ONLINE SUPPLEMENT

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

Mohamed A. Saleh[1,2], Erika I. Boesen[1,3], Jennifer S. Pollock[1,2], Virginia J. Savin[4], and David M. Pollock[1,2,3,5]

[1]Vascular Biology Center, [2]Department of Pharmacology and Toxicology,

[3]Department of Physiology, [5]Department of Surgery,

Medical College of Georgia, Augusta, GA 30912 USA

[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 assay[2] 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 ($\Delta V = (V_{final} - V_{initial})/V_{initial}$), 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 $V=4/3\pi r^3$. The magnitude of ΔV was related to the albumin reflection coefficient, σ_{alb}, by the following equation: $(\sigma_{alb})_{experimental} = (\Delta V)_{experimental} / (\Delta V)_{control}$; the σ_{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 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 diaminobenzamidine (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

1. Misra RP. Isolation of glomeruli from mammalian kidneys by graded sieving. *Am J Clin Pathol*. 1972;58:135-139.
2. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248-254.
3. Savin VJ, Sharma R, Lovell HB, Welling DJ. Measurement of albumin reflection coefficient with isolated rat glomeruli. *J Am Soc Nephrol*. 1992;3:1260-1269.
4. Fujihara CK, Avancini Costa Malheiros DM, de Lourdes Noronha II, De Nucci G, Zatz R. Mycophenolate mofetil reduces renal injury in the chronic nitric oxide synthase inhibition model. *Hypertension*. 2001;37:170-175.