Endothelin-1 Increases Glomerular Permeability and Inflammation Independent of Blood Pressure in the Rat

Mohamed A. Saleh, Erika I. Boesen, Jennifer S. Pollock, Virginia J. Savin, David M. Pollock

Abstract—Endothelin (ET) 1 is a potent vasoactive peptide implicated in the pathogenesis of hypertension and renal disease. The aim of the current study was to test the hypotheses that ET-1 increases albumin permeability of glomeruli isolated from normal rats and that chronic ET-1 infusion will increase glomerular permeability and inflammation independent of blood pressure. Glomerular permeability to albumin was determined from the change in glomerular volume induced by exposing isolated glomeruli to oncotic gradients. Incubation of glomeruli taken from normal rats with ET-1 at a concentration that did not produce direct glomerular contraction (1 nmol/L) significantly increased glomerular permeability to albumin, reaching a maximum after 4 hours. Chronic ET-1 infusion for 2 weeks in Sprague-Dawley rats significantly increased glomerular permeability to albumin and nephrin excretion rate, effects that were attenuated in rats given an ET_A receptor antagonist (ABT-627, 5 mg/kg per day). Urinary protein and albumin excretion and mean arterial pressure (telemetry) were not changed by ET-1 infusion. Acute incubation of glomeruli isolated from ET-1-infused rats with the selective ET_A antagonist significantly reduced glomerular permeability to albumin, an effect not observed with acute treatment with a selective ET_B antagonist. Chronic ET-1 infusion increased glomerular and plasma soluble intercellular adhesion molecule 1 and monocyte chemoattractant protein 1 and elevated the number of macrophages and lymphocytes in renal cortices (ED-1 and CD3-positive staining, respectively). These effects were all attenuated in rats given an ET_A selective antagonist. These data support the hypothesis that ET-1 directly increases glomerular permeability to albumin and renal inflammation via ET_A receptor activation independent of changes in arterial pressure. (Hypertension. 2010;56:942-949.)

Key Words: intercellular adhesion molecule ■ monocyte chemotactic protein ■ macrophage ■ kidney ■ rat

At a physiological level, endothelin (ET) 1 plays an important role in the control of fluid-volume balance and blood pressure. Specifically, ET-1 promotes diuresis and natriuresis within the collecting duct and action through ET_B receptors. Systemically, ET_B receptors clear ET-1 from the circulation and protect against ET_A receptor–dependent vasoconstriction, cell proliferation, matrix accumulation, and inflammation. Recent clinical studies have suggested that ET_A antagonists may be a useful therapeutic approach for proteinuric renal disease, but the precise mechanism of action is not known. ET_A receptors are responsible for a wide range of effects in the kidney including vasoconstriction of renal cortical vessels, mesangial cell contraction and proliferation, stimulation of extracellular matrix production, and inflammation. The ET system has been implicated in a variety of renal diseases including chronic proteinuric disorders, such as diabetes mellitus, hypertension, and glomerulonephritis. Overexpression of ET-1 in the kidney causes renal inflammation and fibrosis. This role is supported by the finding that ET_A or ET_A/B antagonists attenuate development and progression of renal disease in models of these disorders.

Proteinuria and albuminuria represent early signs of glomerular injury, and their presence predicts not only an elevated risk for nephropathy but also cardiovascular disease in general. The mechanistic pathways of albuminuria in chronic kidney disease have not been resolved. A recent phase III clinical trial in patients with diabetic nephropathy demonstrated that avosentan, a modestly selective ET_A antagonist, decreased urinary albumin excretion rate after 12 weeks of treatment. This benefit occurred although nearly all of the subjects in this trial were receiving treatment with angiotensin receptor blockers and angiotensin-converting enzyme inhibitors, indicating an independent antiproteinuric effect of ET receptor antagonism. However, the main adverse effect of the new class of drugs was peripheral edema, especially at high dosages of avosentan. Little is known about the specific mechanisms of ET-1 action in chronic kidney disease, and many of the beneficial effects of ET antagonists have not been distinguished from their blood pressure–lowering effect.

Chemokines such as monocyte chemoattractant protein-1 (MCP-1) and soluble (s) intercellular adhesion molecule-1.
(ICAM-1) are important in attracting inflammatory cells and in their attachment to the endothelium, thus facilitating the early process of macrophage and lymphocyte infiltration into the kidney. MCP-1 may contribute to the development of diabetic nephropathy by facilitating the formation of tubulo-interstitial lesions through macrophage recruitment and activation. Increased ICAM-1 expression along with increased leukocyte trafficking has been described in experimental models of nephropathy. ET-1 has been demonstrated to possess a chemoattractant role for renal and blood macrophages and lymphocytes in an ET receptor–dependent manner.

The current study was designed to determine the direct actions of ET-1 in promoting changes in glomerular permeability and renal inflammation. We hypothesized that chronic ET-1 infusion would stimulate ET receptor expression to increase expression of inflammatory mediators with subsequent infiltration of inflammatory cells into the kidney. We also proposed that ET-1 has a direct effect on glomeruli to increase permeability to albumin.

**Methods**

**Intravenous Infusion of ET-1**

All of the surgical and experimental procedures were performed according to the guidelines for the care and use of animals established by the Medical College of Georgia and was approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Harlan Laboratories), 225 to 250 g, were housed under conditions of constant temperature and humidity and exposed to a 12:12-hour light-dark cycle. ET-1 (2 pmol/kg per minute, American Peptide, Inc) or saline vehicle (0.9% NaCl) was infused intravenously for 14 days via an osmotic minipump (model 2ML2, Alza Scientific) connected to a catheter (phycocerythin 50) placed in the jugular vein, implanted under sodium pentobarbital (50 mg/kg IP) anesthesia. Three groups of rats were studied (n=5 to 9): (1) saline infusion; (2) ET-1 infusion; and (3) ET-1 infusion plus ABT-627 (5 mg/kg per day, Abbott Laboratories) in the drinking water. An additional subset of rats was also given ABT-627 in the drinking water without any ET-1 infusion (n=4). ABT-627 is a selective ET receptor antagonist at this dose in vivo; the dosing was identical to that used in the chronic ET-1 infusion protocol.
drinking water or the ETA receptor antagonist, ABT-627, in the drinking water for 2 weeks. As depicted in Figure 3B, chronic ABT-627 treatment significantly attenuated the elevation of Palb in glomeruli isolated from ET-1–infused rats (0.18 ± 0.02 versus 0.4 ± 0.03; \( P < 0.05 \)). Glomeruli from non–ET-1–infused rats given ABT-627 maintained normal Palb (0.03 ± 0.04; \( n = 4 \)).

Incubation of glomeruli isolated from ET-1–infused rats with ABT-627 in vitro for 15 minutes at 37°C also significantly reduced the elevated Palb (Figure 4A). However, in vitro incubation of glomeruli from ET-1–infused rats with A-192612, a selective ETB antagonist, did not produce any significant changes in Palb (Figure 4B).

Representative transmission electron micrographs of kidneys showing glomerular structures from saline- and ET-1–infused rat glomeruli are depicted in Figure 5A and 5B, respectively. ET-1 infusion appeared to cause some detachment of podocytes and foot process effacement in glomerular tufts relative to saline-infused control rats, suggesting that ET-1 mediates podocyte injury and increases glomerular permeability. We also observed that ET-1 infusion increases nephrin shedding from the glomeruli into the urine. Nephrin is a filtration slit protein expressed in glomerular epithelial cells (podocytes) and acts as a size- and charge-selective filtration barrier. Shedding of nephrin into the tubular fluid and urine is considered a sign of reduced glomerular permeability and, thus, glomerular injury. Nephrin excretion was significantly increased in ET-1–infused rats compared with saline-infused control rats (\( P < 0.05 \); Figure 5C). Chronic treatment of ET-1–infused rats with ABT-627 significantly decreased urinary nephrin excretion (Figure 5C).

Consistent with efficient clearance of ET-1 from the circulation, plasma ET-1 levels were not significantly changed by chronic ET-1 infusion with or without treatment with ABT-627: 1.06 ± 0.17 pg/mL (\( n = 6 \)) in saline-infused rats, 0.88 ± 0.25 pg/mL (\( n = 7 \)) in ET-1 rats, and 1.42 ± 0.51 pg/mL (\( n = 5 \)) in the ET-1 + ABT-627 group. Furthermore, there was no change in 24-hour ET-1 excretion: 0.28 ± 0.03 pg/mL (\( n = 6 \)) in saline-infused rats, 0.25 ± 0.03 pg/mL (\( n = 7 \)) in ET-1 rats, and 1.42 ± 0.51 pg/mL (\( n = 5 \)) in the ET-1 + ABT-627 group.
days after saline infusion, ET-1 infusion, or ET-1 infusion plus ABT-627 treatment. The density of cells expressing the macrophage-specific CD68 antigen and T-cell–specific CD3 antigen in the renal cortex was very low in saline-infused rats (Figure 7A and 7D) and markedly increased in ET-1–infused rats (Figure 7B and 7E). ABT-627 treatment reduced the number of both CD68- and CD3-positive cells in ET-1–infused rats (Figure 7C and 7F). Finally, there were no significant differences in kidney weight between saline and ET-1–infused animals (1.23±0.03 and 1.22±0.02 g, respectively).

Discussion
Since the discovery of ET-1, the mechanisms by which ET-1 contributes to chronic kidney disease have been slow in being resolved. In various animal models of hypertension, ET-1 has been shown to exert proinflammatory, promitogenic, and profibrotic actions in the kidney, which may be at least partially attributable to the hypertensive effects of ET-1. In the present study, we demonstrated that ET-1 induces systemic and local glomerular inflammation, as well as increases in glomerular permeability independent of effects on blood pressure.

In the present study, we showed in vitro that concentrations of ET-1 >1 nmol/L produced glomerular contraction in a dose-dependent manner. The contractile effect of ET-1 is potentially attributable to mesangial cell contraction, as reported by Simonson and Dunn.19 At lower concentrations, we observed a fairly rapid increase in $P_{\text{ab}}$ after only 30 minutes and a further increase during the 5-hour incubation period. The normal podocyte has a highly organized cytoskeleton with microfilaments including actin, α-actinin-4, and myosin that are also in association with focal adhesion-related proteins, such as $\alpha_\beta_{3}$ integrins.20 Together, these results have led us to hypothesize that disruption of structural organization by ET-1 may play a role in alterations of normal podocyte function leading to increased glomerular permeability.

Consistent with our previous study,13 we found that infusion of ET-1 at 2 pmol/kg per minute IV for 2 weeks had no effect on mean arterial pressure. In a similar dose range (1 to 5 pmol/kg per minute), others have also reported no effect of
chronic ET-1 infusion on arterial pressure in rats. However, Sedeek et al and Yao et al reported that chronic ET-1 infusion elevates arterial pressure in rats on a normal salt diet. Therefore, we took care to choose a dose of ET-1 that would allow us to examine the effects of ET-1 independent of hypertension and used telemetry to verify the lack of an effect on arterial pressure. Dao et al reported pressure-independent actions of ET-1 in this model by infusing ET-1 at 1 and 5 pmol/kg per minute for 28 days and observing hypertrophy and small artery hyperplasia, respectively. Therefore, it appears that variations of local ET-1 concentrations are sufficient to produce blood pressure–independent cellular responses in vivo.

Ours is not the first study to report blood pressure–independent effects of ET-1 on renal pathology. Hocher et al demonstrated that transgenic overexpression of the human ET-1 gene in mice resulted in glomerulosclerosis and interstitial fibrosis without the presence of systemic hypertension. In the same animal model, Hocher et al showed that ET-1 induced recruitment of inflammatory cells into the kidney, which contributed progressively to the fibrosis independent of blood pressure. In their study, blood pressure was similar in 3-month–old ET-1 transgenic mice and their corresponding littermates. However, ET-1 transgenic mice exhibit chronic renal inflammation characterized by infiltration of CD4+ T cells and macrophages. Such findings suggest that an activated renal ET system could be a blood pressure–independent risk factor for the progression of renal fibrosis to end-stage renal disease. However, we cannot rule out the possibility of alterations of intraglomerular capillary pressure allowing for albumin or proteinuria in normal Sprague–Dawley rats. These data are in agreement with findings in ET-2 transgenic rats and human ET-1 transgenic mice that display glomerulosclerosis with the absence of proteinuria. The absence of proteinuria suggests that the changes in permeability determined in isolated glomeruli are not sufficient to translate into measurable albuminuria. Recent studies have renewed interest in the role of proximal tubular uptake of albumin in protecting against albuminuria, and changes in Pab, on the order of magnitude that we observed in the chronic ET-1 model, 0.4, are much less than those observed in rats displaying overt proteinuria associated with hyperglycemia. Several studies have established that an increase in glomerular permeability typically occurs before the development of overt proteinuria. Thus, it remains possible that ET-1 infusion for longer periods than 2 weeks may result in significant albuminuria and/or proteinuria.

ETa receptor antagonism using ABT-627 attenuated Pab in glomeruli isolated from ET-1–infused rats even when glomeruli were treated ex vivo for only 15 minutes, confirming the role of endogenous ET-1 and ETa receptor activity within the glomerulus influencing permeability to albumin. It is unlikely that changes in expression (mRNA or protein) of glomerular filtration barrier molecules (ie, nephrin, CD2AP, and podocin) can explain the rapid effect of ETa receptor blockade on Pab. Rather, ETa receptor-mediated cytoskeletal rearrangement is a more likely mechanism of reduced Pab. This hypothesis is supported by previous studies by Morigi et al showing that F-actin redistribution and gap formation occurred when exogenous ET-1 was added to cultured podocytes. Intervention with LU-302146, a selective ETa receptor antagonist, prevented F-actin redistribution and decreased proteinuria or albuminuria in normal Sprague–Dawley rats.
intercellular gap formation induced by Shigatoxin 2. Moreover, the same group has reported that inhibition of Rho kinases, which are crucial for the formation of stress fibers, resulted in a significant inhibition of F-actin rearrangement in response to ET-1. Treatment of non–ET-1 infused rats with ABT-627 had no effect on Palb as one might expect because Palb was no different from 0 in these rats.

Our data further support a specific role for the ET_A receptor in control of Palb because ETB receptor antagonism did not alter the increase in Palb induced by ET-1.33 Treatment of non–ET-1 infused rats with ABT-627 had no effect on Palb as one might expect because Palb was no different from 0 in these rats.

Our data further support a specific role for the ET_A receptor in control of Palb because ETB receptor antagonism did not alter the increase in Palb induced by ET-1. This is consistent with relatively low expression of ETB receptors in glomeruli compared with ET_A receptors.33 It is important to note, however, that this does not exclude a role for the ETB receptor in modulating the progression of glomerular injury and renal disease. Studies by Tazawa et al34 and Pfab et al35 have demonstrated that rats lacking a functional ETB receptor develop more severe renal dysfunction, proteinuria, and renal injury in response to subtotal nephrectomy and streptozotocin-induced hyperglycemia. In these studies, the rat lacking functional ETB receptors also developed disease-induced elevations in blood pressure that could account for the increased proteinuria. These findings, taken together with our current results, suggest that the protective role of ETB receptors is through hemodynamic mechanisms rather than direct ETB-dependent effects on permeability, which we were unable to observe in the permeability experiments.

We observed that chronic ET_A receptor activation increased the excretion of nephrin into the urine, again suggesting that ET_A receptors influence podocyte function and P_ab. Collino et al16 identified a mechanism of nephrin loss that may account for the enhanced glomerular permeability in preeclampsia. These investigators provided evidence for a factor(s) present in serum from preeclamptic patients who trigger production of ET-1 from glomerular endothelial cells and that ET-1, in turn, may induce shedding of nephrin from the surface of podocytes. They went on to conclude that ET-1 activates the podocyte cytoskeleton and modifies surface expression of nephrin, thus depleting it from the plasma membrane and excretion into the urine. Others have shown that upregulation of ET-1 production by podocytes is induced by protein overload, resulting in cytoskeletal changes associated with foot-process effacement, a hallmark of chronic glomerular disease.37 ET-1 released by podocytes thus may contribute to glomerular barrier dysfunction by direct effects on podocytes themselves. Because ET-1 also increases reactive oxygen species,13 ET-1 may elevate glomerular perme-

Figure 7. Representative immunostaining images of kidney cortical sections isolated from rats treated with saline (A and D), ET-1 (B and E), or ET-1+ABT-627 (C and F) for 2 weeks. Sections were stained for CD68-positive cells (monocytes/macrophages; A through C) or CD3-positive cells (T cells; D through F) (indicated by arrows). Numbers of CD68-positive cells are represented in G and H with CD3-positive cell counts in I and J. *P<0.05 vs saline and †P<0.05 vs ET-1 alone. n=4 to 5 rats per group.
ability in chronic settings via superoxide-mediated enhancement of gelatinase synthesis by mesangial cells, reduction of de novo synthesis of proteoglycans, and degradation of the glomerular basement membrane.38

We also observed that infusion of ET-1 for 2 weeks significantly increased glomerular sICAM-1, MCP-1, and plasma MCP-1 levels independent of hypertension or loss of glycemic control (euglycemic clamp test, unpublished observations). These data agree with previous work published by Amiri et al,39 who reported that transgenic mice that overexpress human preproendothelin 1 in endothelial cells exhibit various vascular inflammatory responses, including macrophage infiltration, transcription factor activation (activator protein-1 and nuclear factor-κB), and increases in VCAM-1 expression independent of blood pressure elevation. These data suggest that ET-1 directly triggers the initial phase of renal inflammation through upregulation of systemic MCP-1 and local glomerular ICAM-1 and MCP-1. These inflammatory molecules are known to participate in macrophage infiltration into the kidney.8 Future studies are needed to explore the mechanism of ET-1–induced glomerular ICAM-1 and MCP-1 upregulation. The proinflammatory effects of ET-1 were also blocked by ABT-627 consistent with the proinflammatory effects of ET-1 being ETₐ receptor mediated. We have observed previously that ETₐ blockade in intact rats has no effect on indices of inflammation.12 One challenge will be to determine whether the inflammatory effects of ET-1 contribute to changes in glomerular permeability and, furthermore, proteinuria.

Perspectives

The use of ETₐ selective and ETₐB receptor antagonists is under consideration for treatment of proteinuric renal disease,7,40 but we have little knowledge of how they may confer benefit. We observed that ETₐ receptor activation results in overexpression of circulating and glomerular inflammatory mediators, as well as changes in glomerular permeability to albumin independent of blood pressure. Our results provide mechanistic support for the use of ETₐ selective blockade in chronic kidney disease, but, thus far, the clinical data indicate frequent fluid retention problems of uncertain origin. Future studies are needed to specifically investigate receptor subtype-specific actions in the human kidney.

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Disclosures

None.

References


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All data in the remainder of the manuscript are correct as published.

The authors apologize for the errors in the original manuscript.

These corrections have been made to the current online version of the article, which is available at http://hyper.ahajournals.org/cgi/content/full/56/5/942.
Endothelin-1 increases glomerular permeability and inflammation
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Isolation of glomeruli

Rats were anesthetized with sodium pentobarbital (50 mg/kg; i.p). A blood sample was taken from the abdominal aorta for determination of both plasma sICAM-1 and MCP-1 as outlined below. Kidneys were removed, decapsulated and placed in ice-cold phosphate-buffered saline (PBS; pH 7.4) containing phenylmethylsulfonylfluoride (PMSF, 1mM). Glomeruli were isolated by gradual sieving as described previously.1 The isolated glomeruli were re-suspended in 1 ml PBS and the final glomerular pellet was snap frozen in liquid nitrogen and stored at -80°C.

Glomeruli were re-suspended in lysis buffer (20 mM HEPES, pH 7.4, 10 mM NaCl, 5 mM EDTA, 0.2% Triton X-100, 10 mM sodium fluoride, 1 mM sodium ortho-vanadate, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin) and homogenized by ultrasonic homogenizer (20 s). After centrifugation at 10000 × g for 10 min, the supernatant was used for protein determination using the Bradford assay² and subsequent analysis of glomerular expression of both sICAM-1 and MCP-1 by ELISA as outlined below.

Measurement of glomerular albumin permeability (P_alb)

For measurement of P_alb, glomeruli were isolated from the renal cortex in medium containing 5% bovine serum albumin (BSA), 115 mM NaCl, 5 mM KCl, 10 mM sodium acetate, 1.2 mM dibasic sodium phosphate, 25 mM sodium bicarbonate, 1.2 mM magnesium sulphate, 1 mM calcium chloride and 3.5 mM glucose, pH 7.4, at room temperature.¹

The rationale and methodology for the determination of albumin permeability has been described in detail previously.³ In brief, images of 10–15 glomeruli per kidney preparation (i.e., per rat) were captured using a digital camera through an inverted microscope before and after a medium change from 5% BSA to 1% BSA. The medium exchange creates an oncotic gradient across the basement membrane resulting in a glomerular volume change (∆V= (V_final−V_initial)/V_initial), which was measured off-line by an image analysis program (Digimizer, MedCalc Software bvba, Mariakerke, Belgium). The computer program determines the average radius of the glomerulus in two-dimensional space, and the volume is derived from the formula V=4/3πr³. The magnitude of ∆V was related to the albumin reflection coefficient, σ_alb, by the following equation: (σ_alb)experimental = (∆V)experimental / (∆V)control; the σ_alb of the control glomeruli was assumed to be equal to 1. P_alb is defined as (1−σ_alb), and describes the movement of albumin consequent to water flux. When σ_alb is zero, albumin moves across the membrane with the same velocity as water, and P_alb is 1.0. Conversely, when σ_alb is 1.0, as in control glomeruli, albumin cannot cross the membrane with water, and P_alb is zero.

Immunohistochemical Analysis

Kidneys were perfused with 4% paraformaldehyde in 100 mM dibasic sodium phosphate buffer and immersed in 4% paraformaldehyde solution overnight at room temperature, transferred to 70% ethanol for 24 h, and paraffin embedded. The kidneys were sectioned at a thickness of 4 µm onto Superfrost plus slides. Slides were incubated overnight in the absence or presence of primary antibody to CD68 for monocytes/macrophages (ED-1; Serotec, Kidlington, Oxford, UK) or CD3 (Santa Cruz Biotechnology, Santa Cruz, CA) for T cells in humidity chambers at 4°C, followed by incubation for 30 min with peroxidase-conjugated goat anti-mouse
IgG (Serotec, Kidlington, Oxford, UK) at room temperature. Positive staining was detected with dianinobenzamidine (DakoCytomation, Carpinteria, CA) and counterstained with Mayer’s hematoxylin. The stained sections were viewed on bright-field setting with an Olympus BX40 microscope (Olympus America, Melville, NY) fitted with a digital camera (Olympus DP70; Olympus America). For quantification of T cell and monocytes/macrophage number, CD3 and CD68 positive cells respectively were counted in the entire cortex of a given kidney (magnification x40) in a blinded fashion. Cortical sections were all of similar size.

Transmission electron microscopy
Renal cortical tissue sections were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate (NaCac) buffer, pH 7.4, and post fixed in 2% osmium tetroxide in NaCac, stained en bloc with 2% uranyl acetate, dehydrated with a graded ethanol series and embedded in Epon-Araldite resin. Thin sections were cut with a diamond knife on a Leica EM UC6 ultramicrotome (Leica Microsystems, Inc, Bannockburn, IL), collected on copper grids and stained with uranyl acetate and lead citrate. Cells were observed in a JEM 1230 transmission electron microscope (JEOL USA Inc., Peabody, MA) at 110 kV and imaged with an UltraScan 4000 CCD camera & First Light Digital Camera Controller (Gatan Inc., Pleasanton, CA).

References
Correction

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