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 chemoattractant 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. Littl 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

© 2010 American Heart Association, Inc.

Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.110.156570
(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 tubular-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 chemotactic role for renal and blood macrophages and lymphocytes in an ETA 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 ETA receptors 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 (phycoerythrin 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 (500 nmol/L). All of the data are presented as mean±SEM. Data were compared using unpaired Student’s t test or 1-way ANOVA. Differences were considered statistically significant with P<0.05. Analyses were performed using GraphPad Prism version 5.0 software (GraphPad Software Inc).

**Statistical Analysis**

All of the data are presented as mean±SEM. Data were compared using unpaired Student’s t test or 1-way ANOVA. Differences were considered statistically significant with P<0.05.

**Results**

**Acute Effects of ET-1 on Isolated Glomeruli**

The addition of ET-1 to the 5% BSA incubation medium elicited a significant concentration-dependent contraction of glomeruli isolated from normal rats observed within 5 minutes. The lowest concentration of ET-1 that elicited identifiable contraction (2.7±0.3%) was 10 nmol/L (Figure 1A). No glomerular contraction was observed when 1 nmol/L of ET-1 was added for 5 minutes. Because the calculation of glomerular permeability relies on glomerular volume differences on switching the medium from 5% to 1% BSA, we chose a dose of 1 nmol/L to study the effects of ET-1 on glomerular permeability to preclude volume changes attributed to contractile effects of ET-1 affecting our results.

Incubation of glomeruli with ET-1 (1 nmol/L) caused a time-dependent increase of $P_{\text{ab}}$. As shown in Figure 1B, there was a slight increase in $P_{\text{ab}}$ after 15 minutes (0.10±0.08) compared with controls (0 nmol/L ET-1; 0.00±0.02), but the increase in $P_{\text{ab}}$ was not statistically significant until 30 minutes of incubation (0.26±0.10) and was sustained at 4 hours (0.57±0.07).

**$P_{\text{ab}}$ in Chronic ET-1–Infused Rats**

ET-1 infusion (2 pmol/kg per minute, IV) for 2 weeks did not affect the mean arterial pressure (measured continuously by telemetry; Figure 2). $P_{\text{ab}}$ was significantly increased in glomeruli isolated from ET-1–infused rats ($P<0.05$; Figure 3A). However, there was no effect on protein or albumin excretion (Figure 3C and 3D, respectively). Separate groups of rats were infused with ET-1 and given either normal

**Immunohistochemical Analysis**

Details of the immunohistochemical methods are available in the online Data Supplement. Briefly, harvested renal tissue from each group of rats was prepared and evaluated for CD68- and CD3-positive immunoreactivity for analyses of macrophages and T cells, respectively.

**Plasma and Urinary Analyses**

Commercially available kits for rat sICAM-1 and ET-1 (Quantikine sICAM-1 and Quantiglo ET-1 Immunoassay, respectively, R&D Systems) and MCP-1 (RayBioTech, Inc) were used for accurate determination of these 2 cytokines in plasma and glomerular homogenates (1 μg of total protein loaded). Nephrin concentration was determined in urine via ELISA kit (Exocell, Inc). Urinary protein concentrations were determined using the Bradford colorimetric method (Bio-Rad Laboratories) according to the manufacturer’s instructions. Urinary concentrations of albumin were measured using enzyme immunoassay kits from Cayman Chemical (Cayman Chemical).

**Transmission Electron Microscopy**

Details of the transmission electron microscopy in this study are available in the online Data Supplement. Briefly, renal cortical tissue from each group of rats was prepared, and 4 to 5 sections were analyzed. Ten glomeruli per section were evaluated.

**Isolation of Glomeruli**

Details of glomeruli isolation are available in the online Data Supplement (please see http://hyper.ahajournals.org). Briefly, glomeruli were isolated by gradual sieving, as described previously. Glomeruli were homogenized, and total protein was determined in the supernatant using the Bradford assay.

**Measurement of Glomerular Albumin Permeability**

Please see the online Data Supplement for details of glomerular albumin permeability ($P_{\text{ab}}$) measurement. In brief, the rationale for the determination of albumin permeability has been described in detail previously. $P_{\text{ab}}$ is measured via calculating the volume change created by the oncotic pressure induced by switching the surrounded medium from 5% into 1% BSA. This volume change is mathematically translated into $P_{\text{ab}}$. 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 ETA receptors 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.

---

**Statistical Analysis**

All of the data are presented as mean±SEM. Data were compared using unpaired Student’s t test or 1-way ANOVA. Differences were considered statistically significant with P<0.05. Analyses were performed using GraphPad Prism version 5.0 software (GraphPad Software Inc).

**Results**

**Acute Effects of ET-1 on Isolated Glomeruli**

The addition of ET-1 to the 5% BSA incubation medium elicited a significant concentration-dependent contraction of glomeruli isolated from normal rats observed within 5 minutes. The lowest concentration of ET-1 that elicited identifiable contraction (2.7±0.3%) was 10 nmol/L (Figure 1A). No glomerular contraction was observed when 1 nmol/L of ET-1 was added for 5 minutes. Because the calculation of glomerular permeability relies on glomerular volume differences on switching the medium from 5% to 1% BSA, we chose a dose of 1 nmol/L to study the effects of ET-1 on glomerular permeability to preclude volume changes attributed to contractile effects of ET-1 affecting our results.

Incubation of glomeruli with ET-1 (1 nmol/L) caused a time-dependent increase of $P_{\text{ab}}$. As shown in Figure 1B, there was a slight increase in $P_{\text{ab}}$ after 15 minutes (0.10±0.08) compared with controls (0 nmol/L ET-1; 0.00±0.02), but the increase in $P_{\text{ab}}$ was not statistically significant until 30 minutes of incubation (0.26±0.10) and was sustained at 4 hours (0.57±0.07).

**$P_{\text{ab}}$ in Chronic ET-1–Infused Rats**

ET-1 infusion (2 pmol/kg per minute, IV) for 2 weeks did not affect the mean arterial pressure (measured continuously by telemetry; Figure 2). $P_{\text{ab}}$ was significantly increased in glomeruli isolated from ET-1–infused rats ($P<0.05$; Figure 3A). However, there was no effect on protein or albumin excretion (Figure 3C and 3D, respectively). Separate groups of rats were infused with ET-1 and given either normal

**Immunohistochemical Analysis**

Details of the immunohistochemical methods are available in the online Data Supplement. Briefly, harvested renal tissue from each group of rats was prepared and evaluated for CD68- and CD3-positive immunoreactivity for analyses of macrophages and T cells, respectively.

**Plasma and Urinary Analyses**

Commercially available kits for rat sICAM-1 and ET-1 (Quantikine sICAM-1 and Quantiglo ET-1 Immunoassay, respectively, R&D Systems) and MCP-1 (RayBioTech, Inc) were used for accurate determination of these 2 cytokines in plasma and glomerular homogenates (1 μg of total protein loaded). Nephrin concentration was determined in urine via ELISA kit (Exocell, Inc). Urinary protein concentrations were determined using the Bradford colorimetric method (Bio-Rad Laboratories) according to the manufacturer’s instructions. Urinary concentrations of albumin were measured using enzyme immunoassay kits from Cayman Chemical (Cayman Chemical).

**Transmission Electron Microscopy**

Details of the transmission electron microscopy in this study are available in the online Data Supplement. Briefly, renal cortical tissue from each group of rats was prepared, and 4 to 5 sections were analyzed. Ten glomeruli per section were evaluated.

---

**Isolation of Glomeruli**

Details of glomeruli isolation are available in the online Data Supplement (please see http://hyper.ahajournals.org). Briefly, glomeruli were isolated by gradual sieving, as described previously. Glomeruli were homogenized, and total protein was determined in the supernatant using the Bradford assay.

**Measurement of Glomerular Albumin Permeability**

Please see the online Data Supplement for details of glomerular albumin permeability ($P_{\text{ab}}$) measurement. In brief, the rationale for the determination of albumin permeability has been described in detail previously. $P_{\text{ab}}$ is measured via calculating the volume change created by the oncotic pressure induced by switching the surrounded medium from 5% into 1% BSA. This volume change is mathematically translated into $P_{\text{ab}}$. 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 ETA receptors 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.
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)\(^{17}\) and acts as a size- and charge-selective filtration barrier.\(^{18}\) 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...
ET-1 Induced Inflammation

ET-1 infusion elevated glomerular sICAM-1 and MCP-1 and plasma MCP-1 (Figure 6A, 6B, and 6D) but had no significant effect on plasma sICAM-1 concentration (Figure 6C). Treatment with ABT-627 significantly decreased glomerular and plasma sICAM-1 and MCP-1 concentrations (Figure 6E through 6H).

Figure 7 depicts immunohistochemical analysis of renal cortical tissue for monocyte/macrophage and T cells at 14 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 \( P_{\text{abs}} \) 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, \( \alpha \)-actinin-4, and myosin that are also in association with focal adhesion-related proteins, such as \( \alpha_\text{v}\beta_\text{i} \) 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. Our 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 littersmates. 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 during chronic ET-1 elevation. Moreover, our data indicate that a nonpressor 2-week infusion of ET-1 does not induce proteinuria or albuminuria 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 albuminuria 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 P_{ab} 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, >0.8. 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.

ET_{A} receptor antagonism using ABT-627 attenuated P_{ab} 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 ET_{A} 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 ET_{A} receptor blockade on P_{ab}. Rather, ET_{A} receptor-mediated cytoskeletal rearrangement is a more likely mechanism of reduced P_{ab}. 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 ET_{A} receptor antagonist, prevented F-actin redistribution and decreased phosphorylation of focal adhesion kinase (p-FAK) and paxillin, indicating a role for ET_{A} receptor activity in the regulation of F-actin organization.

**Figure 6.** Effect of ET-1 infusion (A through D) with or without concurrent treatment with ABT-627 (E through H) on glomerular and plasma sICAM-1 and MCP-1 concentrations. n = 5 to 9 per group. *P < 0.05 vs saline and †P < 0.05 vs ET-1 alone.
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 P<sub>ab</sub> induced by ET-1. This is consistent with relatively low expression of ET<sub>B</sub> receptors in glomeruli compared with ET<sub>A</sub> receptors. It is important to note, however, that this does not exclude a role for the ET<sub>B</sub> receptor in modulating the progression of glomerular injury and renal disease. Studies by Tazawa et al and Pfab et al have demonstrated that rats lacking a functional ET<sub>B</sub> 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 ET<sub>B</sub> 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 ET<sub>B</sub> receptors is through hemodynamic mechanisms rather than direct ET<sub>B</sub>-dependent effects on permeability, which we were unable to observe in the permeability experiments.

We observed that chronic ET<sub>A</sub> receptor activation increased the excretion of nephrin into the urine, again suggesting that ET<sub>A</sub> receptors influence podocyte function and P<sub>ab</sub>. Collino et al 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. 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, ET-1 may elevate glomerular permeability.

**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 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.

Acknowledgments

We thank Dr Ahmed Elmarakby (Medical College of Georgia) and Dr Jan Williams (University of Mississippi) for their useful advice on how to isolate glomeruli and measure Pₐb. We also thank Hiram Ocasio for his assistance with telemetry measurements and Janet Hobbs for expert technical assistance in the histology studies.

Sources of Funding

This work was supported by grants from the National Heart, Lung, and Blood Institute (HL69999 and HL64776) and an American Heart Association predoctoral fellowship for M.A.S. A grant from the Egyptian government to M.A.S. also supported this research.

Disclosures

None.

References


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 and David M. Pollock

Hypertension. 2010;56:942-949; originally published online September 7, 2010; doi: 10.1161/HYPERTENSIONAHA.110.156570

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/56/5/942

An erratum has been published regarding this article. Please see the attached page for:
/content/58/4/e31.full.pdf

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2010/09/03/HYPERTENSIONAHA.110.156570.DC1
http://hyper.ahajournals.org/content/suppl/2011/04/22/HYPERTENSIONAHA.110.156570.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
In the *Hypertension* article by Saleh et al (Saleh MA, Boesen EI, Pollock JS, Savin VJ, Pollock, DM. Endothelin-1 Increases Glomerular Permeability and Inflammation Independent of Blood Pressure in the Rat. *Hypertension*. 2010;56:942–949), 2 transposition errors were made by Dr Saleh in (1) the entry of data in the text listing 24-hour ET-1 excretion rate for the 3 groups of rats, and (2) the units for nephrinuria (Figure 5C). While there were no differences between the 3 groups as reported, actual values presented were urine ET-1 concentrations, not excretion rates. The actual excretion rate values were 9.2±1.6, 8.5±0.9, 7.3±1.0 pg/d for the saline, ET-1, and ET-1+ABT-627 groups, respectively. In Figure 5C, nephrinuria should be presented as μg/day, not ng/day.

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.
ONLINE SUPPLEMENT

Endothelin-1 increases glomerular permeability and inflammation independent of blood pressure in the rat

Mohamed A. Saleh\textsuperscript{1,2}, Erika I. Boesen\textsuperscript{1,3}, Jennifer S. Pollock\textsuperscript{1,2}, Virginia J. Savin\textsuperscript{4}, and David M. Pollock\textsuperscript{1,2,3,5}

\textsuperscript{1}Vascular Biology Center, \textsuperscript{2}Department of Pharmacology and Toxicology, \textsuperscript{3}Department of Physiology, \textsuperscript{5}Department of Surgery, Medical College of Georgia, Augusta, GA 30912 USA

\textsuperscript{4}Department of Medicine, Kansas City VAMS, Kansas City, MO 64128 USA

Correspondence:
David M. Pollock, Ph.D.
Vascular Biology Center
Medical College of Georgia
1459 Laney Walker Blvd
Augusta, GA 30907-2500 USA
1-706-721-8517 phone
1-706-721-9799 fax
dpollock@mcg.edu
Supplemental Methods

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 (\( \Delta V = (V_{\text{final}} - V_{\text{initial}})/V_{\text{initial}} \)), 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 \( V = 4/3 \pi r^3 \).

The magnitude of \( \Delta V \) was related to the albumin reflection coefficient, \( \sigma_{alb} \), by the following equation: \( (\sigma_{alb})_{\text{experimental}} = (\Delta V)_{\text{experimental}} / (\Delta V)_{\text{control}} \); the \( \sigma_{alb} \) of the control glomeruli was assumed to be equal to 1. \( P_{alb} \) is defined as (1–\( \sigma_{alb} \)), and describes the movement of albumin consequent to water flux. When \( \sigma_{alb} \) is zero, albumin moves across the membrane with the same velocity as water, and \( P_{alb} \) is 1.0. Conversely, when \( \sigma_{alb} \) 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 diaminobenzamide (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.4

**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

In the *Hypertension* article by Saleh et al (Saleh MA, Boesen EI, Pollock JS, Savin VJ, Pollock, DM. Endothelin-1 Increases Glomerular Permeability and Inflammation Independent of Blood Pressure in the Rat. *Hypertension*. 2010;56:942-949), 2 transposition errors were made by Dr. Saleh in (1) the entry of data in the text listing 24-hour ET-1 excretion rate for the 3 groups of rats, and (2) the units for nephrinuria (Figure 5C). While there were no differences between the 3 groups as reported, actual values presented were urine ET-1 concentrations, not excretion rates. The actual excretion rate values were 9.2±1.6, 8.5±0.9, 7.3±1.0 pg/d for the saline, ET-1, and ET-1+ABT-627 groups, respectively. In Figure 5C, nephrinuria should be presented as µg/day, not ng/day.

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

The authors apologize for the errors in the original manuscript.