Endothelin Inhibits NPR-A and Stimulates eNOS Gene Expression in Rat IMCD Cells

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Abstract — We have shown in previous studies that high extracellular tonicity is associated with increased expression of the type A natriuretic peptide receptor (NPR-A) and reduced expression of the endothelial NO synthase (eNOS) gene in cultured rat inner-medullary collecting duct cells. The vasoactive peptide endothelin has been shown to be avidly expressed in this nephron segment, and to be subject to osmotic regulation. We asked whether endothelin might play a role in the control of basal or osmotically regulated NPR-A or eNOS gene expression in these cells. Although exogenous endothelin had little or no effect on basal expression of eNOS mRNA or protein or NPR-A gene expression, both the type A (BQ610) and type B (IRL1038) endothelin receptor antagonists proved capable of reducing eNOS mRNA and protein expression, and increasing levels of the NPR-A mRNA. Increased extracellular tonicity reduced endothelin mRNA accumulation in these cells (≈15% of control levels); however, exogenous endothelin failed to normalize osmotically increased NPR-A activity or expression, or osmotically suppressed eNOS expression. Collectively, these data demonstrate the presence of a number of independent but highly interactive local regulatory networks governing fluid and electrolyte handling in this distal nephron segment. (Hypertension. 2003;41[part 2]:675-681.)

Key Words: natriuretic peptides ■ endothelin ■ nitric oxide synthase ■ osmotic regulation ■ gene expression

The inner-medullary collecting duct (IMCD) plays a major role in the control of fluid and electrolyte homeostasis. Located in the terminal nephron, the IMCD samples up to 5% of filtered sodium load and bears ultimate responsibility for the regulation of urinary sodium concentration. A number of local and systemic regulatory factors—including mineralocorticoids, prostaglandin E2, endothelin, interleukin-1, and atrial natriuretic peptide—signal events in the IMCD that ultimately result in changes in sodium excretion.

Endothelin is a vasoconstrictor peptide that was originally isolated from porcine endothelial cells. It is produced as 3 isoforms (ET1–3) that bind to 2 receptor subtypes (ETA and ETB). ET is produced in the kidney and is particularly enriched in IMCDs, where it has been shown to promote natriuretic activity. Both ETA and ETB receptors are expressed in IMCDs, leading to the suggestion that ET may function as an autocrine/paracrine regulator of sodium and water transport in this terminal nephron segment. A number of local and systemic factors have been shown to regulate ET expression in IMCDs, including immune cytokines and extracellular toxicity. The latter is particularly intriguing given the high osmolar environment surrounding the IMCD cell.

We have shown previously that increased extracellular toxicity results in increased expression of the type A natriuretic peptide receptor (NPR-A) gene and reduced expression of the endothelial NO synthase (eNOS) gene in IMCD cells. In fact, the reduced basal concentrations of cyclic GMP resulting from the reduction in eNOS expression appears to account, at least in part, for the increase in NPR-A expression. Given the high concentrations of endothelin in this nephron segment, we asked whether changes in endothelin gene expression might account for the alterations in NPR-A and eNOS gene expression noted above. We have determined that endogenous ET does, in fact, inhibit NPR-A and stimulate eNOS expression; however, the osmotic regulation of these 2 genes appears to operate independently of ET.

Methods

Materials
[α32P]-dCTP was purchased from Dupont/New England Nuclear Research Products. Atrial natriuretic peptide (ANP) was purchased from Phoenix Pharmaceuticals Inc. eNOS antibody was from Transduction Laboratories. Cyclic GMP radioimmunoassay kit was purchased from Perkin-Elmer/Life Sciences. RNeasy mini kit was obtained from Qiagen Inc. Primer-it RMT kit, hybridization solution, and Nuctrap push columns were purchased from Stratagene Inc. Other reagents were obtained through standard commercial suppliers.

Isolation and Culture of IMCD Cells
Adult Sprague-Dawley rats were euthanized by carbon dioxide narcosis, followed by bilateral thoracotomy, in compliance with a protocol approved by the University of California at San Francisco.
Committee on Animal Research. Kidneys were excised and bivalved with a scalpel blade. The inner-medullary tissue was dissected free from the outer medulla, minced into 1-cm² fragments, and digested with 1 mg/mL collagenase at 37°C with gentle agitation during each 30-minute cycle. IMCD cells were enriched in the preparation by hypotonic lysis as described previously.17 The cells were resuspended in medium 1 (1:1 mixture of Dulbecco’s Modified Eagles Medium [DMEM] and Ham’s F-12 Medium supplemented with 10% fetal bovine serum [FBS], 42 mM/L sodium bicarbonate, 100 IU/mL penicillin, and 100 μg/mL streptomycin) and seeded on to culture plates. After 24 hours, the cells were placed in K-1 medium (1:1 mixture of DMEM and Ham’s F-12 medium supplemented with 10 mM/L HEPES at pH 7.4, 42 mM/L sodium bicarbonate, 5 μg/mL insulin, 50 mM/L hydrocortisone, 5 μg/mL transferrin, 5 μg/mL triiodothyronine, 100 IU/mL penicillin, and 100 μg/mL streptomycin) and cultured for 3 to 4 days.

Measurement of ANP-Stimulated Cyclic GMP Levels
IMCD cells were grown to ~80% confluence and incubated for varying time intervals under conditions outlined in the individual figure legends. For measurement of ANP-stimulated cyclic GMP accumulation (ANP-s-CGMP), cells were washed 3 times with prewarmed PBS and incubated with 0.5 mL of DMEM containing 0.5 mM/L 3-isobutyl-1-methylxanthine and 10 mM/L HEPES (pH 7.4) for 10 minutes at 37°C. 10⁻⁷ mol/L ANP was added to the medium, and the incubation was continued for another 10 minutes. The reaction was stopped by removal of medium and addition of 0.3 mL of 12% trichloroacetic acid. The extraction was continued for 30 minutes at 4°C. The contents of the plate were collected and centrifuged to pellet particulate material; the supernatant fraction was extracted 4 times with 0.5 mL of water-saturated ether. Cyclic GMP levels were determined by radioimmunoassay, after acetylation of the sample and standard, using a commercial antibody and [³²P]-cGMP as tracer.

RNA Isolation and Northern Blot Analysis
IMCD cells were plated in 100-mm dishes, cultured, and treated with different reagents as indicated in the figure legends. Total RNA was extracted from cells with the RNeasy mini kit according to the instructions provided by the manufacturer. Total RNA was denatured and separated on a gel containing 2.2% formaldehyde, transferred to a nitrocellulose filter, and hybridized to radiolabeled cDNA probe as described previously.18 A 1.2-kb EcoRI fragment of the rat NPR-A cDNA18 and a 4.0-kb EcoRI fragment from the bovine eNOS cDNA (kindly provided by W. Sessa, Yale University, New Haven, Conn)19 were isolated from vector sequence, radiolabeled using the primer-it polymerase chain reaction (PCR) using an upstream sense oligonucleotide that incorporated a HindIII site at its 5’-terminus and a downstream antisense oligonucleotide containing a BglII site at its 3’-terminus. The fragment produced by PCR was cut with HindIII and BglII and ligated into the HindIII/BamH1 sites of a- luciferase, a luciferase reporter plasmid described previously.21

Transfection and Luciferase Assay
Cells were plated in 6-well plates and grown to ~70% confluence. At that time, transfection was performed with Lipofectin Reagent (Life Technologies) using a protocol recommended by the manufacturer. One microgram of ~1197eNOS-LUC with 0.2 μg CMV-β-galactosidase (β-gal) was introduced into each well. The DNA-liposome suspension was incubated in the cultures for 5 to 6 hours at 37°C in Opti-MEMI Reduced Serum Medium (GIBCO BRL). The suspension was then removed and replaced with K-1 medium for the ensuing 24 hours, at which point, cells were treated with different concentrations of sucrose, NaCl, or urea in K-1 medium for defined periods of time. At the end of the incubation, cells were washed 3 times with PBS and lysed with Promega lysis buffer. Luciferase activity was measured using the Luciferase Assay System (Promega). β-Galactosidase activity was assayed using the GalectoLight Plus Chemiluminescence Assay (Tropix). Luciferase levels were normalized for β-galactosidase activity in the individual cultures.

Immunoblot Analysis
Cells were scraped into lysis buffer18 containing 1% Triton X-100. Forty micrograms of total protein was denatured at 100°C for 3 minutes in loading buffer (125 mM/L Tris at pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol), subjected to 8% SDS-PAGE, and transferred onto polyvinylidene difluoride membrane at 28 V overnight in transfer buffer plus SDS (25 mM/L Tris at pH 7.5, 190 mM/L glycine, 20% methanol, 0.05% SDS). The membrane was blocked with 5% nonfat milk in TBST (50 mM/L Tris-HCl at pH 7.5, 150 mM/L NaCl, 0.1% Tween 20) and probed with anti-eNOS, (diluted 1:1000 in TBST). A horseradish peroxidase–conjugated second antibody (diluted 1:1000 in TBST) was used to detect immunoreactive bands by means of the enhanced chemiluminescence Western blotting detection system (Amersham Chemical Corp). Signal was identified and quantified using NIH Image.

Statistical Analysis
Data were evaluated by 1-way ANOVA with Newman-Keuls test for significance.

Results
Both NO and ET are expressed in the IMCD.4–6,16,22 NO has been postulated to function as an intermediate in signaling endothelin activity in a number of nephron segments,23–26 including the IMCD. We asked whether either exogenous or endogenous ET would prove capable of regulating eNOS mRNA levels in the IMCD cell cultures. As shown in Figure 1, after 24 hours of incubation, ET promoted a modest and not statistically significant increase in eNOS mRNA levels. However, because ET is known to be produced in these cells,4–6 we questioned whether high levels of endogenous ET might mitigate any potential response to exogenous peptide. This was confirmed with inhibitor studies presented in Figure 1. BQ610 (ET1 receptor antagonist) effected ~75% inhibition of eNOS gene expression, whereas IRL1038 (ETa receptor antagonist) reduced expression by ~70%. In each case, the inhibition was partially reversed by inclusion of exogenous ET (10⁻⁷ mol/L). The partial reversal may reflect the lower concentration of ET (versus inhibitor) used here. The combination of both inhibitors (BQ610+IRL1038) resulted in ~80% inhibition of eNOS gene expression; however, in this case, the addition of exogenous ET failed to provide a significant reversal of the inhibition. A more detailed time course (1, 4, 8, and 24 hours) of treatment with ET or the ET receptor agonist IRL1620 also failed to demonstrate an increase in eNOS mRNA levels (data not
shown). The inhibition of eNOS expression by both BQ610 and IRL1038 was confirmed at the level of eNOS protein (Figure 2A) and the eNOS gene promoter (Figure 2B) with dose-dependent reductions in each seen after treatment with the individual antagonists.

We next examined the ability of ET to regulate expression of the NPR-A gene that encodes a receptor known to be avidly expressed in IMCD.15,27,28 As shown in Figure 3, administration of ET (10^{-7} mol/L) had virtually no effect on basal NPR-A mRNA levels, essentially mirroring the response of eNOS to ET shown in Figure 1. A similar lack of efficacy was noted with the ET_{A} receptor agonist IRL1620 (data not shown). Treatment with either BQ610 or IRL1038 led to a significant increase (2- to 3-fold) in NPR-A gene transcript levels, whereas co-administration of exogenous ET resulted in partial reversal of the stimulatory effect. Again, the combination of BQ610 and IRL1038 was only marginally more effective in stimulating NPR-A expression than was either agent alone, and the addition of ET in this instance failed to reverse the stimulatory effect.

Previous studies have suggested that endothelin, a vasoconstrictor peptide that is produced at very high levels in the IMCD, is negatively regulated by increased extracellular oncoticity.12–14 In our hands, sucrose and NaCl, but not urea, effected a time-dependent suppression of ET mRNA levels (Figure 4). ET mRNA levels after 24 hours of exposure to the hyperosmotic media were reduced to ~15% and 10% of control levels with sucrose and NaCl, respectively. Urea at equimolar concentrations was without effect.

The opposing effects of the osmotic stimulus on NPR-A versus ET expression and parallel suppression of ET and eNOS expression raised the obvious question as to whether the 3 might be mechanistically linked. We have previously provided data linking the reduction in eNOS expression to the stimulation of NPR-A, so the major question here was the
role that ET might play, if any, in signaling the osmoregulation of the other 2 genes.

Having demonstrated that endogenous ET plays a role in regulating NPR-A gene expression, we asked whether the reduction in ET gene expression seen after osmotic challenge might be linked to the increase in NPR-A activity and gene expression. ET had no effect on ANP-stimulated cyclic GMP production, an index of NPR-A activity, in these cells (Figure 5) paralleling its lack of activity at the level of the NPR-A gene transcript above (Figure 3). This lack of ET-dependent activity was reproduced over a dose range extending from $10^{-8}$ to $10^{-6}$ mol/L (data not shown). Sucrose (150 mmol/L), on the other hand, effected a predictable 3-fold increment in NPR-A activity. When ET was combined with sucrose, there was no reduction in ANP-stimulated cyclic GMP production, indicating that the stimulation seen in the presence of sucrose does not arise exclusively as a consequence of reduction in local ET production. This was confirmed at the level of NPR-A gene expression. At 8 hours (Figure 5B) and 24 hours (Figure 5C) after application of the osmotic stimulus with either NaCl or sucrose, there was a robust increase in NPR-A transcript levels, but in no instance did ET effect a reduction in these levels. Collectively, these data indicate that although osmotic stress reduces ET expression in IMCD cells and endogenous ET appears to play some role in tonically suppressing NPR-A activity and gene expression, the osmotic stimulation of NPR-A cannot be explained through the reduction in ET gene expression.

To determine whether the osmo-dependent reduction in ET expression is responsible for the decrease in eNOS mRNA levels, we examined the ability of exogenous ET to reverse the inhibition of eNOS expression seen after 8 hours of treatment with 75 mmol/L NaCl. As shown in Figure 6A, the NaCl-induced reduction in eNOS mRNA levels was unaffected by exogenous ET, implying that the decrease in ET production in the setting of high extracellular osmolality is not responsible for the coincident reduction in eNOS expression. This was supported by studies looking at eNOS protein accumulation (Figure 6B). Paralleling the RNA studies described above, exogenous ET was incapable of reversing the NaCl-dependent reduction in eNOS protein levels. Although BQ610 or IRL1038 effected a significant reduction ($\approx 40\%$) in eNOS protein levels after 24 hours of treatment, this reduction was substantially less than that seen with NaCl (75 mmol/L), (Figure 6C) implying that the osmo-stimulus encompasses more than simple withdrawal of ET. The ET antagonists were not additive with NaCl in suppressing eNOS; in fact, the combination was no better than NaCl alone implying, perhaps, that the osmo-stimulus operates more distally in the inhibitory signal transduction cascade.

**Discussion**

The present study documents a number of important features relevant to signal transduction systems operating in the IMCD. First, increased extracellular tonicity reduces expression of the ET gene, which typically displays robust expression in IMCD cells under isotonic conditions. Second, en-
Endogenous ET stimulates eNOS and inhibits NPR-A gene expression in these cells. These latter effects appear to be maximally activated under basal conditions in which administration of exogenous ET had little additional activity. Third, the osmotic stimulation of NPR-A and suppression of eNOS gene expression noted previously does not appear to be mechanistically linked solely to the osmotic inhibition of ET gene expression.

ET is known to be produced at high concentrations in the IMCD, perhaps higher than any other location in the organism.\textsuperscript{4–6} It acts to promote sodium excretion in this nephron segment through a mechanism that may involve activation of eNOS and increased NO production.\textsuperscript{7} These effects are believed to be largely independent of its vasoactive properties in the renal vasculature. It has been suggested that the dominant ET receptor in the IMCD is the type B receptor\textsuperscript{23}; however, selective inhibitor studies have demonstrated that type A and type B ET receptors are expressed in IMCD cells,\textsuperscript{8} a model that is more compatible with the findings presented here. The near equivalent efficacy of BQ610 and IRL1038 over a similar dose range implies that ET\textsubscript{\textalpha} and ET\textsubscript{\textbeta} each play an important role in signaling ET activity in this nephron segment, a scenario that is similar to that reported for isolated rabbit liver,\textsuperscript{29} which also expresses both receptor subtypes. The fact that each (at 10\textsuperscript{\textminus}7 mol/L) promotes near maximal inhibition (eNOS) or stimulation (NPR-A) of target gene expression, and the fact that the combination of the 2 antagonists is not additive, suggests that ETA and ET\textsubscript{\textbeta} operate cooperatively in triggering postreceptor signaling cascades. A similar lack of additivity with the ETA and ET\textsubscript{\textbeta} receptor antagonists was reported in the study of Wang et al\textsuperscript{29} mentioned above.

Our earlier studies suggested that the osmotic stimulation of NPR-A expression is owing, at least in part, to suppression of eNOS expression and activity, presumably resulting in a reduction in basal cyclic GMP levels and consequent release of tonic suppression of NPR-A gene transcription.\textsuperscript{16} The current study clearly indicates that osmotic reduction in ET
expression is not linked to the stimulation of NPR-A activity or the reduction in eNOS expression, even though ET antagonists clearly increase NPR-A expression and reduce eNOS expression under conditions of normal extracellular tonicity. This conclusion is predicated on the fact that exogenous ET failed to inhibit osmo-stimulated NPR-A (see Figure 4) or stimulate eNOS gene expression (Figure 5). It should be noted that the latter interpretation needs to be qualified to some degree. If, as noted above, a significant portion of ET-dependent signal transduction flows through eNOS in the IMCD cell, one might anticipate finding a lack of activity with exogenous ET under hyperosmolar conditions. It is also possible that the osmotic and ET-dependent regulatory pathways communicate at a level distal to mRNA accumulation. Although it is clear that NO participates in the osmo-regulation of NPR-A expression, the involvement of ET in this process in the context of the intact nephron may depend on its ability to activate residual eNOS activity. Thus, conditions may exist in vivo in which osmo-suppression of ET is more profound than that for eNOS. In this setting, suppression of ET may contribute more significantly (vis-à-vis eNOS) to the subsequent increase in NPR-A expression.

The current study raises the possibility of a number of regulatory autocrine/paracrine networks within the IMCD, implying a hierarchy of complex control mechanisms governing salt and water balance in this nephron segment.

**Perspectives**

The regulation of signal transduction pathways in the IMCD has obvious implications for the control of sodium homeostasis. Recent studies have demonstrated that high-salt diet significantly reduces immunoreactive ET in the inner medulla of the rat. In addition, several studies have suggested that this maneuver also regulates eNOS levels in this same nephron segment, although the latter findings are to some degree discrepant. In aggregate, available studies argue...
for an important role for the natriuretic peptides, endothelin, and NO in controlling sodium handling in this terminal nephron segment and possibly contributing to disorders characterized by inappropriate sodium retention. The physiological or pathophysiological relevance of the osmoregulation of these signaling pathways will obviously have to be tested in a relevant animal model.

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References
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