An autocrine role for endothelin-1 in the regulation of proximal tubule NHE3

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Background. Chronic metabolic acidosis leads to an increase in NHE3 activity that is mediated by endothelin-1 (ET-1) expression and activation of the proximal tubule endothelin B receptor. Chronic metabolic acidosis increases preproET-1 mRNA abundance in kidney cortex, but the cell responsible has not been identified.

Methods. PreproET-1 mRNA abundance was quantified by competitive reverse transcription-polymerase chain reaction (RT-PCR) on tissue harvested from control rats or rats in which chronic metabolic acidosis was induced by addition of NH4Cl to the drinking water.

Results. Chronic metabolic acidosis leads to an increase in preproET-1 mRNA expression in kidney cortex, proximal tubules, and glomeruli. The increase in preproET-1 expression correlates with the decrease in blood [HCO3−]. ET-1 expression is also increased by acidosis in abdominal aorta, but not in cardiac muscle.

Conclusion. In the renal proximal tubule, chronic metabolic acidosis induces an increase in preproET-1 expression, providing a mechanism for autocrine regulation of proximal tubule NHE3 activity. This response is not unique to the proximal tubule cell, but is also not ubiquitous.

The kidney adapts to an acid load by increasing H+ secretion and HCO3− reabsorption in the proximal and distal nephron, leading to an increase in net acid excretion. In the proximal tubule, the majority of H+ secretion is mediated by the Na+/H+ antiporter isoform, NHE3 [1–4]. Chronic metabolic acidosis increases proximal tubule apical membrane NHE3 activity and protein abundance [3, 5–7]. The endothelins play a key role in acid regulation of proximal tubule NHE3. Endothelin-1 (ET-1) causes phosphorylation and trafficking of NHE3 to the apical membrane, resulting in an increase in NHE3 activity [8–12]. Chronic acidosis elicits an increase in renal cortical preproET-1 mRNA expression [13]. Finally, in mice deficient in the ETB receptor, acid feeding causes a more severe metabolic acidosis and has no effect on proximal tubule apical membrane NHE3 activity [13].

The purpose of the present study was to determine the source of the acid-induced increase in cortical ET-1 synthesis. Possible sources include vascular endothelial cells, glomeruli, and/or the proximal tubule itself. In addition, we addressed the question of whether an acidosis-induced increase in ET-1 expression is specific to the kidney, or whether this is a more generalized effect. Cardiac myocytes and vascular endothelial cells both synthesize ET-1, where it increases contractility in cardiac and vascular smooth muscle cells, respectively [14–17]. In heart failure, up-regulation of myocardial ET-1 expression supports contractility, although the long-term sequella of increased ET-1 expression is further progression of heart failure [18, 19]. As chronic metabolic acidosis impairs heart function, acidosis-induced increases in ET-1 synthesis could contribute [20–22]. Thus, the effect of chronic metabolic acidosis on ET-1 synthesis was also examined in cardiac and vascular tissue.

METHODS
Materials and supplies

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) except collagenase B, which was from Boehringer Mannheim (Indianapolis, IN, USA); guanidium thiocyanate from Fisher Scientific (Pittsburgh, PA, USA); EcoICRI from Promega (Madison, WI, USA); and random primers, Superscript II reverse transcriptase, Taq DNA polymerase, deoxynucleoside triphosphate (dNTP) mix, and low glucose Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 culture media from Gibco BRL (Gaithersburg, MD, USA).
**Experimental animals**

Experiments were performed in male Sprague-Dawley rats, weighing 230 to 320 g, fed regular rodent Chow and drinking water ad libitum. A chronic metabolic acidosis was induced by addition of 0.3 mol/L NH₄Cl to the drinking water for 2 or 7 days. Control animals were administered regular drinking water. Rats were anaesthetized and the kidneys harvested and placed in ice cold phosphate-buffered saline (PBS) buffer (pH 7.4). The cortex was then carefully dissected and used to isolate proximal tubules or glomeruli, as described below, or immediately transferred to ice-cold 4 M guanidinium thiocyanate (GTC), 0.025 mol/L Na₃ citrate, 0.5% sarcosyl, and 0.72% β-mercaptoethanol, pH 7.0, for extraction of total RNA.

For the aorta studies, about 2 cm of abdominal aorta was carefully separated from the surrounding connective tissue, harvested, and immediately placed in ice-cold PBS buffer (pH 7.4). The aorta was placed on an ice block and minced with a razor blade, placed in ice-cold PBS buffer (pH 7.4). The tissue was then pelleted by gravity × 5 minutes, the supernatant removed, and the tissue transferred to 700 µL ice-cold GTC (pH 7.0) and sonicated on ice for 3 to 5 seconds × 3 to 5.

For the studies on cardiac tissue, 2 to 3 mm cubes of left ventricular muscle were harvested and immediately placed in ice-cold PBS buffer (pH 7.4). The tissue was placed on an ice block and cut into 1 to 2 mm pieces, which were then transferred in 3 to 5 mL ice-cold PBS buffer (pH 7.4) to a Dounce homogenizer, and homogenized with 5 to 10 strokes. The tissue was then pelleted by gravity × 5 minutes, the supernatant removed, and the tissue transferred to 700 µL ice-cold GTC (pH 7.0) and sonicated on ice for 3 to 5 seconds × 3 to 5.

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**Isolation of proximal tubules**

In the first protocol, kidneys were harvested and the cortex immediately removed, sliced with a Stadie-Riggs tissue slicer, and transferred to ice-cold, sterile PBS buffer (pH 7.4). Isolation of proximal tubules was carried out as previously described [23, 24]. Briefly, tissue slices were washed three times in Prep-Media (50:50 low glucose DMEM:Ham’s F12) and incubated in 10 mL of 95% O₂/5% CO₂ preequilibrated Prep-Media containing 1.9 mg/mL collagenase B × 10 to 12 minutes at 37°C in a shaking water bath. Collagenase B digestion was stopped by the addition of 10 mL ice-cold Prep-Media, and tissue kept on ice for the remaining steps. The digested tissue was passed through an 80 mesh size stainless steel sieve (sieve openings 180 µm) and the flow-through then placed on a second sieve (170 mesh size, sieve openings 90 µm) (Fisher Scientific). The proximal tubules, which remained on the second sieve, were loosened with a few milliliters of ice-cold Prep-Media, aspirated into a pasteur pipette, washed, and gravity-pelleted in 15 mL ice-cold Prep Media for 10 to 15 minutes. After aspirating off the supernatant, the pelleted tubules were washed three times in 15 mL of ice-cold Prep Media and washed three times in 15 mL ice-cold PBS buffer (pH 7.4). The tubules were recovered from each wash by gravity pelleting. After the last wash, tubules were pelleted by centrifugation at 45g × 3 minutes at 4°C, the supernatant discarded, the proximal tubule fragments transferred to 4 mol/L GTC, and total RNA extracted as described below. In all tubule preparations, we carefully evaluated the purity and integrity of the tubules by microscopy, and did not find evidence of “attached” capillaries or other debris.

In some experiments, kidneys were perfused with an Fe₃O₄ suspension prior to harvest to ensure removal of all glomeruli from the tubule preparation [25]. Briefly, kidneys were slowly perfused via the abdominal aorta with 10 to 15 mL of heparinized PBS buffer (pH 7.4), followed by 10 mL of a 2% Fe₃O₄ suspension in PBS, in which the iron particle size was <5 µm and the suspension sonicated × 5 minutes just before use. Ligation of the aorta both proximal and distal to the renal arteries prevented perfusion of the general circulation, and a small nick in the vena cava permitted runoff of perfused fluid. Following perfusion, kidneys were harvested, dissected, and the proximal tubules isolated as described above, with the following modification. After sieving through the 80 mesh sieve, the flow through, containing proximal tubule fragments and some glomeruli, was exposed to a magnet × 1 minute (Dynal MPC-1 Magnet) (Dynal, Oslo, Norway), thereby removing the Fe₃O₄-loaded glomeruli from the tubule suspension. The suspension was then placed on the second (170 mesh size) sieve and processed as described above.

**Isolation of glomeruli**

Glomeruli were isolated by differential sieving, using a previously published protocol modified as indicated [26, 27]. Briefly, kidneys were harvested as described above without perfusion, the cortex removed and diced into small pieces (1 to 2 mm³), collagenase B digested, and passed through an 150 mesh size sieve (sieve openings 106 µm), which was placed on top of a 60 mesh size sieve (sieve openings 250 µm) for stability. The flow through, containing the glomeruli, was placed on a 200 mesh size sieve (sieve openings 75 µm). The glomeruli, which now remained on top of the sieve, were loosened by repeated applications of 1 to 3 mL of ice-cold PBS onto the sieve, aspirated into a pasteur pipette, and washed and gravity-pelleted in 10 to 15 mL ice-cold PBS buffer (pH 7.4) for 10 to 15 minutes three times as described above. Following the third wash, the glomeruli were pelleted by centrifugation at 45g × 3 minutes at 4°C, the supernatant discarded,
and the glomeruli transferred to 4 mol/L GTC for RNA extraction.

**Extraction of total RNA and quantification of preproET-1 mRNA**

Total RNA was extracted using phenol/chloroform, as previously described [28]. PreproET-1 mRNA abundance was quantified by competitive reverse transcription-polymerase chain reaction (RT-PCR) using a 321 bp heterologous sequence generated from rat NHE3 as the internal standard [13, 29]. The DNA template-containing vector (pCRII-TOPO) was cut with EcoI CRI and cRNA was transcribed using a RNA transcription kit with T7 RNA polymerase (Stratagene, La Jolla, CA, USA).

cDNA was generated from total RNA and internal standard cRNA using random primers and Superscript II reverse transcriptase. The amount of total RNA varied depending on the tissue and batch of internal standard, and is indicated in the figure legends. The concentration of internal standard varied from 0.16 to 100 pg/μL. For annealing of random primers, 4 μL of total RNA and 4 μL of internal standard cRNA were supplemented with 1 μL of random primers (1 μg/μL) and 3 μL of diethyl pyrocarbonate (DEPC)-treated water, making a final volume of 12 μL for each sample. After annealing of random primers to the RNA (at 70°C × 10 minutes), samples were cooled on ice × 10 minutes and supplemented for reverse transcription with 2 μL ice-cold 10× PCR buffer [200 mmol/L Tris-HCl (pH 8.4) and 500 mmol/L KCl], 1 μL of 50 mmol/L MgCl2, 1 μL of 10 mmol/L dNTP solution, 2 μL of 0.1 mol/L dithiothreitol (DTT), 1 μL of Superscript II reverse transcriptase, and 1 μL of H2O. Reverse transcription was performed by incubation at 25°C × 10 minutes, followed by 42°C × 50 minutes. The reaction was stopped by incubation at 70°C × 15 minutes, followed by 95°C × 5 minutes.

For PCR, 2 μL of cDNA containing sample was cooled on ice × 10 minutes and then supplemented with 5 μL ice-cold 10× PCR buffer (see above), 1.5 μL of 50 mmol/L MgCl2, 1 μL of 10 mmol/L dNTP solution, 10 pmol of each primer, 0.5 μL of Taq DNA polymerase, and 38 μL of DEPC-treated water making a final volume of 50 μL for each sample. The preproET-1 sense primer was 5’-ATGGATTATTTTCCGTTGAT-3’ (bp 1 to 20) and the antisense primer was 5’-GGGAGTTGAGCCCCAGA TGA-3’ (bp 212 to 231) [30, 31]. After denaturation of DNA (at 94°C × 3 minutes), 32 PCR cycles were performed (each cycle 94°C × 1.5 minutes, 50°C × 1.5 minutes, and 72°C × 1.5 minutes), followed by final extension (at 72°C × 10 minutes). The PCR products were electrophoretically analyzed using a 2.2% agarose gel and fluorescence intensity quantified using NIH Image 1.54 software. Results are normalized for total mRNA abundance (see below) and expressed as a ratio of acid-fed/control animals.

**Measurement of mRNA abundance**

Dilutions of total RNA (2.5 and 1.25 ng/μL) of each RNA sample were denatured in 2.2 mol/L formaldehyde and 15× standard sodium citrate (SSC) (1 × SSC: 0.15 mol/L NaCl and 0.015 mol/L Na3 citrate, pH 7.0) at 65°C × 10 minutes, and dot blotted to a nylon transfer membrane (HybondN+ N+ nucleic acid transfer membranes) (Amersham, London, England) using a vacuum driven dot blot apparatus (Convertible® filtration manifold system) (Gibco BRL). RNA was cross-linked to the nylon membrane at 80°C × 2 hours, and prehybridized by incubation at 42°C × 2 hours in 20 mL of a solution containing 4× SSC, 50 mmol/L Na3PO4 (pH 7.0), 1× Denhardt’s solution, and 0.2% sodium dodecyl sulfate (SDS). Hybridization was performed at 42°C overnight in 10 mL of the prehybridization solution containing 1.5× 106 cpm of 32P-radiolabeled oligo-dT. After hybridization, the membrane was washed in 2× SSC and 0.1% SDS at room temperature × 5 minutes, followed by a second wash at 37°C × 30 minutes. The membrane was then exposed to film and labeling quantified by densitometry.

**Statistics**

Statistical significance was determined by Student t test, with P < 0.05 considered to be statistically significant. All data sets passed the normality test.

**RESULTS**

**Animal characteristics**

Rats administered 0.3 mol/L NH4Cl drinking water developed a chronic metabolic acidosis within 2 days (plasma [HCO3–], acid-fed 20.0 ± 0.6 mmol/L vs. control 29.0 ± 0.4 mmol/L; P < 0.001) (Fig. 1). After 7 days of NH4Cl ingestion, plasma [HCO3–] in acid-fed animals returned to control values (acid-fed 29.3 ± 1.4 mmol/L vs. control 29.8 ± 0.4 mmol/L) (Fig. 1). Thus, animals were able to correct the metabolic acidosis and restore acid-base balance to normal in spite of continued ingestion of acid. This result is similar to that previously reported by us in rats [5].

**Effect of acidosis on preproET-1 mRNA abundance in rat kidney cortex**

PreproET-1 mRNA abundance was quantified by competitive RT-PCR as described in the Methods section. Results are normalized for the abundance of total mRNA by dot blot analysis and expressed as a ratio of preproET-1 mRNA abundance in acid-fed vs. control animals.
After 2 days of NH₄Cl ingestion, renal cortical preproET-1 mRNA abundance was increased in acidotic animals to 192 ± 18% of control animals (Fig. 2). After 7 days of NH₄Cl intake, cortical preproET-1 mRNA abundance in acid-fed animals was not different than in control animals (Fig. 2). This result is different than that previously obtained in mice, in which preproET-1 mRNA abundance was increased 2.4-fold after 7 days of NH₄Cl administration [13]. However, in rats plasma [HCO₃⁻] returned to normal by 7 days, whereas plasma [HCO₃⁻] was less than control in acid-fed mice at 7 days.

The dependence of the increase in preproET-1 expression on acidemia is further demonstrated when the magnitude of regulation is compared to the change in plasma [HCO₃⁻]. As shown in Figure 3, when each animal pair is considered as a data point, the acid feeding-induced increase in cortical preproET-1 mRNA expression is related to the magnitude of the acidosis, and is only increased when acidemia is present. A similar analysis performed on data obtained from isolated proximal tubules showed the same general relationship, but with somewhat more scatter in the data (P = 0.112).

Source of preproET-1 expression in kidney cortex

To more specifically identify the source of the acid-induced expression of cortical ET-1, preproET-1 mRNA abundance was measured in proximal tubules isolated by sieving. Similar to the studies in whole cortex, preproET-1 mRNA abundance was increased 113 ± 40% in acid-fed compared to control rats at 2 days, and was not different from control animals at 7 days (Fig. 4).

Glomeruli are a known source of ET-1 within the cortex, reaching expression levels comparable to that of the inner medullary collecting duct [30, 32]. In isolated glomeruli harvested from rats fed acid × 2 days, preproET-1 mRNA abundance was increased 89 ± 28% compared to glomeruli harvested from control rats (Fig. 5), similar to the increase in expression observed in whole cortex and proximal tubule suspensions.

Given that glomeruli contaminate proximal tubule preparations, and that ET-1 expression in glomeruli is regulated by acidosis, it was possible that the observed increase in ET-1 expression in proximal tubules was attributable to contaminating glomeruli. To address this, a second set of studies were performed in proximal tubules suspensions where glomeruli were removed.
Fig. 4. Endothelin-1 (ET-1) expression in isolated proximal tubules. PreproET-1 mRNA abundance was quantified by competitive reverse transcription-polymerase chain reaction (RT-PCR) in proximal tubules harvested from control and acid-fed rats at 2 and 7 days. Total amount of RNA used was 160 to 238 ng (2 days) and 112 to 198 ng (7 days). Results are plotted on the y-axis as % of control (N = 6 for both 2 and 7 days). ∗P < 0.05.

Fig. 5. Endothelin-1 (ET-1) expression in isolated glomeruli. PreproET-1 mRNA abundance was quantified by competitive reverse transcription-polymerase chain reaction (RT-PCR) in glomeruli harvested from control and acid-fed rats at 2 days. Total amount of RNA used was 72 to 104 ng. Results are plotted on the y-axis as % of control (N = 6). ∗P < 0.05.

by Fe₃O₄ perfusion. In these glomeruli-free isolated proximal tubules, preproET-1 mRNA abundance was increased 170 ± 64% in tubules from acid-fed as compared to control rats at 2 days (Fig. 6).

PreproET-1 mRNA abundance in aorta and heart

The above data demonstrate that metabolic acidosis stimulates ET-1 expression in at least two cell types in the kidney. To determine whether this is a generalized effect of acidosis on all ET-1 synthesizing cells, or is specific to the kidney, we examined the effect of metabolic acidosis on ET-1 synthesis in cardiac myocytes and vascular cells in the abdominal aorta.

After 2 days of acid feeding, preproET-1 mRNA abundance in abdominal aorta was increased by 196 ± 55% in acid-fed as compared to control rats (Fig. 7), similar to the acid-induced increase found in kidney cortex, isolated proximal tubules, and glomeruli at the same time point. However, after 2 days of acid feeding, preproET-1 mRNA abundance in left ventricular heart tissue was not different from controls (+7 ± 13% in acid-fed compared to control animals) (Fig. 7).
DISCUSSION

Chronic metabolic acidosis induces a series of cellular responses, many of which serve to return blood pH to normal values and some of which are harmful [33]. In the renal proximal tubule, chronic metabolic acidosis induces an increase in transepithelial H+ ion secretion, ammonia synthesis, and citrate reabsorption. These effects are mediated by changes in the activities of a number of enzymes and transporters. One of these, NHE3, is the apical membrane Na/H antiporter that mediates the majority of H+ secretion into the lumen of the proximal tubule. Its activity and protein abundance on the apical membrane are increased by acid [3, 5]. ET-1 has similarly been demonstrated to increase apical membrane NHE3 activity [9, 10]. In cultured opossum kidney (OKP) cells, incubation in acid media and ET-1 each increase apical membrane NHE3 activity and protein abundance, although the effect of ET-1 is more rapid [6, 8, 11, 34]. This observation led to the hypothesis that ET-1 may mediate the effect of acidosis on NHE3. Indeed, acid feeding increases renal cortical preproET-1 mRNA expression in mice, and, in mice lacking the ETB receptor, acid feeding induces a more severe metabolic acidosis and has no effect on NHE3 activity [13].

The present results provide further information regarding increased expression of preproET-1. The endothelins generally do not function as circulating hormones, but rather function locally as paracrine or autocrine factors. In theory, acidosis could induce ET-1 production in neighboring endothelial cells, in glomeruli, or in the proximal tubule itself. The present results demonstrate that ET-1 likely functions as both an autocrine and a paracrine factor. Acid increases preproET-1 expression in the proximal tubule, which can then bind to ETB receptors and activate NHE3. In addition, acidosis leads to increased ET-1 production in glomeruli. The present studies do not address which glomerular cell type is responsible; endothelial cells may be the most likely source. ET-1 produced in glomeruli could flow from Bowman’s space into the proximal tubule lumen, where it could activate NHE3. In addition, acid regulation of aortic preproET-1 expression suggests that endothelial cell expression is acid regulated, and raises the possibility that capillary endothelial cells could serve as an additional source of ET-1 production.

In preliminary studies in tissue culture, we have confirmed the ability of acid pH to increase proximal tubule synthesis and secretion of ET-1 (unpublished observations). OKP cells, a proximal tubule epithelial cell line, were demonstrated to secrete ET-1 and big ET-1 (an ET-1 precursor). Lowering media pH increased the rate of secretion of both ET-1 and big ET-1. Similarly, in other preliminary studies, we have shown that lowering media pH increases expression of a preproET-1 promoter/luciferase reporter gene construct in OKP cells (unpublished observations). Taken together, these studies confirm that the proximal tubule is able to respond to decreased pH by increasing preproET-1 gene expression and ET-1 secretion.

The time course of the observed effect on ET-1 expression is also of interest. We have previously shown in rats that chronic acid feeding initially induces a decrease in blood pH and [HCO3−], but that these parameters return to normal within 7 to 14 days [5]. In spite of the apparent loss of acidemia, apical membrane NHE3 protein abundance continues to rise. The present studies demonstrate that increased ET-1 expression is only seen early when acidemia is present, and specifically is not elevated at 7 days of acid feeding.

Thus, NHE3 protein abundance continues to increase when there are no measurable changes in blood pH or preproET-1 expression. We have, however, previously shown that the ETB receptor (and presumably ET-1) is required for any acid-induced increase in NHE3 [13]. We believe that this apparent paradox is related to the fact that rats eat and drink at night when they are active, and sleep during the day when we obtain the blood and RNA samples. Thus, the animals may be acidotic after ingesting the acid at night, with plasma composition returning toward normal during the day. As the animals increase their capacity for acid excretion, they are able to return bicarbonate concentration to normal by daytime. Thus, from 7 days onward, blood pH, plasma [HCO3−], and preproET-1 expression may be altered only at night, providing the stimulus for increased apical membrane NHE3 abundance.

The observation that acidosis increases preproET-1 expression in glomeruli and proximal tubule epithelial cells suggests that this effect is not unique to a single cell type. This was confirmed by demonstrating that preproET-1 expression was increased in aorta. The most likely source of ET-1 in the aorta is the endothelial cell, which may also be the source of ET-1 in glomeruli.

Cardiac myocytes also express ET-1, and ET-1 has been postulated to be pathogenic in these cells [14–19]. Thus, if acidosis increased ET-1 production in these cells, it could contribute to the cardiac dysfunction that has been reported in this condition [20–22]. However, our studies showed no effect of acidosis on myocardial preproET-1 expression.

CONCLUSION

These results demonstrate that the renal proximal tubule, glomerulus, and aorta increase ET-1 production in response to chronic metabolic acidosis. This effect is seen only when blood [HCO3−] is low. ET-1 produced in the glomerulus and proximal tubule binds to ETB receptors on the proximal tubule, causing trafficking of NHE3 to the apical membrane and increasing renal acidification.
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