Calcium regulation of endothelin-1 synthesis in rat inner medullary collecting duct

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Calcium regulation of endothelin-1 synthesis in rat inner medullary collecting duct. Am J Physiol Renal Physiol 293: F601–F606, 2007. — ET-1 is an important regulator of systemic blood pressure and is found in the collecting ducts (IMCD) and in other renal nephron cells (17). Mice with collecting duct-specific disruption of the ET-1 promoter that confer unique calcium responsiveness. This suggests that IMCD-specific enhancer elements exist within the ET-1 promoter that confer unique calcium responsiveness.

Numerous studies showed that renal medullary and urinary ET-1 production is increased by salt or water loading, suggesting that collecting duct-derived ET-1 is stimulated in response to expanded extracellular fluid volume or some related process (reviewed in Ref. 10). However, the factors regulating collecting duct ET-1 production are not well understood. Circulating hormones that modify renal Na and/or water reabsorption are potential candidates for regulating collecting duct ET-1 synthesis. However, none of these hormones has been shown to mediate an increased ET-1 under volume-expanded states. Atrial natriuretic peptide, which is elevated during volume expansion, either modestly decreased (12) or did not change (22) ET-1 release by inner medullary collecting duct (IMCD). Hormones which are decreased during volume expansion could potentially exert a tonic inhibitory effect on IMCD ET-1 production, but current data do not support this. ANG II infusion does not alter urinary ET-1 excretion (8). Vasopressin does not change ET-1 release from porcine or rat IMCD (11, 22). Aldosterone, which would be expected to exert an inhibitory effect on IMCD ET-1 production, rapidly induced ET-1 mRNA accumulation in a mouse IMCD cell line (mIMCD-3 cells) (5); this suggests that ET-1 may serve as a negative feedback regulator of aldosterone-stimulated Na reabsorption. Another possibility is that renal nerve activity could inhibit collecting duct ET-1 production; decreased renal nerve activity, as occurs during volume expansion, could mediate increased collecting duct ET-1 release. However, neither epinephrine (11) nor norepinephrine (unpublished observations by our group) had any effect on ET-1 release by rat IMCD. Hence, there is no evidence to date that circulating hormones or renal nerves mediate the volume expansion-associated increase in collecting duct ET-1 production.

Physical factors that are altered during volume expansion might also mediate changes in collecting duct ET-1 production. High Na intake has been demonstrated to increase medullary toxicity which, in turn, augments thick ascending limb ET-1 production, leading to increased endothelial nitric oxide synthase activity and inhibition of chloride transport (6, 7). The effect of extracellular toxicity on collecting duct ET-1 production is, however, controversial. Media made hypertonic with NaCl or other relatively impermeant solutes have been reported to increase Madin-Darby canine kidney (MDCK; a distal nephron cell line) ET-1 production (14). In contrast, media hypertonicity due to NaCl or mannitol reduced collecting duct ET-1 synthesis in our hands and in others (13, 29). Increased tubule flow rate, as occurs during Na or water loading, is an interesting possibility for stimulating collecting duct ET-1 synthesis.

ENDOTHELIN-1 (ET-1) is an autocrine inhibitor of collecting duct Na and water reabsorption. This nephron segment produces and binds relatively large amounts of the peptide (reviewed in Ref. 9). In vitro studies indicate that ET-1 can inhibit collecting duct water reabsorption (30) and Na-K-ATPase activity (34), as well as reduce epithelial Na channel activity in distal nephron cells (17). Mice with collecting duct-specific disruption of the ET-1 gene are hypertensive and have impaired renal Na and water excretion (1). Similarly, mice with collecting duct-specific knockout of the endothelin B receptor gene develop hypertension as well as reduced ability to excrete an acute Na load (4). Decreased collecting duct ET-1 production is associated with hypertension in animal models, while urinary ET-1 excretion has been reported to be reduced in patients with essential hypertension (reviewed in Ref. 10). Taken together, the above data indicate that collecting duct-derived ET-1 is an important regulator of systemic blood pressure and renal salt and water excretion.
release. Recent studies indicate that elevated luminal flow rate can be detected by collecting duct cells through bending of primary cilia leading to increased intracellular calcium concentration (18, 19, 27). Direct examination of the effect of luminal flow on collecting duct ET-1 production has not been successfully performed, most likely due to technical difficulties in detecting changes in ET-1 production. However, examination of the effects of intracellular calcium, as well as related signaling pathways [calmodulin (CaM)], could be more readily accomplished and could well provide clues as to how extracellular fluid volume might regulate collecting duct ET-1 synthesis. Consequently, the current study was undertaken to examine whether, and how, changes in intracellular calcium concentration, and associated signaling pathways, might modify collecting duct ET-1 production.

**MATERIALS AND METHODS**

*Materials.* Types 1 and 2 collagenase were obtained from Worthington (Lakewood, NJ); penicillin, streptomycin, and glutamine from Invitrogen (Carlsbad, CA); culture dishes from BD Falcon (Franklin Lakes, NJ); pGEM-T cloning vector from Promega (Madison, WI); G-50 columns from Roche (Indianapolis, IN); and A23187, ionomycin, KN-62, KN-93, actinomycin D, and W-7 from Calbiochem (San Diego, CA). All other reagents and materials were obtained from Sigma (St. Louis, MO) unless stated otherwise.

*Cell culture.* Rat IMCD fragments were isolated by removing the inner medulla, mincing, and incubating in HBSS containing 1 mg/ml type I collagenase + 0.1 mg/ml DNase at 37°C for 40 min. Our protocols were submitted to, and approved by, the University of Utah Institutional Animal Care and Use Committee. The digest was shaken vigorously several times during the incubation, osmotically shocked by addition of 1.5 volumes water, and centrifuged at 400 g for 5 min. The pellet was washed in HBSS + 10% BSA, centrifuged, resuspended in renal epithelial growth media (REGM; Cambrex, Waterbury, CT) and plated on plastic culture plates. Cells were grown at 37°C in 5% CO2 until confluence. After being confluent for no more than a day, cells were growth arrested in DMEM/F12 without phenol red + 1,000 U/ml penicillin, 1,000 μg/ml streptomycin, and 0.292 mg/ml glutamine for 24 h and then studied immediately. IMCD cell culture was modified for transfection experiments as described in that section.

Primary cultures of rat aorta endothelial cells were prepared by a modification of the method of Kwan et al. (15). Briefly, the vasculature was perfused via cardiac puncture with HBSS, the thoracic aorta was removed, sliced open lengthwise, cut into 2-mm sections, and incubated in 0.2% type II collagenase/HBSS at 37°C with gentle agitation for 15 min. The aortic pieces were then removed and the supernatant was centrifuged at 1,500 rpm for 5 min. The resulting pellet was resuspended in 10% BSA/HBSS and recentrifuged at 800 rpm for 5 min. The final pellet was resuspended in RPMI 1640/10% FBS, aliquoted onto 24-well culture plates previously coated with 0.2% gelatin, and grown in at 37°C in 5% CO2 until confluent (~5–6 days).

**ET-1 release.** In general, IMCD or aortic endothelial cells were incubated with vehicle or test compound for 1 h in growth arrest media. The media was then discarded and fresh media plus vehicle, agonist, or antagonist was added for 4 h at 37°C in 5% CO2. After incubation, media was transferred to a siliconized microfuge tube and stored frozen. On the day of analysis, media was vacuum concentrated to dryness and ET-1 content was determined using a QuantIGlo ET-1 enzyme immunoassay (R&D Systems, Minneapolis, MN). Luminescence was measured using a Molecular Devices LMaxII Luminometer and Softmax Pro 4.7 data analysis program. Cells were dissolved in 0.1 N NaOH and protein content was determined using the Bradford protein assay.

**RNA analysis.** Riboprobes were made from primary cultures of rat IMCD. RNA was reverse transcribed and the resultant cDNA was used as template for PCR amplification of the ET-1 coding region using primers designed to amplify 552 bp of rat ET-1 cDNA–ET-1 promoter-pGL3 construct (sequenced to ensure authenticity). The ET-1-pGL3 construct was subsequently gel-isolated, blunt ended, recircularized by ligation, and ultimately transfected into bacteria. All constructs were sequenced to ensure authenticity.

Serial deletions of the 5' promoter region of the ET-1pGL3 construct were generated in a series of separate restriction enzyme reactions using the following enzymes: SauI (−1725), SauII (−1026), NhelI (−366), and MluI (−75). The fragment to be deleted was liberated from the vector using the pGL3 multiple cloning site cutter KpnI. The shortened ET-1pGL3 constructs were subsequently gel-isolated, blunt ended, recircularized by ligation, and ultimately transformed into bacteria. All constructs were sequenced to ensure authenticity.

**Transient transfection assays.** DNA constructs were transiently transfected into primary cultures of IMCD or aortic endothelial cells using Lipofectamine 2000 Reagent (Invitrogen). Cells were grown on 24-well tissue culture plates to greater than 90% confluence. Transfections were carried out for 18 h according to the manufacturer’s protocol, using pRL-TK Renilla luciferase as a control for transfection efficiency. The various compounds to be tested were added 24 h posttransfection and allowed to incubate overnight. The following day, the culture medium was changed and the cells were placed back into a 37°C, 5% CO2 incubator, being added for only a total of 4 h before analysis of promoter activity. However, due to the relatively long...
half-life of luciferase, it was necessary to expose the cells to test compounds overnight. Cells were lysed in passive lysis buffer (Promega) and subjected to freezing and thawing to ensure complete lysis. Luciferase activity in the cell lysates was determined using the Dual-Luciferase reporter assay system (Promega) to allow sequential determination, in the same sample, of both the firefly luciferase activity from the ET-1 promoter constructs and the transfection efficiency from the pRL-TK Renilla luciferase. All assays were carried out in a DRC-1 single sample luminometer (DIGENE Diagnostics). All reported firefly luciferase values were normalized for transfection efficiency using the pRL-TK, Renilla luciferase value.

The transfection efficiency was 27% in >90% confluent primary cultures of IMCD cells. Optimal conditions for transfection were determined using the pEGFP-C1 vector (Clontech), containing a CMV promoter-driven coding region for green fluorescent protein. For optimization, the DNA concentrations in the transfection system were varied from 0.5 to 5 μg and Lipofectamine 2000 from 2 to 12 μl. After 24 h, the percentage of cells transfected for each condition was analyzed by fluorescent microscopy.

Statistics. All data were analyzed by ANOVA using the Bonferroni correction. Data are expressed as means ± SE. P < 0.05 was taken as significant.

RESULTS

ET-1 release. Basal ET-1 release from primary cultures of rat IMCD cells was 2.1 pg ET-1/mg total cell protein over 1 h, 7.7 ± 0.5 pg ET-1/mg total cell protein over 4 h, and 15.7 ± 0.8 pg ET-1/mg total cell protein over 24 h. These determinations were made from cells 24 h following growth arrest; cells were growth-arrested to facilitate evaluation of the effects of potential stimulators or inhibitors of ET-1 release in the absence of confounding effects of added growth factors or serum. For purposes of comparison, ET-1 release from proliferating cells was determined and found to be 23.5 ± 1.6 pg ET-1/mg total cell protein over 4 h, approximately three times that seen when growth-arrested.

The effects of blocking various components of calcium signaling on IMCD ET-1 release were evaluated. Chelation of intracellular calcium with BAPTA markedly reduced IMCD ET-1 release (Fig. 1). In addition, inhibition of CaM with W7 reduced ET-1 release to 45% of control levels (Fig. 1). Inhibition of CaM kinases (CaMKs) with KN-93 or KN-62 also decreased IMCD ET-1 release, although KN-93 was a more effective inhibitor than KN-62 (Fig. 1). The spectrum of KN-93 and KN-62 inhibition of CaMKs has not been fully ascertainment, hence it is not possible to say which CaMKs are primarily involved. Treatment with calcium ionophores resulted in significant cell toxicity at concentrations that are well tolerated by other cell types (0.1–1 μM A23187 or 0.1–1 μM ionomycin); ET-1 production was markedly reduced, but these results were deemed uninterpretable. Similarly, 1–5 μM EGTA caused variable cell detachment, obviating data interpretation.

ET-1 mRNA metabolism. The above data indicated that calcium/CaM significantly regulated IMCD ET-1 release. To see whether such regulation occurred at the mRNA level, steady-state ET-1 mRNA content was assessed. BAPTA markedly reduced ET-1 mRNA, while W7 and KN-93 decreased mRNA content by −40–50% (Fig. 2). This inhibitory effect of W7 was not due to alterations in mRNA stability since ET-1 mRNA half-life was not altered (Fig. 3). Thus calcium/CaM increases ET-1 release, at least in part, through increases in mRNA content, the latter being due to augmented ET-1 gene transcription and not reduced mRNA degradation.

ET-1 promoter activity in IMCD cells. To more directly determine how ET-1 production is regulated at the transcriptional level, rat ET-1 promoter-firefly luciferase DNA constructs were generated and transfected in rat IMCD cells. Five different sized ET-1 promoter regions were studied, extending from +189 (numbered 3’ to the transcriptional start site) to −75 (minimal promoter), −366, −1026, −1725, and −3048 (all numbered 5’ to the transcriptional start site). The minimal promoter drove very little luciferase activity (Fig. 4). The −366 region exhibited increased activity while the −1026 region further increased luciferase activity (Fig. 4). There was a marked increase in reporter activity using the −1725 region; extending this to −3048 caused a comparable increase in reporter activity compared with −1725 (Fig. 4).

To directly examine whether ET-1 promoter activity is regulated by CaM/CaMK, the effect of W7 on activity of the −366, −1026, and −3048 constructs was examined. This range of promoter constructs covers all degrees of promoter activity seen, from modest (−366), to moderate (−1026), to maximal (−3048). Since no difference was seen between the −1725 and −3048 constructs, only one size was utilized. W7
markedly decreased promoter activity of the −3048, but not activity of the smaller sized promoters (Fig. 5A).

**ET-1 production and promoter activity in rat aortic endothelial cells.** The finding that ET-1 promoter regions 5′ to −1026 conferred markedly enhanced activity in IMCD is in contrast to that reported in other cell types, particularly endothelial cells, where the first 150 bp 5′ to the transcriptional start site contain the main regulatory regions (see DISCUSSION). To determine whether IMCD can regulate ET-1 promoter activity in a manner different to other cell types, and particularly in our hands under similar experimental conditions, ET-1 production and ET-1 promoter activity, and their regulation by CaM, were assessed in primary cultures of rat aortic endothelial cells. Baseline ET-1 release from aortic endothelial cells was 1.5 ± 0.2 pg ET-1·mg total cell protein −1·4 h −1 (n = 6), ~20% of that released by IMCD cells. In contrast to IMCD, treatment with W7 did not affect ET-1 release by aortic endothelial cells (1.8 ± 0.2 pg ET-1·mg total cell protein −1·4 h −1, n = 6). Also, in contrast to IMCD, transfection of the −366, −1026, and −3048 ET-1 promoter-reporter constructs revealed no difference in firefly luciferase activity (relative to Renilla luciferase) in endothelial cells (Fig. 5B). In addition, W7 had no effect on promoter activity for any of the three constructs (Fig. 5B). Thus, at least compared with endothelial cells, ET-1 promoter activity is uniquely regulated by CaM in IMCD. Furthermore, regions of the ET-1 promoter 5′ to −366 confer unique regulation of ET-1 gene transcription in IMCD.

**DISCUSSION**

One of the more extensively studied factors in regulation of ET-1 production is calcium. Despite this, the effects of calcium (as well as CaM and/or CaMK) on ET-1 synthesis appear to be quite variable, differing between and even within cell types. In human mesangial cells, BAPTA has no effect on basal or thrombin-stimulated ET-1 mRNA; similarly, a calcium inonophore (A23187) had no effect on ET-1 production by rat pulmonary artery smooth muscle cells (32). In human umbilical vein endothelial cells (HUVEC), thrombin-induced increases in ET-1 mRNA were inhibited by BAPTA, EDTA,
W7, or KN-62, while A23187 increased basal and thrombin-stimulated ET-1 mRNA levels (21). In contrast, another group found that ionomycin and A23187 decreased ET-1 production by HUVEC (23). In rat aortic endothelial cells, heparin inhibition of ET-1 mRNA expression was blunted by W7 (33). Hence, it was not possible to predict what effect, if any, calcium would have on collecting duct ET-1 synthesis (although one study found that A23187 modestly increased ET-1 production by porcine IMCD (22)). The current study found that ET-1 synthesis by IMCD is markedly calcium/CaM/CaMK dependent, providing strong evidence that this system can play an important role in modulating collecting duct ET-1 release, and ultimately ET-1 actions.

The mechanism by which calcium and CaM regulate IMCD ET-1 production is clearly at the transcriptional level. Reduction or inhibition of these factors reduced steady-state mRNA levels but did not affect mRNA stability. Most importantly, CaM inhibition greatly reduced activity of the −3048 ET-1 promoter construct. Notably, CaM inhibition did not reduce activity of the −1026 or −366 ET-1 promoter fragments. Furthermore, maximal ET-1 promoter activity was conferred by regions distal to the first proximal 1-kb region of the promoter. These findings have no precedent. Although no data are available in collecting duct cells, ET-1 promoter elements primarily involved in regulation of gene transcription by other cell types are located within the first 150 bp 5′ to the transcriptional start site. For example, bovine aortic endothelial cells transfected with ET-1 promoter constructs exhibited maximal promoter activity in the proximal 143-bp promoter fragment; regions containing the proximal 0.2, 0.7, 1.7, and 4.4 promoter fragments did not confer increased (or decreased) activity (16).

Similarly, the proximal 120–143 bp of the ET-1 promoter had maximal activity in transfected mesangial cells, compared with 1.7- and 4.4-kb proximal promoter regions (3). To our knowledge, the effect of CaM inhibition or calcium on ET-1 promoter activity has not been well studied. A putative calcium response element has been described at the transcriptional level. Therefore, calcium/CaM/CaMK may be involved in ET-1 synthesis by IMCD, depending on the cell type and the region of the promoter that is being analyzed.

The finding that calcium/CaM regulates IMCD ET-1 production may be an important clue as to how extracellular fluid volume is coupled to collecting duct ET-1 synthesis. Collecting duct principal cells express primary cilia that extend into the lumen and have been implicated as flow sensors (18, 19). Studies using MDCK cells showed that bending the primary cilia increases intracellular calcium concentration, while removal of the primary cilia abolishes flow sensing (25, 26). In addition, mutation of genes leading to loss of primary cilia abolishes regulation of calcium entry into collecting duct cells (27). Since Na or water loading increase collecting duct luminal fluid flow rate, and likely cause increased ET-1 production by this nephron segment, it seems quite possible that primary cilia are involved in coupling flow rate to ET-1 release, and likely through alteration of intracellular calcium concentration. Notably, increased flow rate or shear stress can increase ET-1 production by nonrenal cell types (2, 20, 24, 31), although the role of calcium in this response has not been clarified. Studies are needed that will examine the direct effect of flow on collecting duct ET-1 production and whether such an effect is calcium/CaM dependent, although completing such studies will be technically quite challenging.

In summary, the current study demonstrates that IMCD ET-1 production is markedly regulated by calcium/CaM, that this occurs at the transcriptional level, and involves pathways that are at least relatively unique to collecting duct cells. Further elucidation of this system may shed substantial light on how renal Na and water excretion, and systemic blood pressure, are regulated.

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REFERENCES

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