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Endothelin up-regulation and localization following renal ischemia and reperfusion

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Endothelin up-regulation and localization following renal ischemia and reperfusion.

Background. Endothelin (ET), a potent vasoconstrictor, is known to play a role in ischemic acute renal failure. Although preproET-1 (ppET-1) mRNA is known to be up-regulated following ischemia/reperfusion injury, it has not been determined which component of the injury (ischemia or reperfusion) leads to initial gene up-regulation. Likewise, although ET-1 peptide expression has been localized in the normal kidney, its expression pattern in the ischemic kidney has not been determined. Therefore, the purpose of this study was twofold: (*a*) to determine whether ischemia alone or ischemia plus reperfusion is required for the up-regulation of ppET-1 mRNA to occur, and (*b*) to localize ET-1 peptide expression following ischemia in the rat kidney to clarify better the role of ET in the pathophysiology of ischemia-induced acute renal failure.

Methods. Male Lewis rats underwent clamping of the right renal vascular pedicle for either 30 minutes of ischemia (group 1), 60 minutes of ischemia (group 2), 30 minutes of ischemia followed by 30 minutes of reperfusion (group 3), or 60 minutes of ischemia followed by three hours of reperfusion (group 4). The contralateral kidney acted as a control. ppET-1 mRNA up-regulation and ET-1 peptide expression were examined using the reverse transcription-polymerase chain reaction and immunohistochemistry, respectively.

Results. Reverse transcription-polymerase chain reaction yielded a control (nonischemic) value of 0.6 ± 0.2 densitometric units (DU) of ppET-1 mRNA in the kidney. Group 1 levels (30 min of ischemia alone) were 1.8 ± 0.4 DU, a threefold increase ($P < 0.05$). Group 2 levels (60 min of ischemia alone) increased almost six times above baseline, 3.5 ± 0.2 DU ($P < 0.01$), whereas both group 3 and group 4 (ischemia plus reperfusion) did not experience any further significant increases in mRNA levels (1.9 ± 0.4 DU and 2.8 ± 0.6 DU, respectively) beyond levels in group 1 or 2 animals subjected to similar ischemic periods. ET-1 peptide expression in the ischemic kidneys was significantly increased over controls and was clearly localized to the endothelium of the peritubular capillary network of the kidney.

Conclusions. Initial ET-1 gene up-regulation in the kidney occurs secondary to ischemia, but reperfusion most likely contributes to sustaining this up-regulation. The marked increase of ET-1 in the peritubular capillary network suggests that ET-induced vasoconstriction may have a pathophysiological role in ischemic acute tubular necrosis.

Key words: ischemic renal failure, reperfusion injury, acute tubular necrosis, gene up-regulation, vasoconstriction.

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Endothelin (ET), a 21-amino acid peptide, is the most potent vasoconstrictor discovered to date [1]. Shortly after its discovery in 1988, it was suggested that ET might be an important mediator of acute renal failure (ARF) because of its intense vasoconstrictive properties [2]. Several lines of evidence supporting this hypothesis have been reported. First, it was shown that exogenous ET infused into the kidney was able to decrease renal blood flow and glomerular filtration rate (GFR) to create conditions that can lead to ARF [3, 4]. Shibouta et al, using a rat model of bilateral renal artery occlusion, reported that in addition to renal dysfunction and histologic evidence of proximal tubular necrosis, there was a concomitant rise in renal tissue levels of ET [5]. This established a link between a stimulus known to cause ARF, namely ischemia/reperfusion (I/R) injury [6, 7], and a rise in ET production. Subsequently, several groups demonstrated that the administration of either monoclonal antibodies to ET or ET receptor antagonists resulted in the improvement of both renal function and renal histology in the setting of I/R injury [8–11].

With a role for ET in the pathophysiology of ARF now established [12–15], we became interested in clarifying ET's impact on ischemia-induced ARF. Although it is known that renal I/R injury leads to an increased synthesis of ET via ET gene up-regulation [16], no one to date has determined whether the initial up-regulation of the ET gene is due to the ischemic or reperfusion component of the I/R injury. All studies we reviewed have examined ET gene up-regulation following a period

of ischemia and reperfusion [4, 5, 16], despite the fact that they can be examined as separate events. This distinction could be important in order to determine the appropriate timing of pharmacologic blockade of ET in clinical practice. Moreover, although the sites of ET synthesis have been determined in the normal human kidney [17], localization of ET production in the kidney following an ischemic insult has not been determined. Localizing the actual sites of ET-1 expression following ischemia will help to explain further its role in the pathophysiology of ARF.

Therefore, the purpose of this study was twofold: (*a*) to determine whether ischemia alone or ischemia plus reperfusion is required for the up-regulation of prepro-ET-1 (ppET-1) mRNA to occur by examining specific periods of ischemia and reperfusion for their individual and combined influences on ET-1 gene up-regulation; and (*b*) to localize ET-1 peptide expression following ischemia in the rat kidney to clarify better the role of ET in the pathophysiology of ischemia-induced ARF.

METHODS

Model of renal ischemia and reperfusion

Male Lewis rats, 250 to 300 g, were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN, USA) and were cared for in accordance with the Case Western Reserve University Animal Research Center regulations. All animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and underwent a midline laparotomy to expose the right kidney. The right renal pedicle was dissected free from surrounding tissues, and an atraumatic vascular clamp was placed across the renal artery, vein, and ureter for either 30 or 60 minutes of ischemia. To determine the effects of ischemia alone on ET-1 gene expression, group 1 animals ($N = 5$) underwent 30 minutes of ischemia without reperfusion, and group 2 ($N = 9$) underwent 60 minutes of ischemia also without reperfusion. To determine the additional impact of reperfusion on the same ischemic insults, group 3 animals ($N = 5$) underwent 30 minutes of ischemia followed by 30 minutes of reperfusion, and group 4 ($N = 5$) underwent 60 minutes of ischemia followed by a three-hour period of reperfusion. The contralateral kidney, which was not rendered ischemic in any of the test groups, acted as an internal control for each animal. After the assigned treatment period was completed, both the control and the ischemic kidney were removed and processed for further analysis.

Reverse transcription-polymerase chain reaction analysis for ppET-1 mRNA

Kidneys for reverse transcription-polymerase chain reaction (RT-PCR) analysis of ppET-1 mRNA were dissected free from surrounding tissue and were snap frozen in liquid nitrogen and stored at −70°C until ready for analysis. Determination of ppET-1 mRNA followed a method from Rhoten et al [18]. Briefly, 30 mg of tissue was homogenized in 600 μl of RNA lysis thiocyanate lysate buffer (Quiagen Inc., Chatsworth, CA, USA) using a hand-held RNase-free tissue grinder (Tekmar Inc., Cincinnati, OH, USA). The homogenate was then centrifuged at 15,000 × g in a microcentrifuge for three minutes. Total RNA was then extracted from the supernatant using the RNeasy total RNA kit from Quiagen. First strand complementary DNA was synthesized with 1 μg total RNA as a template for ET analysis and a downstream primer of the rat ppET-1 gene (National Biosciences Inc., Plymouth, MN, USA; Fig. 1B). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (0.1 μg) was used as an internal control. A reverse transcription reaction was performed (25 mM $MgCl_2$, 10 × PCR buffer II, 10 mM of each of the following: dATP, dCTP, dGTP, and dTTP, 1 μl of murine leukemia virus reverse transcriptase, and 1 μl of RNase inhibitor) at 42°C for 40 minutes. Next, PCR was performed by adding 25 mM $MgCl_2$, 10 × PCR buffer II, 0.75 μl of the upstream and downstream primers for rat ppET-1 (Fig. 1) or GAPDH (sequences not shown), 0.5 μl of AmpliTaq DNA polymerase, and RNase free water to the prior reverse transcription reaction mixture for two minutes at 95°C (template melting step), 35 cycles of melting for one minute at 95°C, annealing for one minute at 60°C, extending for one minute at 72°C, and one final extension for seven minutes at 72°C. (Note that the reactions for ppET-1 and GAPDH mRNA were carried out in separate microcentrifuge tubes. This was not multiplex PCR.) Samples were then vacuum dried, separated on a 2% agarose gel with 0.5 μg/ml ethidium bromide, and analyzed by scanning densitometry using the National Institutes of Health Image program for the Macintosh computer.

A) UPSTREAM PRIMER

CCT CCT CTT CTT CTG ATC CCT TTG

B) DOWNSTREAM PRIMER

TGT CTT TTT GGT GAG CAC ACT GG

Fig. 1. Polymerase chain reaction (PCR) primers for rat preproendothelin-1 gene. (*A*) Twenty-four nucleotide sequence for rat forward (upstream) primers of endothelin-1 (ET-1) cDNA representing positions 157 to 180 of the gene. (*B*) Twenty-three nucleotide sequence for rat backward (downstream) primers of ET-1 cDNA, representing positions 536 to 514 of the gene. Both primers were analyzed in MacVector 5-1 using rat ET-1 cDNA sequence from National Biosciences Inc.

Immunohistochemical staining for ET-1

Five animals from group 2 (60 min of ischemia alone) had both kidneys removed and coated in OCT-tissue

TEK II (Miles Laboratories, Naperville, IL, USA). These fresh samples were then frozen in liquid nitrogen cooled 2-methylbutane and stored at −70°C. The kidneys were sectioned at 5 μm on a cryostat, placed on slides, and refrozen overnight. Immunohistochemical staining was carried out according to the method of Rhoten et al [18]. Briefly, thawed slides were fixed in acetone for 10 minutes and were then washed in Dulbecco's phosphate-buffered saline (DPBS). To block endogenous peroxidases, the slides were washed in 60 ml of a 3% solution of hydrogen peroxide, methanol, and distilled water for 20 minutes. Slides were again rinsed in DPBS. To block nonspecific binding, the slides were washed with 10% normal goat serum for 30 minutes. After a third DPBS rinse, the slides were incubated with the primary antibody (mouse anti–ET-1 monoclonal antibody; QED Bioscience, Plymouth, MN, USA) at 1:100 and 1:200 dilutions in 1% normal goat serum in DPBS solution for one hour. The slides were then incubated with the horseradish peroxidase-labeled secondary antibody (goat anti-mouse IgG H + L antibody; Kirkegard and Perry Laboratories Inc., Gaithersburg, MD, USA) at 1:100 and 1:200 dilutions for one hour. Slides were developed using a diethylaminobenzidine solution, counterstained with methyl green and were then dehydrated for mounting in successive concentrations of ethanol (70%, 95%, and 100%) followed by xylene clearing. Following mounting (Eukitt reagent; Calibrated Instruments, Inc., Hawthorne, NY, USA), photomicrographs were taken of all slides with a Nikon Optiphot camera using Agfapan ASA25 film with a green filter. All photos were exposed and developed identically to allow semiquantitative comparisons of relative staining intensity.

Statistical analysis

Statistical analysis of our data was performed using the InStat program for Macintosh computers obtained from Graphpad Software (San Diego, CA, USA). The data presented are the mean ± SEM. P less than 0.05 is considered significant.

RESULTS

PreproET-1 mRNA levels

The purpose of these experiments was to determine whether ischemia alone or ischemia plus reperfusion is responsible for the initial up-regulation of ppET-1 mRNA. Therefore, a RT-PCR reaction was performed on total RNA extracted from the ischemic and control (nonischemic) kidneys from all four experimental groups to isolate ppET-1 mRNA. A representative gel produced from the control and ischemic kidney analysis (Fig. 2A) clearly demonstrates two isolated bands: a 380 bp mRNA product, representing ppET-1, and a second product at 452 bp, representing GAPDH. GAPDH acts as a housekeeping gene to ensure that the up-regulation of the ppET-1 gene was not simply a global gene up-regulation secondary to the ischemic injury. It also normalizes all samples for differences in input RNA. The RT-PCR reaction was a linear assay, as seen in Figure 2B. In this type of analysis, as the total amount of input RNA is increased, the signal intensity of the corresponding gel band increases. This allows semiquantitative analysis of

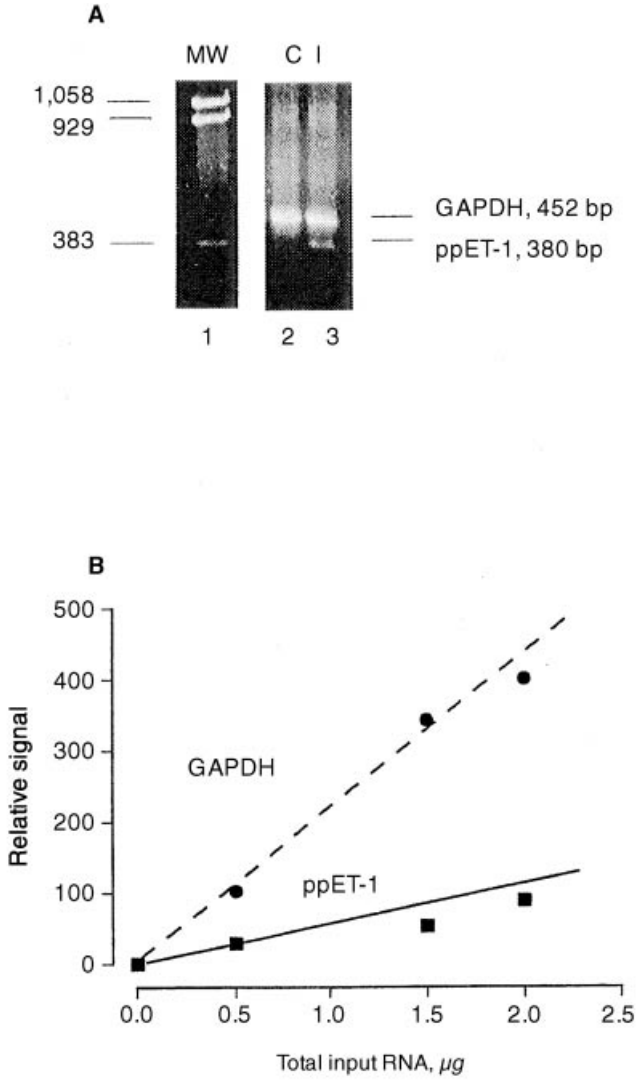

Fig. 2. Results of the RT-PCR analysis of preproendothelin-1 (ppET-1) mRNA levels. (*A*) Representative agarose gel demonstrating products of PCR amplification of rat renal ppET-1 mRNA. Lane 1 denotes the molecular weight (MW) of known products for comparison to experimental samples. Lane 2 denotes control (C) kidney—no ischemia. A weak band representing ppET-1 is seen at 380 bp. Lane 3 shows ischemic (I) kidney. Note the increased signal intensity in the 380 bp band representing an increase in ppET-1 mRNA. Ischemia acts as a potent stimulus to up-regulate ppET-1 mRNA. (*B*) Graph of the results of scanning densitometry of the gel shown in *A*, demonstrating that the mRNA produced was directly proportional to the amount of total input RNA, verifying that the assay was linear and could be subjected to semiquantitative analysis. Symbols are (●) GAPDH; (■) ppET-1.

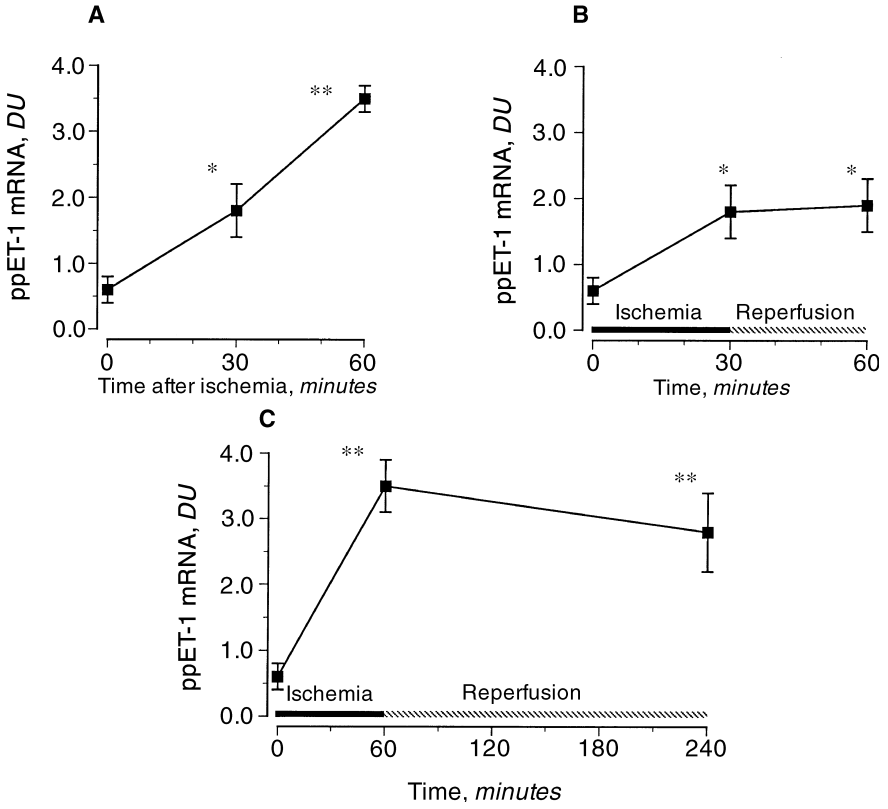

Fig. 3. Densitometric analysis of rat renal preproendothelin-1 mRNA following various ischemia/reperfusion injuries. All values of ppET-1 mRNA are expressed on the *y*-axis in densitometric units (DU). (*A*) Kidneys exposed to 30 minutes of ischemia (group 1) demonstrated an increase in ppET-1 mRNA three times above nonischemic controls (*$P < 0.05$). Extending the ischemia time to 60 minutes increased the levels to almost sixfold above baseline levels (**$P < 0.01$). Moreover, the doubling of ppET-1 levels seen by increasing the time of ischemia from 30 to 60 minutes was also statistically significant ($P < 0.05$). (*B*) Thirty minutes of ischemia plus 30 minutes of reperfusion (group 3) essentially maintained but did not lead to any significant increases in the levels of ppET-1 mRNA when compared with levels observed after 30 minutes of ischemia alone. Likewise (*C*), the mRNA levels of ppET-1 following 60 minutes of ischemia with 180 minutes of reperfusion (group 4) did not increase the levels of ppET-1 mRNA transcription over levels achieved with 60 minutes of ischemia alone. These studies implicate ischemia as the primary stimulus that initially up-regulates the ppET-1 gene.

the stained gel bands to be performed. When we examined the gel bands from our various experimental groups, utilizing densitometric analysis, we found the results that are outlined in Figure 3.

PreproET-1 mRNA levels in normal rat kidneys were 0.6 ± 0.2 densitometric units (DU). This represents the baseline expression of ppET-1 mRNA in the rat kidney (refer to the 0-min time on Fig. 3A). In our first set of experiments, which were designed to examine the impact that ischemia alone has on ppET-1 mRNA transcription levels, group 1 kidneys (exposed to 30 min of ischemia; Fig. 3A) exhibited a rise in ppET-1 mRNA levels to 1.8 ± 0.4 DU. This rise represented a significant ($P <$ 0.05) threefold increase in mRNA expression, indicating that a minimal ischemic event without reperfusion can evoke a significant increase in ppET-1 mRNA transcription. When we increased the kidney's ischemic period to 60 minutes (group 2; Fig. 3A), the mRNA levels increased to 3.5 ± 0.2 DU, ($P < 0.01$), almost six times that of control kidneys. Notably, when we compared group 1 to group 2, the doubling of ppET-1 mRNA levels (1.8 ± 0.4 DU to 3.5 ± 0.2 DU), resulting from the additional 30 minutes of ischemia, was also statistically significant ($P < 0.05$). This trend points to the obvious concern of always minimizing the amount of time an organ is exposed to ischemia in the controlled setting (that is, clamp time in vascular surgery).

In our second set of experiments, we wished to determine what additional impact the specific reperfusion times we chose would add to ppET-1 mRNA synthesis. Therefore, group 3 kidneys were exposed to 30 minutes of ischemia followed by 30 minutes of reperfusion. This experimental group serves two purposes: First, it allows a direct comparison to our findings in group 1 (30 min ischemia alone), and second, it represents an equivalent amount of time (60 min total) when compared with group 2 (60-min ischemia alone). Group 3 treatment resulted in 1.9 ± 0.4 DU of ppET-1 mRNA. Although this amount of mRNA up-regulation is significant when compared with our controls ($P < 0.05$), it is not significantly different from group 1 levels (1.8 ± 0.4 DU; Fig. 3B). Finally, group 4 kidneys were exposed to a 60-minute period of ischemia followed by a three-hour period of reperfusion. The ppET-1 mRNA level in these kidneys was 2.8 ± 0.6 DU (Fig. 3C). Again, although this level of expression was still almost six times that observed in normal kidneys, it was not significantly different from the ppET-1 mRNA levels noted in group 2 (animals exposed to the same 60 min of ischemia without reperfusion).

Localization of ET-1 expression in the ischemic kidney

Endothelin-1 peptide expression has been localized via immunohistochemistry to several areas in the normal

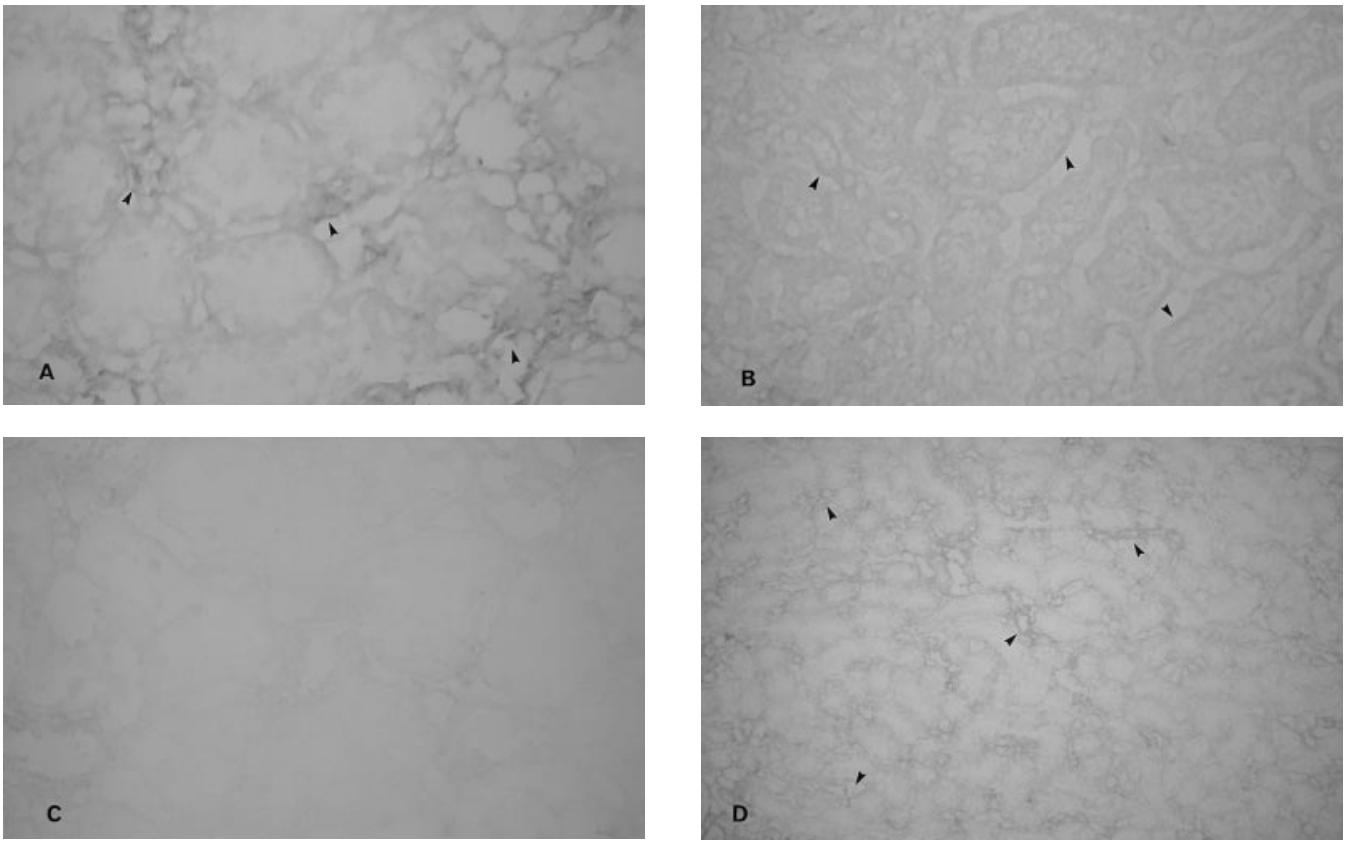

Fig. 4. Photomicrographs demonstrating ET-1 peptide expression in the cortex of the rat kidney following ischemia. (*A*) Magnification (×40) of a kidney exposed to 60 minutes of ischemia. ET-1 expression is seen in the endothelium of the peritubular capillary network (arrows, dark reaction product). (*B*) Magnification (×40) of the normal (nonischemic) kidney of the same animal as in panel A. Minimal staining is present in portions of the PTCN (arrows) surrounding the convoluted tubules. Control specimens were also tested with nonimmune serum in an ischemic kidney (*C*) to illustrate that immunoreactivity was specific for ET-1. (*D*) Finally, a low-power (×10) magnification) view of the ischemic treated kidney shows the relatively universal expression of ET-1 in the PTCN throughout the cortex of the ischemic kidney (arrows point to a few of the many peritubular capillaries expressing the ET-1 peptide).

kidney, including the endothelial lining of interlobular and arcuate arteries, glomerular capillaries, and some convoluted tubular epithelium [17]. However, the localization of ET-1 following an ischemic event has yet to be reported. We postulated that because of the significant increase in ET-1 gene up-regulation that we found following ischemia, there would be a concomitant increase in ET-1 peptide expression. Because ischemic injury to the kidneys primarily affects the renal tubules [6], we hypothesized that the tubular epithelium or surrounding peritubular capillaries would be the primary site(s) of this increased expression of ET-1 peptide. Indeed, we found markedly higher levels of ET-1 peptide in the endothelium of the cortical peritubular capillaries of ischemically injured kidneys (Fig. 4A, arrows, dark reaction product) compared with low levels of peritubular capillary ET-1 in control (nonischemic) kidneys (Fig. 4B, arrows point to peritubular capillaries with minimal staining). When we examined the medullary regions of these same kidneys (not shown), we did not note any significant increases in basal ET-1 expression. In contrast to the intensely stained capillaries, the ET-1 staining in the cortical tubular epithelium of ischemic kidneys was only slightly elevated or normal (Fig. 4A) when compared with controls. In addition, although not shown, we did note typical staining for ET-1 in the glomerular capillaries, interlobular and arcuate arteries, and veins in both control and ischemic kidneys. However, the amount of ET-1 peptide present was not significantly different between them.

To demonstrate that the enhanced staining pattern seen in the ischemic kidney truly represents ET-1 expression and not nonspecific antibody binding, the secondary antibody was applied alone to ischemic kidney sections. As illustrated in Fig. 4C, there was essentially no staining along the peritubular capillary network (PTCN), thereby providing a negative control. In addition, a low-power view of the ischemic kidney (Fig. 4D) illustrates that the peritubular capillary expression of ET-1 takes place uniformly throughout the cortex of the kidney following

a global ischemic insult. Taken together, these results indicate that renal ischemia increases ET-1 peptide expression uniformly throughout the endothelium of the cortical PTCN of the renal convoluted tubules, whereas baseline medullary expression of ET-1 seems unaffected.

DISCUSSION

Endothelin has been implicated as a contributing factor to ischemia-induced ARF [12–15]. The ET gene has been shown to be up-regulated by some of the same conditions seen in ARF, namely, hypoxia, hypotension, and I/R injury [4, 12–14, 16, 19]. Prior research has shown that after 45 minutes of ischemia, followed by a two-hour period of reperfusion, ppET-1 mRNA levels are increased [16]. However, this study does not clearly delineate which component of the injury—ischemia, reperfusion, or a combination of the two—leads to the initial increase in ET synthesis. Our studies, in a rat model of ischemic ARF, clearly indicate that ischemia alone initiates ppET-1 mRNA up-regulation in the kidney. After only 30 minutes of ischemia without reperfusion, ppET-1 mRNA expression had almost tripled, and by 60 minutes, it was over sixfold higher than control levels. When we added 30 minutes of reperfusion to a 30-minute ischemic insult (group 3), we saw no significant increase in ppET-1 levels. This trend was confirmed in our animals exposed to 60 minutes of ischemia and three hours of reperfusion; the ppET-1 mRNA levels were again not statistically different from kidneys exposed to 60 minutes of ischemia alone. These data implicate ischemia as the primary initiating stimulus for up-regulation of the ppET-1 gene. Although we did not see continued increases in ppET-1 mRNA in either of our reperfusion groups, the importance of the reperfusion injury cannot be dismissed. ppET-1 mRNA expressed in vascular endothelial cells is very unstable and has a half-life of only approximately 15 minutes [20]. Therefore, unless reperfusion continues to stimulate ppET-1 mRNA synthesis, we should have seen a decrease in the ppET-1 mRNA levels in group 4 after three hours of reperfusion, which we did not. Thus, reperfusion certainly plays a role in this injury cascade.

Our novel observation that ischemia alone increases ppET-1 mRNA synthesis may have important implications in regard to the clinical use of pharmacologic blockade against ET-induced vasoconstriction. In the context of renal vascular surgery or kidney donation for transplantation (both cadaveric and living related donors), in which varying periods of ischemia to the kidney are anticipated, an ET receptor antagonist could be used to protect against ARF and acute tubular necrosis (ATN). We have shown that ischemia alone is able to up-regulate the ppET-1 gene and that ET-1 peptide expression is clearly present in the cortical PTCN after 60 minutes of ischemia without any reperfusion. This peptide, once produced, will bind with intense affinity to ET receptors that have also been shown to be up-regulated or that at least demonstrate increased affinity for ET in models of ischemic ARF [21, 22]. This binding is significant and considered by some almost irreversible [15, 23]. Thus, if an ET receptor antagonist is not present and bound to receptors prior to ET peptide formation, its effectiveness as a potential agent to ameliorate vasoconstriction could be diminished. Therefore, because ET can be produced without reperfusion, ET antagonists may be most effective if given prior to the period of ischemia.

Endothelin's role in the pathophysiological process of ARF appears to be due primarily to its intense vasoconstrictive properties in the kidney. ET has classically been shown to cause vasoconstriction in both the afferent and efferent arterioles of the glomerular tuft when administered exogenously [3, 12, 24]. The vasoconstriction in this area, along with the vasoconstriction sometimes seen in the arcuate and interlobular arteries [17], has been the postulated cause of ET-induced ARF. However, the endogenous site(s) of ET-1 peptide expression in the kidney following an ischemic event has not yet been clearly delineated. Determination of these sites could further clarify the pathophysiology of ET-1 in ARF. Our immunohistochemical analysis of the kidney revealed increased expression of ET-1 in the endothelium of the cortical PTCN following an ischemic event. The PTCN represents a continuation of the efferent arteriole of the glomerulus into a capillary bed that surrounds the convoluted tubules [25]. Expression of ET-1 in the vascular bed, which directly surrounds and supplies the convoluted tubules, has potentially significant consequences. By restricting blood flow in the PTCN, ET-1 would cause hypoxia to the adjacent cells lining the tubules. Prolonged hypoxia from ongoing vasoconstriction in this bed could, in turn, lead to cell death and to the typical tubular epithelial sloughing seen in ATN. Consequently, our localization of ET-1 to the PTCN further supports the hypothesis of ET-1 as a possible pathophysiological factor in ATN.

Supporting evidence for ET acting in the PTCN and causing ATN may also come from ET receptor antagonist studies performed by others. The use of the ET_A receptor antagonist BQ 123 in both isolated perfused kidney models and *in vivo* models of renal ischemia led to improved renal blood flow, preservation of GFR, and a significant decrease in proximal tubular necrosis [9–11]. The improvements in renal blood flow and GFR have previously been shown to be due to the effects that ET has on the preglomerular and postglomerular vasculature, namely the afferent and efferent arterioles of the glomerulus and the mesangial cells [12–14]. Endothelin, by increasing afferent and efferent arteriolar resistance, reduces nephron plasma flow rate and diminishes single-

nephron GFR [3, 12, 13]. Moreover, our localization of ET to the postglomerular PTCN would be consistent with the dramatic improvement in histology seen in the proximal tubules of kidneys treated with ET receptor antagonists [8–11]. The presence of ET in the PTCN may also be related to other changes that typically accompany ATN. For example, during ATN, tubular reabsorption of sodium decreases [6, 7]. Supporting a contributory role for ET in this process are the reports that the infusion of ET or endogenous ET production stimulated by I/R injury has been shown to increase the fractional excretion of sodium [9, 10, 14, 15]. Conversely, the addition of a nonselective ET_{A+B} or a specific ET_A receptor antagonist led to increased net tubular reabsorption of sodium and a decreased fractional excretion of sodium in the same model [9, 10]. Once again, our localization studies place ET in a position in which it could directly exert these physiologic changes.

One putative role for ET in the PTCN may be on the recovery phase of ATN when epithelial cells regenerate and renal function returns. ET-1 is known to be a potent mitogen for several types of renal cells. Ong et al showed that hypoxia induces ET-1 gene up-regulation and that the ET-1 peptide subsequently produced was able to act as an autocrine growth factor in human proximal tubular cells *in vitro* [26]. The peritubular location of ischemia-induced ET-1 expression demonstrated in our *in vivo* experiments would be an ideal location for an autocrine growth factor to aid in the tubular epithelial cell regeneration process. Endothlin produced in the capillary network could be passed across the basement membrane of the tubular cell, where it exerts its mitogenic effect leading to tubular regeneration. The passage of ET, in this fashion, has previously been suggested by Borczuk, Berman and Factor, who noted some interesting immunohistochemical staining patterns in patients who died of complications associated with ARF [27]. Examination of postmortem kidneys from such patients revealed an increased interstitial staining pattern for ET-1 in the medulla, whereas a strong cortical tubular staining pattern was seen in normal kidney sections. These investigators speculated that the variation in staining for ET in the ARF kidneys could be due to the shedding of proximal tubular epithelium, which eventually passes into the medulla, where it forms a tubular cast. The staining pattern for ET-1 in our experiments may be related and indicate that ET-1 produced in the PTCN crosses into the tubular system and, along with cellular debris from ATN, eventually accumulate in the medulla. However, because we did not allow our animals to survive and progress into ATN, we cannot support or refute the hypothesis of Borczuk et al.

In conclusion, our data indicate that ischemia leads to a significant increase in ET-1 peptide expression and localizes this expression to the endothelium of the cortical peritubular capillaries of the ischemic kidney. This finding further implicates ET's role in ischemia-induced renal failure and may explain some of the impaired tubular function and tubular regeneration seen during the various phases of ATN. In addition, we have shown that the ppET-1 gene can be up-regulated by ischemia alone, but that reperfusion likely plays a role in sustaining ongoing gene up-regulation in the time frames that we examined. These points support the hypothesis that ET plays an important role in the pathophysiology of ARF and/or ATN. Moreover, they indicate that the most optimal time to administer ET receptor antagonists may be prior to the ischemic event.

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APPENDIX

Abbreviations used in this article are: ARF, acute renal failure; ATN, acute tubular necrosis; DPBS, Dulbecco's phosphate-buffered saline; DU, densitometric unit; ET, endothelin; GFR, glomerular filtration rate; I/R, ischemia/reperfusion; ppET-1, preproendothelin-1; PTCN, peritubular capillary network; RT-PCR, reverse transcription-polymerase chain reaction.

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