. Clin. Invest.*, **103**, 953–961 (1999).
- 10) Jackson E. K., Oates J. A., Branch R. A., *Circ. Res.*, **49**, 180–185 (1981).
- 11) Imanishi M., Kawamura M., Akabane S., Matsushima Y., Kuramochi M., Ito K., Ohta M., Kimura J., Takamiya M., Omae T., *Hypertension*, **14**, 461–468 (1989).
- 12) Needleman P., Turk J., Jakschik B. A., Morrison A. R., Lefkowitz J. B., *Ann. Rev. Biochem.*, **55**, 69–102 (1986).
- 13) Williams C. S., Mann M., Dubois R. N., *Oncogene*, **18**, 7908–7916 (1999).
- 14) Harris R. C., McKanna J. A., Akai Y., Jacobson H. R., Dubois R. N., Breyer M. D., *J. Clin. Invest.*, **94**, 2504–2510 (1994).
- 15) Hartner A., Goppelt-Strube M., Hilgers K. F., *Hypertension*, **31**, 201–205 (1998).
- 16) Vio C. P., Cespedes C., Gallardo P., Masferrer J. L., *Hypertension*, **30**, 687–692 (1997).
- 17) Kammerl M. C., Richthammer W., Kurtz A., Kramer B. K., *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **282**, R1613–R1617 (2002).
- 18) Peti-Peterdi J., Warnock D. G., Bell P. D., *J. Am. Soc. Nephrol.*, **13**, 1131–1135 (2002).
- 19) Renard S., Voilley N., Bassilana F., Lazdunski M., Barbry P., *Pflugers Arch.*, **430**, 299–307 (1995).
- 20) Alvarez de la Rosa D., Li H., Canessa C. M., Fyfe G. K., Zhang P., *Annu. Rev. Physiol.*, **62**, 573–594 (2000).
- 21) Garty H., Palmer L. G., *Physiol. Rev.*, **77**, 359–396 (1997).
- 22) Verrey F., *Am. J. Physiol. Renal Physiol.*, **277**, F319–F327 (1999).
- 23) Verity E. M., Omar A. I., Randy W. L., Russell F. H., Thomas J. S., Christie P. T., *Mol. Endo.*, **15**, 575–588 (2001).
- 24) Brooks H. L., Allred A. J., Beutler K. T., Coffman T. M., Knepper M. A., *Hypertension*, **39**, 470–473 (2002).
- 25) Snyder P. M., Olson D. R., Thomas B. C., *J. Biol. Chem.*, **277**, 5–8 (2002).
- 26) Taubman M. B., Berk B. C., Izumo S., Tsuda T., Alexander R. W., Nadal-Ginard B., *J. Biol. Chem.*, **264**, 526–530 (1989).
- 27) Andreucci J. J., Grant D., Cox D. M., Tomc L. K., Prywes R., Goldhamer D. J., Rodrigues N., Bedard P. A., McDermott J. C., *J. Biol. Chem.*, **277**, 16426–16432 (2002).
- 28) Huang C., Ma W. Y., Hanenberger D., Cleary M. P., Bowden G. T., Dong Z., *J. Biol. Chem.*, **272**, 26325–26331 (1997).
- 29) Liu G., Ma W. Y., Bode A. M., Zhang Y., Dong Z., *J. Biol. Chem.*, **278**, 2124–2130 (2003).