Role of prostaglandins in collecting duct-derived endothelin-1 regulation of blood pressure and water excretion

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Running head: PGE2 in collecting duct ET-1 knockout

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Collecting duct (CD)-derived endothelin-1 (ET-1) exerts natriuretic, diuretic and hypotensive effects. In vitro studies have implicated cyclooxygenase metabolites, and particularly PGE2, as important mediators of CD ET-1 effects. However, it is unknown whether PGE2 mediates CD-derived ET-1 actions in vivo. To test this, CD ET-1 knockout (KO) and control mice were studied. During normal salt and water intake, urinary PGE2 excretion was unexpectedly increased in CD ET-1 KO mice as compared to controls. Salt loading markedly increased urinary PGE2 excretion in both groups of mice, however the levels remained relatively higher in knockout animals. Acutely isolated inner medullary CD (IMCD) from knockout mice also had increased PGE2 production. The increased IMCD PGE2 was COX2-dependent since NS398 blocked all PGE2 production. However, increased CD ET-1 KO COX2 protein or mRNA could not be detected in inner medulla, or IMCD, respectively. Inner medullary COX1 mRNA and protein, and IMCD COX1 mRNA, levels were unaffected by Na intake or CD ET-1 KO. Knockout mice on a normal or high Na diet had elevated blood pressure as compared to controls; this difference was not altered by indomethacin or NS398 treatment. However, indomethacin or NS398 did increase urine osmolality and reduce urine volume in knockout, but not control, animals. In summary, IMCD COX2-dependent PGE2 production is increased in CD ET-1 KO, indicating that CD-derived ET-1 is not a primary regulator of IMCD PGE2. Further, the increased PGE2 in CD ET-1 KO mice partly compensates for loss of ET-1 with respect to maintaining urinary water excretion, but not in blood pressure control.

Key words: cyclooxygenase, inner medullary, sodium
INTRODUCTION

The collecting duct (CD) is the predominant site of endothelin-1 (ET-1) production and binding in the kidney (6). Recent studies have implicated CD-derived ET-1 in the regulation of systemic blood pressure and renal Na and water excretion. CD-specific knockout of ET-1 causes impaired ability to excrete a Na load, as well as salt-sensitive hypertension (1). CD-specific knockout of the ETB receptor also results in hypertension and reduced Na excretion after an acute salt load (5). In addition, CD-specific knockout of ET-1 causes reduced ability to excrete an acute water load associated with heightened sensitivity to the cAMP-elevating effects of vasopressin (AVP) (4). Thus CD-derived ET-1 functions as a natriuretic, diuretic and hypotensive factor.

Perhaps the most extensive studies on mediation of collecting duct ET-1 actions have involved analysis of the role of PGE2. ET-1 stimulates PGE2 accumulation by acutely isolated rat IMCD, an effect that is mediated by activation of the ETB receptor (7). Similarly, ET-1 increases PGE2 levels in acutely isolated rabbit IMCD (12). ET-1 inhibition of ouabain-sensitive $^8$Rb uptake in acutely isolated rabbit IMCD was abolished by pretreatment with ibuprofen; taken together with the above observations, this suggested that ET-1 inhibition of Na/K ATPase activity in IMCD is PGE2-dependent (12). In contrast, the role of PGE2 in mediating ET-1 inhibition of collecting duct water transport is less evident. ET-1 inhibition of AVP-dependent cAMP accumulation in acutely isolated rat IMCD, in the presence of phosphodiesterase blockade, was shown to be unaffected by indomethacin pretreatment (7). Furthermore, ET-1 inhibition of AVP-stimulated osmotic water permeability (Pf) in isolated perfused rat IMCD was not affected by prior exposure to indomethacin, while PGE2 inhibition of AVP-stimulated Pf was unaffected by pretreatment with ET-1 (8). While not related to a direct collecting duct effect, PGE2 has been shown to reduce ET-1-induced vasoconstriction of medullary descending vasa recta in rat, thereby potentially increasing medullary blood flow (9). Thus, taken together, in vitro
studies suggest that PGE2 may mediate, at least in part, ET-1 inhibition of collecting duct Na transport, as well as potentially modulating ET-1 affects on neighboring cells in the medulla (e.g. vasa recta). However, there is no in vivo evidence for a role of PGE2, or other cyclooxygenase metabolites, in mediating collecting duct ET-1 actions. Consequently, the current study was undertaken, using mice with collecting duct-specific knockout of ET-1, to assess the in vivo relevance of PGE2 in mediating CD-derived ET-1 actions.
MATERIALS AND METHODS

Transgenic mouse lines. All experiments were performed with approval from the Institutional Animal Care and Use Committees at the University of Utah. CD ET-1 KO and littermate control mice were generated utilizing the Cre/loxP system, as previously described (1, 5). Briefly, mice with exon 2 of the ET-1 gene flanked by loxP-sites (floxed) (provided by Dr. Masashi Yanagisawa at the Howard Hughes Institute at University of Texas Southwestern Medical Center) were mated with AQP2-Cre mice, containing 11 kb of the mouse AQP2 gene driving expression of Cre recombinase. Female AQP2-Cre mice were mated with male floxed ET-1 mice; female offspring heterozygous for both AQP2-Cre and floxed ET-1 were bred with males homozygous for floxed ET-1. Animals homozygous for floxed ET-1 and heterozygous for AQP2-Cre (CD ET-1 KO) were used in this study. Littermates that were homozygous for the floxed ET-1 gene, but without Cre, were used as control mice. Genotyping was performed as previously described (1). All mice were studied at 3-4 months of age. All reagents used in studies were obtained from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

Chronic telemetry studies. Mice had catheters inserted into the right carotid artery, tunneled subcutaneously, and the attached radiotransmitter localized to the back. Continuous recording of BP and pulse was performed using telemetry (Data Sciences International, Arden Hills, MN). Two days after the surgery, mice were placed into Hatteras metabolic cages (Cary, NC) and acclimated for 3 days. Mice were fed 10 ml of a normal Na (0.3%) gelled diet that contained all nutrients and water as previously described (1). Hemodynamic values were not recorded during this conditioning period. After the 3-day acclimation period, BP and pulse were determined for 2 consecutive days. Daily gel intake and body weights were measured. Systolic, diastolic and mean BPs, as well as pulse rate, were averaged over the course of each day. At the end of this baseline period, mice were given vehicle alone, NS398 (15 mg/kg/day intraperitoneally (I.P.)) or indomethacin (5 mg/kg/day I.P.), while remaining on the gelled normal Na intake. After 3 days of NS398, indomethacin or vehicle treatment
on a normal Na diet, mice were placed on a high Na diet for 7 days. The high Na diet consisted of 10 ml of gelled diet containing 1% Na plus normal saline to drink. Daily weights and telemetry were obtained.

*In vivo water and solute excretion studies.* Mice were acclimated to metabolic cages and a normal Na gelled diet, then given 2 more days of a normal Na diet. Urine was collected on day 2 of the normal Na diet. Mice were then given vehicle alone, NS398 (15 mg/kg/day I.P.) or indomethacin (5 mg/kg/day I.P.) for 3 days while being maintained on the normal Na diet. Urine was collected on the 3rd day of NS398 or indomethacin treatment. Urine volume was measured and urine osmolality determined using an Osmett II (Precision Systems, Natick, MA).

*In vivo PGE2 and COX studies.* Mice were acclimated to metabolic cages and a normal Na gelled diet, then given 2 more days of a normal Na diet followed by 3 days of a high Na diet as described above. Urine during day 2 of a normal Na diet and day 3 of a high Na diet was collected and analyzed for PGE2 and volume. Urine PGE2 was determined using an enzyme immunoassay kit and the manufacturer's protocol (Cayman Chemicals, Ann Arbor MI). Both kidneys from each mouse were used for determination of inner medullary COX1, COX2 and ß-actin protein levels (see description below).

*In vitro PGE2 determination.* CD ET-1 KO and control mice were treated with 2 days of a normal Na diet, followed by 3 days of a high Na diet (diets as described above). Mice were sacrificed at the end of the normal or high Na diet periods. IMCD were acutely isolated in a manner similar to that previously described (7). Briefly, inner medulla were minced in Kreb’s buffer with 1mg/ml collagenase (Invitrogen, Carlsbad, CA ) and 0.1 mg/ml DNase, and incubated at 37°C for 30 min. IMCD fragments were washed, resuspended in HBSS, and pre-incubated in vehicle alone or 25 µM NS398 for 30 min at 37°C. Cells were gently pelleted by spinning at 3,000 rpm in a table top microfuge for 1 minute, the cell pellets gently resuspended in HBSS with vehicle alone, 25 µM NS398 or 10 µM indomethacin and placed back in a 37°C water bath for an additional 30 minutes. Cells were pelleted at 6,000 rpm for 1 min, aliquots of
the supernatant removed, and PGE2 measured as above. The cell pellet was used for
determination of protein by the Bradford assay (BioRad, Hercules, CA). PGE2 levels were
normalized to total cell protein.

Western analysis. Renal inner medulla from normal or high Na diet CD ET-1 KO and
control mice were homogenized in 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1%
Nonidet P-40, 0.5% sodium deoxycholate and protease inhibitor cocktail tablets (Catalog No.
11697498001, Roche Diagnostics, Indianapolis, IN). Samples were centrifuged at 4,000g and
the supernatant was centrifuged at 17,000g for 30 min. Protein concentration was determined
using Coomassie reagent. Samples were solubilized in Laemmli buffer containing 0.5% lithium
dodecyl sulfate and run on a denaturing NUPAGE 4-12% Bis-Tris mini-gel using the MOPS
buffer system (Invitrogen). Proteins were transferred to PVDF plus nylon membranes by
electroelution. Blots were incubated overnight at 4°C with primary antibody, then incubated with
1:2000 donkey anti-rabbit horseradish peroxidase-conjugated donkey anti-rabbit IgG for 1 hr
(GE Healthcare, Piscataway, NJ). Antibody binding was visualized using the enhanced
chemiluminescence system (Amersham International, UK). Primary antibodies utilized were
1:5000 rabbit anti-mouse COX1, 1:5000 rabbit anti-mouse COX2 (Catalog Nos. 160109 and
1601269, respectively, both from Cayman Chemicals), and 1:10,000 rabbit anti-human ß-actin
(Abcam, Cambridge, MA). All blots were reprobed for ß-actin.

Real-time PCR. IMCD from CD ET-1 KO and control mice were isolated as described
above. Total RNA was prepared using RNeasy minicolumns with on-column DNase I treatment
(Qiagen, Valencia, CA). Samples were reverse transcribed and real-time PCR performed using
a Smart Cycler (Cepheid, Sunnyvale, CA). Primer sequences for mouse COX-1 cDNA were COX-
1 F 5' - CAC TGG TGG ATG CCT TCT CT -3' and COX-1 R 5' - CCG TAC AGC TCC TCC AAC
TC -3', which yielded a product size of 226 bp and amplify between 1405-1631 (Gene Bank
NM_008969). Primer sequences for COX-2 cDNA were COX-2 F 5' - CCC TGA AGC CGT ACA
CAT CA -3' and COX-2 R 5' - TGT CAC TGT AGA GGG CTT TCA ATT -3', which yielded a
product size of 80 bp and amplify between 1470-1490 (Gene Bank NM_011198). The primers for GAPDH were GAPDH F 5'-TGG CCT CCA AGG AGT AAG AA-3' and GAPDH R 5'-CTG GGA TGG AAA TTG TGA GG-3', which yield a product size of 110 bp. The ratio of COX1 or COX2 to GAPDH cDNA was determined for each sample.

*Cyclic AMP studies.* CD ET-1 KO and control mice were given 2 days of a normal Na diet and then sacrificed. IMCD were prepared as described above; all incubations were done in Kreb's buffer. IMCD were pre-incubated with vehicle alone, 10 µM indomethacin or 25 µM NS398 as described above, rinsed, then incubated with 10 nM AVP with vehicle alone or with COX inhibitors at 37°C. Cells were extracted with ethanol and cAMP measured by ELISA (Assay Design, Ann Arbor, MI) using a SpectraMax Plus Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). Total cell protein was measured by the Bradford assay. Cyclic AMP levels were normalized to total cell protein.

*Statistics.* Comparisons between floxed ET-1 and CD ET-1 KO mice were analyzed by one way analysis of variance with the Bonferroni correction. p<0.05 was taken as significant. Data are expressed as mean ± SEM.
RESULTS

CD ET-1 KO and control mice were analyzed at 3-4 months of age. There were no differences between the two groups with respect to body weight or gender distribution (males = females within groups). On a normal Na diet, urinary PGE2 excretion was significantly elevated in CD ET-1 KO animals as compared to controls (Figure 1). When placed on a high Na diet for 3 days, urinary PGE2 excretion markedly increased in both groups as compared to a normal Na diet. However, while on a high Na diet, urinary PGE2 excretion remained greater in CD ET-1 KO mice as compared to controls. The 3-day high Na diet time period was based on the time at which maximal sodium retention was observed in CD ET-1 KO mice after changing to a high Na intake (1). In order to determine the mechanism by which urinary PGE2 is elevated in CD ET-1 KO mice as compared to controls, inner medullary COX1 and COX2 protein levels were determined (Table 1). COX1 protein expression was not detectably different between CD ET-1 KO and control mice maintained on a normal Na diet; further, COX1 protein expression was unchanged in either group by 3 days of a high Na diet. COX2 protein expression was also not different between CD ET-1 KO and control mice on a normal Na diet; 3 days of high Na intake increased COX2 protein levels, but these were not different between CD ET-1 KO and control animals. Since failure to detect a difference in inner medullary COX protein expression could relate to the presence of non-collecting duct cells, acutely isolated IMCD were studied. Despite combining inner medulla from 5 mice, COX1 and COX2 protein could not be detected in isolated IMCD. Consequently, COX1 and COX 2 mRNA steady-state levels were determined in acutely isolated IMCD (Table 1). No differences in IMCD COX1 mRNA levels were detected between CD ET-1 KO and control mice on a normal or high Na diet; similarly, IMCD COX2 mRNA levels were not different between CD ET-1 KO and control mice on either Na intake. Since mRNA levels do not necessarily reflect protein expression or enzyme activity, production of PGE2 by acutely isolated IMCD, and the effect of specific COX isoform inhibition, was assessed. PGE2
production was significantly increased in IMCD from CD ET-1 KO mice as compared to controls when both groups had been maintained on a normal Na diet (Figure 2). PGE2 production was also higher in IMCD from CD ET-1 KO as compared to control mice after both groups had been given 3 days of high Na intake (Figure 2). Similar to the urinary PGE2 excretion data, high Na intake increased IMCD PGE2 production (as compared to a normal Na diet) in IMCD from both groups of animals. NS398, a specific COX2 inhibitor, abolished PGE2 production by IMCD from both groups to a comparable extent as that seen with indomethacin, thereby strongly supporting the notion that the elevated PGE2 production in collecting ducts from CD ET-1 KO mice is COX2-dependent.

Since PGE2 has been shown to antagonize the hydroosmotic effects of AVP, the effect of blocking cyclooxygenase activity, using either non-specific COX blockade or specific COX2 inhibition, on urine osmolality and volume were assessed (Figure 3). CD ET-1 KO and control mice had similar urine osmolality and volume on the normal Na gelled diet (fluid and solute intake precisely matched since they consume all the gel and are given no other food or water). After administration of either NS398 or indomethacin, urine volume fell, and urine osmolality increased in CD ET-1 KO mice. However, neither NS398 nor indomethacin altered urine volume or osmolality in control animals. To determine if the cyclooxygenase inhibitor-impaired water excretion in CD ET-1 KO mice, as compared to controls, was due to altered AVP-induced cAMP accumulation, acutely isolated IMCD were studied from animals on a normal Na diet. AVP increased IMCD cAMP accumulation to a greater extent in CD ET-1 KO IMCD as compared to control mice (Figure 4). NS398 or indomethacin, which both abolished all PGE2 production (see above), markedly increased cAMP levels in IMCD from both groups of mice, and to a comparable extent. However, AVP responsiveness remained greater in CD ET-1 KO mice as compared to controls. Notably, these studies were done in the absence of phosphodiesterase inhibition, thereby allowing any potential alteration in cAMP formation or degradation to be detected.
Since PGE2 can exert natriuretic and antihypertensive effects, the effect of blocking cyclooxygenase activity, using either non-specific COX blockade on COX2-specific inhibition, on systemic blood pressure was determined (Figure 5). Animals were maintained on the gelled diets to assure comparable intakes. On a normal Na diet, systolic BP was about 13 mm Hg greater in CD ET-1 KO as compared to control mice. Indomethacin increased systolic BP in the two groups of mice and to a comparable degree. Similarly, NS398 increased systolic BP in both groups of mice by comparable amounts. Administration of a high Na diet increased BP in CD ET-1 KO mice, but not in control mice, as has been previously reported (1).
DISCUSSION

Based on the finding that ET-1 increases collecting duct PGE2 production, and that PGE2 can exert natriuretic and diuretic effects, it was expected that CD-specific knockout of ET-1 would result in relative collecting duct PGE2 deficiency, and that such deficiency would explain, at least in part, the hypertension and impaired water excretion in CD ET-1 KO animals. However, this proved to not be the case. Urinary PGE2 excretion and IMCD PGE2 production were unexpectedly increased in CD ET-1 KO mice. This indicates that CD-derived ET-1 is not the primary factor driving collecting duct PGE2 production. In fact, the absence of CD-derived ET-1 apparently results in a compensatory increase in collecting duct PGE2 production. Further, this compensation seems to be mainly in response to ET-1 deficiency-induced impaired water excretion, but not to CD-derived ET-1-dependent blood pressure control.

Although changes in COX1 and COX2 protein and mRNA expression in CD ET-1 KO mice could not be detected, the current study suggests that the apparent compensatory elevation in IMCD PGE2 production in CD ET-1 KO mice is COX2-dependent. This conclusion is supported by the findings that NS398 causes similar increases in IMCD PGE2 and AVP-induced cAMP accumulation as compared to indomethacin, while NS398 reduced water excretion to a comparable extent as indomethacin (see discussion below). The biologic significance of COX2 in the collecting duct is uncertain. COX2 mRNA has been localized to mouse IMCD, although COX1 mRNA levels were relatively greater (11). In addition, acutely isolated rat IMCD PGE2 production was more extensively inhibited by COX2 than COX1 blockade (10). COX2 regulation of collecting duct water transport has not been extensively investigated. In one study, bilateral ureteral obstruction increased COX2, and decreased AQP2, protein expression in rat inner medulla; administration of NS398 ameliorated the fall in AQP2 levels (3). Taken together, these studies support the notion that collecting duct COX-2 may regulate water transport, and elevated COX2-derived PGE2 may help counterbalance the
antidiuretic effect associated with ET-1 deficiency in the collecting duct. These studies do not prove that increased COX2 per se is responsible for the compensatory elevation in PGE2 seen in CD ET-1 KO mice (changes in PGE2 synthase or prostaglandin receptors could be involved); rather, these studies show that the increase in PGE2 is through pathways that depend upon COX2.

The mechanisms by which increased collecting duct PGE2 might ameliorate the relative anti-diuretic effect of CD ET-1 KO are speculative. Our group has previously reported that AVP-induced cAMP accumulation, in the presence of phosphodiesterase inhibition, is augmented in IMCD from CD ET-1 KO animals (4). The current study demonstrated that such increased AVP responsiveness is not abolished by complete blockade of PGE2 production. Notably, blockade of PGE2 production markedly increased cAMP levels, confirming that PGE2 can reduce cAMP content in IMCD. Further, these studies were done, for the first time, in the absence of phosphodiesterase inhibition in order to help exclude a potential effect on cAMP phosphodiesterase activity. Taken together, the current data suggest that the elevated IMCD PGE2 content in CD ET-1 KO mice likely impacts AVP-dependent water reabsorption at a point beyond regulation of cAMP levels. Such a finding is consistent with previous studies which have demonstrated that PGE2 may reduce cell surface AQP2 expression by stimulating retrieval from the plasma membrane (13).

PGE2, particularly in the renal medulla, has been shown to regulate BP; medullary infusions of COX1 or COX2 inhibitors in rats increase systemic BP (11). It is interesting, therefore, that inhibition of PGE2 formation, whether non-specifically with indomethacin or through COX2 inhibition, while raising BP in both groups of mice, did not result in a greater elevation in BP in CD ET-1 KO mice as compared to controls. The reasons for this are speculative, however one possibility is that the regulation of BP is more dependent upon medullary interstitial cell, as opposed to collecting duct, PGE2 production. While not possible to examine, it is conceivable that the increase in PGE2 levels seen in CD ET-1 KO mice reflect
collecting duct, but not interstitial cell, PGE2 production. Interstitial cells produce and express relatively large amounts of PGE2 and COX isoenzymes, respectively (2), and have been implicated in control of systemic BP (reviewed in (14)). Clearly, this is an area in need of further investigation.

In summary, while ET-1 can stimulate IMCD PGE2 production, the current study demonstrates that CD-derived ET-1 is not the primary determinant of collecting duct PGE2 synthesis. Further, elevated COX-2 dependent collecting duct PGE2 production apparently partially compensates for the antidiuretic effect of CD ET-1 KO. This apparent compensation is likely mediated by a PGE2 effect distal to AVP-stimulated cAMP accumulation. Thus, the diuretic, natriuretic and hypotensive effects of CD-derived ET-1 are mediated by other pathways.
ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

**Figure 1.** Urinary PGE2 excretion in control and CD ET-1 KO mice. Values are shown after 2 days on a normal Na diet and after 3 days on a high Na diet. N=6 each data point. *p<0.025 vs. control animals on a similar Na diet; #p<0.001 vs. same genotype on a normal Na diet.

**Figure 2.** PGE2 production by IMCD acutely isolated from control and CD ET-1 KO mice previously treated with 2 days of a normal Na diet or 3 days of a high Na diet. Cells were treated for 30 minutes with vehicle alone, a COX2-specific inhibitor (NS398, 25 µM) or 10 µM indomethacin, followed by measurement of PGE2 release over the next 30 minutes. N=12 each data point. *p<0.025 vs. control animals on a similar Na diet; #p<0.001 vs. same genotype on a normal Na diet; $p<0.001 vs. vehicle alone in same genotype and on same Na diet.

**Figure 3.** Urine osmolality (Panels A and B) and volume (Panels C and D) in control and CD ET-1 KO mice on a normal Na diet and treated for 3 days with vehicle alone, indomethacin (5 mg/kg/day I.P.) (Panels A and C) or NS398 (10 mg/kg/day I.P.) (Panels B and D). N=6 each data point. *p<0.01 vs. control treated with same COX inhibitor and vs. CD ET-1 KO treated with vehicle alone. **p<0.025 vs. control treated with same COX inhibitor and vs. CD ET-1 KO treated with vehicle alone.

**Figure 4.** AVP-stimulated cAMP accumulation in acutely isolated IMCD from control and CD ET-1 KO mice. Cells were treated for 30 minutes with vehicle alone, a COX2-specific inhibitor (NS398, 25 µM) or 10 µM indomethacin, followed by addition of 10 nM AVP for 10 minutes. N=6 each data point. *p<0.05 vs. control animals treated with same agents; #p<0.001 vs. same genotype treated with AVP and vehicle alone.
Figure 5. Systolic BP in control and CD ET-1 KO mice. Animals were initially given (days 1-2) a normal Na diet. Indomethacin (5 mg/kg/day I.P.) (Panel A) or NS398 (15 mg/kg/day I.P.) (Panel B) were started on day 3 and continued after animals were changed to a high Na diet on day 6. N=12 each data point. *p<0.001 vs. control animals on same day; **p<0.01 vs. control animals on same day, #p<0.05 vs. same COX inhibitor and normal Na diet in same genotype.
Table 1. Western and real-time PCR analysis of COX1 and COX2 protein and mRNA expression in inner medulla from control and CD ET-1 KO mice. Data are normalized to β-actin (Western) or to GAPDH (PCR) and are expressed in relative densitometry (Western) or relative fluorescence (PCR) units. N=6 each data point. *p<0.01 vs. COX2 values in mice with same genotype on a normal Na diet.

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