Novel Mechanism for Regulation of Endothelin Synthesis: Role of Extracellular pH

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Key Words

Endothelin-1 • Collecting duct • pH • mRNA translation • Furin convertase • Preproendothelin-1 • Big endothelin-1

Abstract

Background/Aims: Several studies suggest that pH may be an important regulator of renal collecting duct endothelin-1 (ET-1) synthesis; the current study was undertaken to examine this interaction. **Methods:** Rat inner medullary collecting duct (IMCD) cells were exposed to varying extracellular pH and ET-1 biosynthetic pathways assessed. **Results:** Exposure of IMCD to varying pH revealed a strong direct correlation between media pH and both mature ET-1 and Big ET-1 release. A direct correlation also existed between cellular Big ET-1 content and pH. Varying media pH did not alter ET-1 mRNA content nor did it affect activity of a transfected 3.2 kb ET-1 promoter. Media pH did not affect ET-1 degradation nor did it alter IMCD cell total protein synthesis (^3H -leucine incorporation). Attempts to measure ET-1 mRNA translation rate using His-Tag labeled ET-1 cDNA were not successful. **Conclusion:** pH regulates IMCD ET-1 production through regulation of either mRNA translation or preproET-1 cleavage. This represents a novel mechanism for regulation of ET-1 synthesis.

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Introduction

Endothelin-1 (ET-1) is a pluripotent 21-amino acid peptide that is produced by, and regulates the function of, virtually every organ system. Regulation of ET-1 synthesis and actions is complex and largely depends upon the target cell type. One of the most highly studied sites of ET-1 production and action is the kidney; within the kidney, the collecting duct has emerged as being of substantial importance [1]. ET-1 modulates several transport processes within the collecting duct; in particular, recent investigations have strongly implicated ET-1 in the regulation of collecting duct H^+ and HCO_3^- transport [2]. Several studies indicate that ET-1 mediates, at least in part, the renal response to metabolic acidosis [3-6]. This effect is due to ET-1 actions on both the proximal and distal nephron, however, since the distal nephron (and particularly the collecting duct) is the site of final regulation of renal acid excretion, it has been of great interest to understand how extracellular pH might regulate ET-1 synthesis, with subsequent control of collecting duct acid/base transport processes. Unfortunately, relatively little is known about pH regulation of ET-1 production. Wesson demonstrated that renal interstitial ET-1 content increased after dietary acid loading [5]. Subsequent studies by this group showed that reduced extracellular pH stimulated ET-1 secretion by renal microvascular endothelial cells

[7]. Interestingly, aortic endothelial cell ET-1 secretion was not affected by pH [7]. It was hypothesized that pH-stimulated ET-1 derived from microvascular endothelial cells could interact with neighboring tubule cells (presumably including collecting duct) and consequently stimulate acid excretion. While these observations are intriguing, it is notable that the collecting duct produces far more ET-1 than any other cell type in the kidney [1]. Hence, it seems important to understand the direct effects of pH on collecting duct ET-1 synthesis. Consequently, the current study was undertaken to examine the effect of varying extracellular pH on ET-1 production by collecting duct cells. We report that, unexpectedly, decreased extracellular pH inhibits collecting duct cell ET-1 production. Notably, this pH effect appears to represent a novel mechanism for control of cellular ET-1 synthesis.

Materials and Methods

Cell culture

IMCD cells were cultured as previously described [8]. Briefly, rat IMCD fragments were isolated by removing the inner medulla, mincing, and incubating in HBSS containing 1 mg/ml type I collagenase (Worthington, Lakewood, NJ) + 0.1 mg/ml DNase (Sigma, St. Louis, MO) at 37°C for 40 min. The digest was osmotically shocked by addition of 1.5 volumes water, and centrifuged at 400 x g for 5 min. The pellet was washed in HBSS + 10% BSA, centrifuged, resuspended in Renal Epithelial Growth Media (REGM, Cambrex, Watersville, MD), and plated on plastic culture plates. Cells were grown at 37°C in 5% CO₂ until confluence. After confluent for no more than a day, cells were growth arrested in DMEM:F12 without REGM for 24 hours, then studied immediately.

ET-1 and Big ET-1 assay

IMCD cells in 24 well plates were incubated in DMEM:F12 without bicarbonate in a 37°C incubator without CO₂. The pH was adjusted to 6.8, 7.1, 7.4 or 7.7 using HCl or NaOH unless specified otherwise. Cells were exposed to 300 µl media at varying pH's for 1, 4 or 24 hr. Media was dried and ET-1 content determined using a QuantiGlo ET-1 enzyme immunoassay (R&D Systems, Minneapolis, MN). Luminescence was measured using a Molecular Devices LMaxII Luminometer. Big ET-1 content was determined using a TiterZyme EIA Big ET-1 kit (Assay Designs, Ann Arbor, MI) and a Molecular Devices SpectraMax Plus 384 Spectrophotometer. Cells were dissolved in 0.1 N NaOH and protein content determined using the Bradford protein assay. For ET-1 stability, 10 pg of ET-1 was placed in test tubes containing 300 µl media adjusted to the above pHs for 4 hr, after which media ET-1 content was assayed as above.

Cell associated ET-1 and Big ET-1 were determined using the same assays as above. Upon completion of the experimental

incubation, cells were washed twice, water added and cells freeze/thawed three times. Cells were scraped to a siliconized tube and debris spun out. Values were normalized by averaging the protein concentration of a random selection of untreated wells from the same plate.

³H leucine incorporation

³H leucine incorporation was measured as described previously [9]. IMCD cells were exposed to varying pH for 4 hr. Media was removed and cells incubated for 20 minutes in HBSS + 1 µCi/ml ³H leucine at room temperature, rinsed with cold HBSS and solubilized in 0.1% SDS. Protein was precipitated following the addition of 5 mg BSA with 10% tricarboxylic, then collected on GF/C filters (Whatman Ltd, Maidstone, England) for determination of CPM.

RNA analysis

Riboprobes were made from primary cultures of rat IMCD as previously described [8]. RNA was reverse transcribed and the cDNA PCR amplified using primers designed to amplify 552 bp of rat ET-1 cDNA - ET-1 5'-primer 5'- GGC TTT CCA AGG AGC TCC AGA -3' and ET-1 3'-primer 5'- ATC AAC TTC TGG TCT CTG TAG AG -3'. The product was cloned into pGEM-T cloning vector (Promega, Madison, WI) and the insert sequenced. The probe was digested to give the antisense strand and riboprobes made using ³²P-UTP and T7 RNA polymerase. Radioactive probe was used at ~10ng/ml with a specific activity ≥10⁹ dpm/µg. Total RNA was isolated from IMCD after exposure to varying pH for 4 hr. RNA was electrophoresed, transferred to a nylon membrane and the blot prehybridized at 60°C in 50% formamide, 5X SSC, 5X Denhardt's, 1% SDS and 100 µg/ml sheared denatured salmon sperm DNA. Fresh solution was added for hybridization along with radioactively-labeled probe and incubated overnight at 60°C. The blot was washed and subjected to autoradiography and densitometry. A human U1 small nuclear riboprotein (snRNP) riboprobe was used for normalizing loading [10]. RNA product sizes were: ET-1 - 2.1 kb and snRNP - 0.75 kb.

ET-1 promoter-luciferase activity

3.2 kb of the ET-1 upstream promoter region encompassing 189 bp of the 5'-untranslated region of the mRNA was utilized as previously described [8]. The DNA construct was transiently transfected into IMCD cells using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA). Transfections were carried out for 18 hr according to the manufacturer's protocol, using pRL-TK Renilla luciferase as a control for transfection efficiency. The cells were placed in growth arrest media for 24 hr. Subsequently, DMEM:F12 at varying pH was added for 4 hr. Cells were lysed and luciferase activity 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, Gaithersburg, MD). All reported firefly luciferase values were normalized for transfection efficiency using the pRL-TK, Renilla

Fig. 1. Effect of varying media pH on ET-1 (Panel A) and Big ET-1 (Panel B) release by IMCD cells. For ET-1 release: N=6 for 1 and 24 hr time points, n=16 for 4 hr time point. For Big ET-1 release (4 hr incubation with varying media pH): N=11 each data point). *p<0.05, **p<0.01, #p<0.005, \$p<0.001 - all vs. pH 7.4 at same time point.

luciferase value. The optimal transfection efficiency IMCD cells was 27% as determined using the pEGFP-C1 vector (Clontech, Mountainview, CA).

mRNA translation assay

A preproET-1 expression vector with 6X HisTag at the C-terminus was made in order to test translational effects of the varying pH conditions. PCR primers were used to amplify the mouse preproET-1 cDNA from transcriptional start through the 3' UTR (1385 bp, based on Genebank accession no. M64711): preproET-1 F 5'- cgc gac gct tcg etc cgg -3' and preproET-1 R 5'- aaa ggt ggg tag act cat att ca -3'. This fragment was cloned into pTarget expression vector (Promega). A 6X HisTag was inserted at the C-terminus using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Expression of preproET-1 was confirmed using the TNT coupled reticulocyte lysate system (Promega) followed by PAGE analysis.

The vector was transfected into IMCD cells as described above. On the day after transfection, fresh media at varying pH, with or without a furin convertase inhibitor, 50 μ M decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Alexis Biochemicals, San Diego), was added and incubated for 24 hr. In a separate set of studies, media pH was varied only for 4 hr and only after 24 hr exposure to furin convertase inhibitor or vehicle alone. At the conclusion of the incubations, cells were lysed with Cytobuster (Novagen, Madison, WI) + 1% NP-40 and scraped. Debris was spun out and His-Tagged proteins isolated using a His-Bind column as per manufacturer protocol (Novagen). Eluted sample was concentrated by centrifugal filtration. 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 MES buffer system (Invitrogen). Proteins were transferred to PVDF plus nylon membrane (Amersham International, UK) by electroelution. The blots were blocked with 5% nonfat dry milk + TBST at room temperature and washed with TBST. Blots were then incubated overnight at 4°C with a HisTag mouse monoclonal antibody (Novagen) in 5% BSA + TBST. The blots were next incubated with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody for 1 hr in TBST. After final washes, antibody binding was visualized using the enhanced chemiluminescence system (Amersham). The protein product from the TNT coupled reticulocyte lysate using the expression vector pTarget-preproET-1 HisTag was used as a positive control.

Statistics

All data were analyzed by ANOVA using the Bonferroni correction. Data are expressed as mean $\pm$ SEM. p<0.05 was taken as significant.

Fig. 2. Effect of varying media pH (4 hr exposure) on cell-associated ET-1 (Panel A) and Big ET-1 (Panel B) by IMCD cells. N=5 each data point. *p<0.05 vs. pH 7.4.

Results

All studies were done on primary cultures of rat IMCD cells that were confluent for one day, then placed in growth arrest media for one day. Studies were done in growth arrest media since most growth factors have a marked stimulatory effect on IMCD ET-1 release [1]. Exposure to pH 6.8 or 7.1 decreased ET-1 release in a dose-dependent manner (Figure 1A). This effect was

Fig. 3. Effect of varying media pH (4 hr exposure) on ET-1 mRNA levels (Panel A, Northern analysis) and ET-1 promoter activity (Panel B, transient transfection analysis) in IMCD cells. Steady-state ET-1 mRNA levels are normalized to sNRP mRNA levels and data are expressed as percent of mRNA levels at pH 7.4 (N=6 each data point). For promoter analysis, IMCD cells were transiently transfected with a 3.2 kb mouse ET-1 promoter-luciferase reporter construct along with a renilla control 24 hr before varying media pH for 4 hr. For these latter experiments, N=12 each data point.

Fig. 4. Effect of varying media pH (4 hr exposure) on ET-1 immunoreactivity (Panel A) and ³H-leucine incorporation into IMCD cells (Panel B). In Panel A, a fixed amount of ET-1 was incubated with varying media pH in a test tube for 4 hr, then ET-1 content determined by the same ET-1 assay as used for determination of released and cell-associated ET-1 (N=6 each data point). In Panel B, IMCD cells were exposed to varying media pH for 4 hr, then ³H-leucine incorporation into cell protein determined as an index of total cell protein synthesis (N=6 each data point).

manifest as early as 1 hr of acid (HCl) exposure (due to limits in assay sensitivity, it is not possible to measure ET-1 release prior to this time point) and was evident at 4 and 24 hr of acid exposure (Figure 1A). Increasing pH to 7.7 for 4 hr (NaOH) increased ET-1 release (Figure 1A). Similar results were obtained using H₂SO₄ as the acid and NH₄OH as the base (data not shown). To determine if pH affected endothelin converting enzyme (ECE) activity, Big ET-1 release was determined. Big ET-1 release was also decreased at pH 6.8 and increased at pH 7.7 (4 hr incubation) (Figure 1B). Since ECE converts big ET-1 to ET-1, these studies indicate that pH did not affect ECE activity.

To determine if pH affected secretion of ET-1 or Big ET-1, cell-associated peptide activity was assayed. Relatively little ET-1 remained cell-associated (about 10% of that released) and, while not statistically significant, tended to be reduced by lower pH (4 hr exposure) (Figure 2A). Cell-associated Big ET-1 was also markedly lower than secreted Big ET-1 (about 5%); Big ET-1 cell content was decreased at pH 6.8 and increased at pH 7.7 (4 hr exposure) (Figure 2B). Hence, pH did not apparently affect Big ET-1 or ET-1 secretion.

To determine if pH regulation of ET-1 release

occurred at the transcriptional and/or mRNA level, the effect of pH on ET-1 steady-state mRNA content and ET-1 promoter activity was determined. Varying pH did not alter IMCD cell ET-1 mRNA content (4 hr exposure) (Figure 3A). Cells were transiently transfected with a 3.2 kb ET-1 promoter-luciferase reporter (this sized construct previously shown to confer maximal ET-1 promoter activity [8]). Varying pH did not affect ET-1 promoter activity (Figure 3B).

To determine if pH affected ET-1 stability or degradation, a fixed amount of ET-1 was incubated for 4 hr at varying pH in a test tube, then measured by immunoassay. Changing pH had no effect on ET-1 stability or immunoreactivity (Figure 4A). To evaluate whether pH globally affected IMCD protein synthesis, ³H-leucine incorporation was assessed. Varying pH did not alter ³H-leucine incorporation (Figure 4B), suggesting no non-specific inhibition of ET-1 synthesis.

The above results indicated that pH regulates ET-1 synthesis either at mRNA translation or preproET-1 cleavage to Big ET-1. No assay is currently available to measure preproET-1, hence we attempted to transfect IMCD cells with preproET-1 cDNA coupled to a His-epitope tag and assay for the effect of pH on cellular

His-Tag content. No His-Tag was detectable at any pH (despite transfection of about 30% of cells on a 10 cm dish). Since preproET-1 cleavage has been reported to be furin protease-dependent, the effect of furin convertase inhibition (50 μ M decanoyl-Arg-Val-Lys-Arg-chloromethylketone) on His-Tag levels was determined. Furin inhibition yielded a very small band for His-Tag that was poorly reproducible (data not shown). No appreciable effect of media pH, whether cells were exposed to varying pH for 4 or 24 hr, was noted. However, given the extreme difficulty in detecting a signal, the failure to see a pH effect is likely not meaningful.

Discussion

The current study demonstrates that extracellular acidification reduces, and alkalization increases, IMCD ET-1 production. The biosynthetic pathway for ET-1 involves gene transcription, mRNA translation to preproET-1, conversion of preproET-1 to big ET-1 (in part by furin convertase), and cleavage of big ET-1 to mature ET-1 by several endothelin converting enzymes (ECE) [1]. The majority of studies indicate that ET-1 synthesis is controlled at the mRNA level, primarily through modulation of gene transcription, and to a lesser extent through regulation of mRNA stability (due to AUUUA regions in the 3' UTR) [11, 12]. However, the pH effect observed in the current study was not dependent on transcription or mRNA stability in that pH did not affect ET-1 promoter activity or mRNA levels. There is some evidence that extracellular ET-1 may be controlled by modulation of ECE activity or via a non-constitutive secretory pathway [13], however pH did not affect ECE activity as evidenced by a similar direction and magnitude of pH-induced changes in big ET-1 as compared to mature ET-1. pH also did not alter secretion of Big ET-1 or ET-1. pH also did not affect ET-1 degradation, consistent with previous reports demonstrating lack of an effect of pH on ET-1 secondary structure [14]. Furthermore, no non-specific effect of pH on total IMCD protein synthesis was observed. The only remaining possibilities for pH regulation of ET-1 synthesis were at the level of mRNA translation or preproET-1 cleavage to Big ET-1. We were unable to determine which of these latter two processes are responsible, however either would represent previously unreported mechanisms for regulation of ET-1 production in any cell type.

PreproET-1 is cleaved at paired basic amino acids to form Big ET-1. The subtilisin-like pro-protein convertase pathway has been implicated, at least in part,

in this process - most notably furin convertase, and also PC7 [15-17]. The furin propeptide is cleaved in an acidic environment, most likely within the trans-Golgi network and endosomal compartments, with formation of active furin capable of cleaving a host of substrates [18]. Such acid activation, if involved in ET-1 formation, would increase ET-1 production, not decrease it as seen in the current study. Furthermore, it seems rather unlikely that pH regulation of ET-1 production would primarily target furin or other prepropeptide cleaving enzymes since these enzymes generally have multiple protein targets - such a system would have a high degree of non-specificity.

pH regulation of mRNA translation has not been extensively studied. pH can affect cytoskeleton structure which, in turn, could affect mRNA translation by altering actin interaction with protein synthetic machinery [19]. Cap-dependent mRNA translation is also pH dependent [20], although this would not be able to confer specific mRNA regulation. Most notably, preprogastrin mRNA translation is increased by luminal gastric acid, however the mechanism by which this occurs is unknown [21]. Regulation that is specific to a given mRNA is well known to be conferred by the 5'- and 3'-UTRs [22]; this seems like the most probable means by which pH might regulate mRNA translation. Examination of such regulation is clearly required, however it will present substantial challenges; we were unable to accurately measure ET-1 mRNA translation rates in intact cells, despite multiple efforts to optimize such detection (including using a specific inhibitor of furin convertase to increase His-tagged preproET-1 detection [16]).

The current study does not address the biologic significance of pH-inhibited IMCD ET-1 production. The studies were performed at pH's likely to be encountered under physiologic conditions. Optimal cell functions occur at pH 7.4, including trafficking, gene transcription and RNA translation, hence this pH was chosen as a reference. Notably, ECE-1 functions optimally at pH 7.1, but this is only one aspect of the ET-1 biosynthetic pathway [1, 13]. The pH range of 6.8-7.7 is very relevant to kidney function. These ranges reflect the entire range of pH that would be encountered in the plasma under pathologic conditions. In addition, this pH range reflects part of the range of urinary pH seen during acid or alkaline loading. Urine pH varies from 5.0 to 9.0, however collecting cells are protected from these extreme urine pH changes by very low apical membrane proton permeability as well as a variety of transporters. It is very likely, therefore, that collecting duct cell pH varies within the range that we studied.

As discussed earlier, metabolic acidosis-stimulated renal acid excretion is partially dependent upon renal ET-1 [3-6]. Such ET-1 generation has been hypothesized to be due to acid stimulation of renal microvascular endothelial cells [23]. Interestingly, this latter study presented evidence that acid-stimulated ET-1 production was transcription-dependent. It is evident, therefore, that very different mechanisms are operative in pH regulation of ET-1 synthesis in IMCD and renal microvascular endothelial cells. Why such differential regulation of ET-1 production should occur is speculative and will require further investigation.

In summary, the current study describes a novel mechanism for regulation of ET-1 production via a

pathway independent of gene transcription, mRNA levels, ECE activity, ET-1 secretion or ET-1 degradation. In particular, the findings most strongly point to the possibility of regulation of ET-1 production at the mRNA translation level. Given that ET-1 has been implicated in a plethora of physiologic and pathologic processes, identification of a new mechanism for modulation of ET-1 production is of substantial potential biologic importance.

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