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Chronic endothelin-1 infusion elevates glomerular sieving coefficient and proximal tubular albumin reuptake in the rat

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Abstract

Aim—We have previously found that chronic endothelin-1 (ET-1) infusion in Sprague-Dawley rats increases glomerular permeability to albumin (P_{alb}) as assessed *in vitro* independent of blood pressure with no observed albuminuria. In this study, we hypothesized that ET-1 increases glomerular albumin filtration with accompanied increase in albumin uptake *via* the proximal tubule, which masks the expected increase in urinary albumin excretion.

Main methods—Nonfasting Munich-Wistar Fromter rats were surgically prepared for *in vivo* imaging (n=6). Rats were placed on the microscope stage with the exposed kidney placed in a cover slip-bottomed dish bathed in warm isotonic saline. Rats were then injected i.v. with rat serum albumin conjugated to Texas Red that was observed to enter capillary loops of superficial glomeruli, move into Bowman's space, bind to the proximal tubular cell brush border and reabsorbed across the apical membrane. Glomerular sieving coefficient (GSC) was calculated as the ratio of conjugated albumin within the glomerular capillary *versus* that in Bowman's space. Rats were again studied after 2 wk of chronic ET-1 (2 pmol/kg/min; i.v. osmotic minipump).

Key findings—Glomerular sieving coefficient was significantly increased in rats following chronic ET-1 infusion (0.025± 0.005 *vs.* 0.017 ± 0.003, $p<0.05$). Mean fluorescence intensity for conjugated albumin within proximal tubules was increased by ET-1 infusion: 118.40 ± 6.34 *vs.* 74.27 ± 4.45 pixel intensity ($p<0.01$).

Significance—These data provide *in vivo* evidence that ET-1 directly increases glomerular permeability to albumin and that albuminuria is prevented by increased PT albumin uptake in the rat.

Keywords

Endothelin; intravital 2-photon microscopy; glomerular sieving coefficient; albumin reuptake; proximal tubule; kidney; rat

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Introduction

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 (Perkins et al., 2007). The mechanistic pathways of albuminuria in chronic kidney disease (CKD) have not been resolved. Concerning the proteinuric role of ET-1, a recent phase III clinical trial in patients with diabetic nephropathy demonstrated that avosentan, a modestly selective endothelin-A (ET_A) antagonist, decreased urinary albumin excretion rate after 12 weeks of treatment (Wenzel et al., 2009). These studies have been confirmed with a more selective ET_A blocker (Kohan et al., 2011). Furthermore, most of the subjects in these studies were already receiving treatment with angiotensin receptor blockers and/or angiotensin converting enzyme inhibitors indicating an independent anti-proteinuric effect of endothelin receptor antagonism. Despite these observations in both animals and humans, little is known about the specific mechanisms of ET-1 action in CKDs and many of the beneficial effects of ET antagonists have not been distinguished from their blood pressure lowering effect.

We have recently reported that a non-pressor 2-week infusion of ET-1 increases glomerular permeability to albumin (P_{alb}), however, this dose does not induce proteinuria or albuminuria in normal Sprague-Dawley rats (Saleh et al., 2010a). 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 (Russo et al., 2007). In addition, changes in P_{alb} on the order of magnitude that we observed in the chronic ET-1 model, 0.4 (Saleh et al., 2010a), are much less than those observed in rats displaying overt proteinuria associated with hyperglycemia, >0.8 (Fabris et al., 2001; Saleh et al., 2010b; Saleh et al., 2011). Several studies have established that an increase in glomerular permeability typically occurs prior to the development of overt proteinuria (Melnick et al., 1981; Bjorn et al., 1995; Sharma et al., 2002; Doublier et al., 2003).

The current study was designed to 1) confirm whether chronic ET-1 causes glomerular albumin leakage in an intact kidney, and 2) whether proximal tubular re-uptake of albumin could account for a lack of proteinuria following chronic ET-1 infusion in the rat. Our approach used intravital 2-photon microscopy to visualize glomerular permeability *in vivo*. Although the clinical relevance of proteinuria (especially albuminuria) has been well documented, the quantitative and mechanistic role of each “barrier” component, including the proximal tubule, in albuminuria remains an area of considerable excitement and debate (Molitoris, 2010). Over the past 5 years, the long-standing controversy regarding the quantitative role of the proximal tubule in the development of albuminuria has reached new heights, with supporting evidence coming from many different genetic, molecular, and technical approaches. The use of two-photon microscopy offers a unique approach to improving the understanding of the roles of glomerular filtration barrier and proximal tubule in albumin filtration and reabsorption. It allows for direct visualization and quantification of the glomerular filtration and its associated S1 proximal tubule segment in Munich Wistar rats with surface glomeruli. It also permits quantification and relating of changes in structure and function longitudinally over the course of a disease process within the same glomerulus, and the corresponding S1 proximal tubule segment. Because the final composition of albumin in the urine is affected by both glomerular filtration and proximal tubule reabsorption, both processes must be considered (Sandoval et al., 2012). Our hypothesis predicts that chronic ET-1 increases glomerular albumin filtration with an accompanied increase in albumin uptake *via* the proximal tubule, which masks the expected increase in urinary albumin excretion.

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Methods

Chronic ET-1 infusion

All animal studies were conducted in compliance with the American Physiological Society guidelines with all protocols and procedures being reviewed and approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. Male Munich-Wistar rats 200 to 250 g (Simonsen Laboratories, Gilroy, CA) were used in this study. To facilitate ET-1 or vehicle infusion, rats were anesthetized with sodium pentobarbital 50 mg/kg, i.p. (Ovation Pharmaceutical, Deerfield, IL) and a catheter (PE-90) was placed in the jugular vein and then tunneled subcutaneously to an osmotic minipump (model 2ML2; Alza Scientific, Palo Atlo, CA) placed subcutaneously at the dorsum of the neck. The minipump contained ET-1 (American Peptide Inc., CA) dissolved in 0.9% NaCl infused at a rate of 2 pmol/kg/min for a period of 14 days (Saleh et al., 2010a). Only one group of rats were studied (n= 6 rats) which glomerular sieving coefficient and fluorescent albumin proximal reuptake were evaluated before starting ET-1 infusion and after two weeks of the continuous infusion of ET-1.

Animal preparation for imaging

Nonfasting Munich Wistar Fromter rats were anesthetized with 50 mg/kg; i.p. sodium pentobarbital (Ovation Pharmaceutical, Deerfield, IL). First a catheter was inserted in the femoral vein to enable the delivery of fluorescent conjugated albumin. To expose the kidney for intravital imaging an incision was made on the left flank. The rat was placed on the microscope stage with the exposed kidney in contact with the inner surface of 50-mm glass-bottom dish (Willco Wells BV, Netherlands). Animals were covered by a water-circulating warming blanket in order to stabilize the temperature during imaging, In addition, the microscope stage was warmed using 2 ReptiTherm heating pads (Zoo Med Laboratories, San Luis Obispo, CA) one under the upper torso/head and one under the lower abdomen. The temperature of the animal was maintained between 36.5 and 37.5°C. The x60 water immersion objective (Nikon Plan Apo, NA 1.2) was heated using an objective heater (Warner Instruments, Hamden, CT). The mean arterial blood pressure was from 80 to 112 mmHg as measured via a femoral arterial line using Lab Chart 6 (AD Instruments, Colorado Springs, CO).

Two-photon microscopy

Unless specified otherwise, two-photon microscopy was conducted on the Bio-Rad MRC 1024 confocal/two-photon microscope as described previously (Dunn et al., 2002; Yu et al., 2007). The illumination source was a tunable Tsunami Ti:Sapphire laser from Spectra-physics (Mountain View, CA). The microscope was an inverted Nikon Eclipes TE200. The band pass filters used for the red and green channels were 605/90 and 525/50, respectively. The excitation wavelength was set at 800 nm for all experiments. The anesthetized rat was placed on the microscope stage with the exposed kidney in direct contact with the cover glass (see *Animal preparation for imaging*). A 60X Nikon Plan Apo water immersion objective (NA 1.2) was used for imaging. All images were collected at a constant pixel dwell time, which yielded a 1.1-s frame time for the frame size of 512 × 512. All images for a given study were collected at approximately the same focal plane at a depth of approximately 10–20 um.

Proteins and Probes

Rat serum albumin (Fraction V; Sigma-Aldrich) was conjugated to Texas Red Sulfonyl Chloride (Invitrogen, Carlsbad, CA) to yield a stoichiometric ratio of 4:1 moles

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dye:albumin, according to the manufacturer's instructions for amine-reactive probes as previously reported (Russo et al., 2007).

Glomerular sieving coefficient (GSC) determination

Images were analyzed using MetaMorph v.7.1 (Molecular Devices, Downingtown, PA), and calculations were done on a Microsoft Excel spreadsheet. The fluorescence intensity in the plasma was recorded in a capillary loop, which had the brightest fluorescence in the field.

Plasma fluorescence was measured at the outer margins of the capillary lumen, a red blood cell free zone. We selected 3 areas in the urinary space of Bowman's capsule where there were no blood vessels and where fluorescence intensity was minimal. The average of the 3 areas was calculated. We recorded the number of pixels (area) and average fluorescence intensities. Calculations of GSCs were based on average intensity values after subtracting background readings for plasma and Bowman's space.

Albumin proximal reuptake measurement

Still frames from two-photon movies at 45 min post injection taken with a 60X water immersion lens with a 1.2 numerical aperture were captured. Mean pixel intensities were generated following background subtraction, thresholding of the endosomal pool and quantitative analysis on ten random fields per rat, as previously reported(Sandoval and Molitoris, 2008).

Statistical analysis

All data are presented as mean ± SEM. Data was compared using paired Student's *t*-test. Differences were considered statistically significant with $p<0.05$. Analysis was performed using GraphPad Prism Version 5.0 software (GraphPad Software Inc, La Jolla, CA).

Results

GSC was calculated as the intensity of fluorescent albumin in Bowman's space divided by the intensity of fluorescent albumin in glomerular capillaries. The background intensity for all locations was subtracted prior to calculating GSC. As shown in Figure 1, GSC was significantly increased in rats following chronic ET-1 infusion (0.025 ± 0.005 *vs.* 0.017 ± 0.003, $p<0.05$). Intensity of the fluorescent signal was also quantified for proximal tubular re-uptake of albumin by measurement of the fluorescence intensity within the S1 segment. As depicted in Figure 2, mean fluorescence intensity for conjugated albumin with Texas Red in proximal tubules was increased by ET-1 infusion: 118.4 ± 6.3 *vs.* 74.3 ± 4.4 pixel intensity ($p<0.01$).

Discussion

The current study provides mechanistic insight into our recent observations that chronic infusion of exogenous ET-1 increases glomerular permeability to albumin, but has no effect on proteinuria. The explanation for these apparent contradictory findings appear to be the result of a limited, yet significant increase in glomerular permeability to albumin while the proximal tubules have the capacity to take up the additional filtered albumin. Thus, our current study substantiates previous findings that attribute a greater role to the proximal tubules in maintaining albumin homeostasis under both physiologic and pathologic conditions.

Over the past 21 years since ET-1 discovery, a prominent role of ET-1 in CKD has been increasingly established. In various hypertensive animal models, there is considerable

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evidence that ET-1 contributes to renal inflammation, cell proliferation, and fibrosis mainly dependent of its hypertensive effects mediated by the ET_A receptor. Furthermore, a series of studies originating from the Benigni lab (Benigni et al., 1993; Bruzzi and Benigni, 1996) and more recently, our own (Saleh et al., 2010b; Saleh et al., 2010a; Saleh et al., 2011), provide compelling evidence that ET-1 contributes to glomerular podocyte dysfunction as a means of producing proteinuria. Recent clinical trials also demonstrate that ET_A receptor blockade can produce effective anti-proteinuric effects. However, the degree to which this occurs independent of blood pressure lowering is not clear. To address this question, we have used the chronic ET-1 infusion model in the rat where blood pressure is unchanged, yet there is an increase in glomerular permeability to albumin (P_{alb}) in isolated glomeruli as well as increases in early inflammatory markers such as plasma and glomerular monocyte chemoattractant protein-1 (MCP-1) and soluble intercellular adhesion molecule-1 (sICAM-1) (Saleh et al., 2010a).

We believe that there is a strong relationship between ET-1 and podocytes either functionally or morphologically. We have found that ET-1 infusion appeared to cause some detachment of podocytes and foot process effacement in glomerular tufts relative to saline-infused control rats *via* using transmission electron micrographs suggesting that ET-1 mediates podocyte injury and increases glomerular permeability (Saleh et al., 2010a). We also observed that ET-1 infusion increases nephrin shedding from the glomeruli into the urine (nephrinuria). Nephrin is a filtration slit protein expressed in podocytes (Ruotsalainen et al., 1999) and acts as a scaffold of the glomerular filtration barrier (Garg et al., 2007). Shedding of nephrin into the tubular fluid is a clear marker of glomerular injury and hence increase in glomerular permeability. We found that nephrin excretion was significantly increased in ET-1-infused rats compared to saline-infused control rats (Saleh et al., 2010a). In addition, we noticed 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_{alb}. Morphologically, others have shown that up- regulation 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 (Morigi et al., 2005). Moreover, it has been reported that inhibition of Rho kinases in podocytes, which are important for the formation of stress fibers, resulted in a significant inhibition of F-actin rearrangement in response to ET-1 (Morigi et al., 2006).

Results of the present study along with those of our previous investigation demonstrate that ET-1 has important effects on glomerular permeability independent of blood pressure. We have also observed that ET_A receptor blockade reduced aluminuria and proteinuria in rats with diabetes (type 1 model) (Saleh et al., 2010b). Importantly, these previous studies also included experiments where isolated glomeruli were incubated with ET_A receptor antagonists *ex vivo*, and we were able to observe reductions in glomerular albumin permeability. These experiments also provide further evidence for actions of ET-1 within the glomerulus independent of blood pressure lowering. Guo et al demonstrated that ablation of as few as 20% of podocytes resulted in albuminuria that resolved over 1–2 weeks after the re-establishment of normal podocyte morphology (Guo et al., 2012). They also showed that immediately after the onset of albuminuria, proximal tubule cells underwent a transient burst of proliferation without evidence of tubular damage or increased apoptosis, resulting in an increase in total tubular cell numbers which may explain the increased proximal uptake of albumin in our study (Guo et al., 2012).

In a model of streptozotocin induced diabetes, Gagliardini and colleagues used fractional clearance of graded-size Ficoll molecules to determine the effects of ET receptor blockade on glomerular dynamics. Avosentan, a modestly selective ET_A antagonist, had no effect on the ultrafiltration coefficient, but was able to prevent podocyte loss and partially lowered

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proteinuria and albuminuria. This could be due to the effect of the ET_A receptor antagonist on glomerular hemodynamics as opposed to a direct effect on permselectivity. In addition, avosentan increased peritubular capillary perfusion, which could enhance proximal tubular protein reabsorption (Gagliardini et al., 2009). While it is possible that ET-1 could have a direct effect to enhance proximal tubular uptake of filtered albumin, it is possible that the increased filtered load of albumin is still below the capacity of the proximal tubule to reabsorb albumin, but this will need to be further investigated.

Infusion of ET-1 for longer time periods and/or at higher concentrations in the rat could potentially have a more profound impact on glomerular permeability that could result in significant albuminuria and/or proteinuria. The level at which glomeruli are exposed to ET-1 in our model versus models of overt proteinuria are not known. Future studies will have to provide more detailed characterization of this model.

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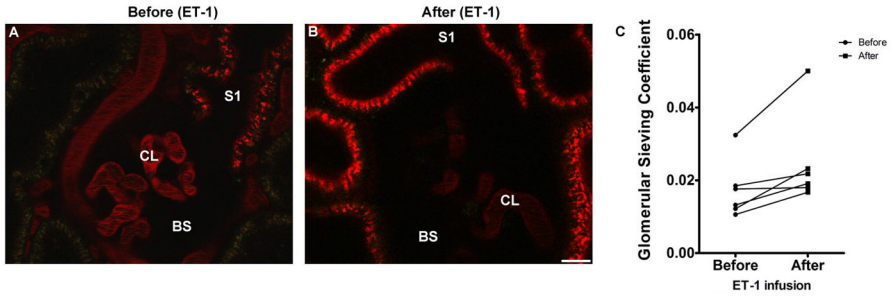

Figure 1. Long term ET-1 treatment increases glomerular albumin permeability

Representative images of Munich Wistar Frömter glomeruli using 2-photon intravital microscopy before (panel A) and after ET-1 treatment (panel B). Although it is not possible to visually distinguish relative differences in Bowman's space (BS) albumin content by the naked eye, the fluorescence intensity ratio between BS and capillary loop (CL) can be readily determined. Despite having been given an identical amount of Texas Red rat serum albumin initially, and imaged at the same time post injection, plasma levels of albumin following ET-1 treatment decreased more rapidly and yielded a higher fluorescence ratio, i.e., glomerular sieving coeficients (GSC). Panel C shows average GSC for individual rats taken before and after chronic ET-1 infusion. For the 6 rats, a total of 28 glomeruli were studied before and after ET-1 infusion, (Bar=15μm; CL=capillary loops).

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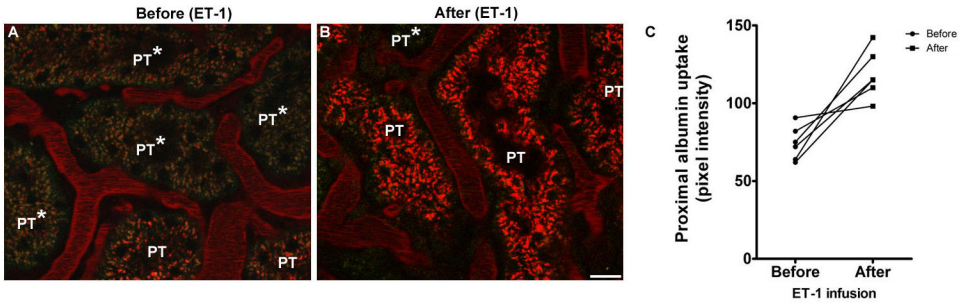

Figure 2. ET-1 exposure increases albumin reabsorption by proximal tubule (PT) segments in ET-1 treated rats

Representative images of proximal tubule (PT) segments from Munich Wistar Frömter rats observed via 2-photon intravital microscopy before (panel A) and after (panel B) ET-1 treatment. Images taken before ET-1 exposure show some heterogenous accumulation of filtered albumin in PT segments; with some segments exhibiting decreased accumulation (PT*). In images taken after ET-1 infusion, accumulation of albumin was seen in a greater percentage of PT segments, with the number of PT segments showing little or no albumin accumulation (although still present) decreasing in number. Average pixel intensity values for background-subtracted images for individual rats before and after ET-1 treatment in are presented in panel C. (Bar=15μm).

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