Microcirculation: nexus of comorbidities in diabetes

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Generalized capillary dysfunction is a morbid element in the metabolic syndrome, and it is likely involved in its complications. We tested the hypothesis that vast amounts of serum albumin previously observed in kidneys of rats with the metabolic syndrome were caused, in part, by leakage from renal peritubular capillaries. We report herein large scale leaks of plasma fluid in peritubular capillaries of rats with the metabolic syndrome. This finding was directly demonstrated in vivo, and the presence of leaked albumin residue confirmed the leak in postmortem kidney specimens. Moreover, renal interstitial fibrosis and tubular atrophy were found in a distribution similar to the leaked renal albumin in obese rats. We suggest that there is an important link between peritubular capillary damage and interstitial fibrosis, represented as tubulointerstitial disease in the metabolic syndrome. We propose that maintenance of the peritubular microcirculation may improve renal outcomes in diabetes and the metabolic syndrome.

diabetic nephropathies; capillary permeability

GENERALIZED CAPILLARY DYSFUNCTION is an important pathogenic mechanism of nephropathy and other complications of uncontrolled diabetes (6, 27). One logical inference from this notion is that glomerular and peritubular capillaries are similarly damaged in diabetic nephropathy. However, the focal point of research on diabetic nephropathy has been the glomerulus (15, 38, 39), and the prospect of significant injury to postglomerular capillaries has received less attention. Investigators have proposed that postglomerular microvessels are damaged in diabetic nephropathy (6), and some experimental support has been advanced (2, 16), prompting the suggestion that damage to peritubular capillaries alters tubular function (16). However, peritubular vasculopathy has the potential of being far more detrimental, as compromised delivery of oxygen and nutrients is potentially lethal to renal tubules (11, 19). In fact, chronic peritubular capillary insufficiency might be a major contributor to tubular death and to interstitial fibrosis (1), both common features of progressive nephropathy in the metabolic syndrome (7, 8).

Capillary leaks of hearts, kidneys, and retinas have been described with various degrees of precision in diabetic rats (21, 31, 37), affirming the view that capillary damage is at least a component, if not an important source, of diabetes complications. We recently found large quantities of rat serum albumin in kidneys of male rats with nephropathy of the metabolic syndrome (7) and hypothesized a severe leakage of blood plasma into the renal interstitium, most likely from postglomerular capillaries. Hence, we searched for postglomerular capillary leakage of macromolecules in male rats with nephropathy of the metabolic syndrome. We studied F1 generation hybrid male rats, the product of Zucker diabetic fatty (ZDF) and spontaneous hypertensive heart failure rats (ZS rats) which prominently depict all common features of the metabolic syndrome (7). We used two-photon intravitral microscopy to reveal massive capillary leaks into the renal interstitium of relatively young obese rats.

MATERIALS AND METHODS

Animals. The research involving animals adhered to the American Physiological Society’s Guiding Principles in the Care and Use of Laboratory Animals. The investigative protocols were approved by the Institutional Animal Care and Use Committee at Indiana University. Obese (OM) and lean (LM) males were F1 generation from a cross between ZDF and spontaneously hypertensive heart failure (SHHF) rats (ZS; Charles River Laboratories, Willlington, MA) (7). The rats were acclimatized for 1 wk before imaging. Rat urine was collected overnight to measure albumin/creatinine ratios, whereas fasting blood obtained at termination was used to measure triglyceride, cholesterol, and glucose. These analyses were performed by the clinical laboratory at the Indianapolis VA Hospital with a Beckman CX44CE clinical system. In preparation for imaging, rats were anesthetized with ketamine/acepromazine (50/2 mg/kg), and their left kidney was exposed by a flank incision. The right femoral vein was then cannulated for administration of fluorescents. Following renal imaging, the anesthetized rats were killed by decapitation, and abdominal fat pads, hearts, livers, and kidneys were harvested, weighed, fixed overnight in 4% paraformaldehyde, and submerged in PBS unstained for postmortem imaging under the two-photon microscope at ×63 magnification.

Intravitral imaging of kidneys. The anesthetized rats were placed on the microscope stage while kept warm by a heating pad set at 37°C. Hoescht 33342 (600 μg in 0.5 ml of 0.9% sodium chloride) was injected first, followed by albumin (2.5 mg) conjugated with either FITC or rhodamine, and 10,000 molecular weight dextran (1.6 mg), conjugated with either rhodamine or FITC. The albumin and dextran probes were mixed in a volume of 0.5 ml of 0.9% sodium chloride (all fluorescent probes were from Molecular Probes/Invitrogen, Carlsbad, CA). The blue Hoescht 33342 dye was used to locate nuclei and define a focal plane, albumin to image blood space, and dextran to label tubular space (9, 24). The timing for image collection was initiated on injection of the Hoescht 33342 dye. The kidneys were imaged in multiple cortical regions to minimize any focal responses, and images were captured approximately every 4 min. The specific dye intensity measurement was performed in images obtained at 12 min (Fig. 1).

Intravitral imaging was conducted by laser-scanning two-photon microscopy with a Bio-Rad MRC1024MP microscope (Bio-Rad Microscience, Hercules, CA) and a multiphoton laser (Spectra-physics, Mountain View, CA). The same transmission, gain, and offset were used for all images in all rats. The rats were then killed, and the kidneys were harvested and fixed in 4% paraformaldehyde. The software for image analysis was MetaMorph (Molecular Devices, Sunnyvale, CA), employed for analysis of specific fluorescence in interstitium, capillaries, and tubule lumens.

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**Fluorescence studies.** Kidneys from lean and obese rats were fixed in 4% paraformaldehyde overnight and then sectioned into 200-μm slices with a Vibratome (Vibratome, St. Louis, MO). The sections were immersed in PBS with 0.2% Triton X-100 for 5 min, washed three times with PBS, blocked for 15 min in PBS with 0.2% bovine serum albumin, and then incubated with the primary antibody rat endothelial cell antigen (RECA-1, 1:100, from Cell Sciences, Canton, MA) (35) in PBS for 30 min at 37°C, washed in PBS, and then fluorescent secondary antibody, Alexa 546 goat-anti-mouse-546 (1:250, Molecular Probes, Carlsbad, CA), was applied for 30 min at 37°C, followed by washing. The sections were viewed with the Zeiss LSM510-UV confocal microscope system.

Visualization of renal albumin was also accomplished by confocal microscopy. Sheep anti-albumin (AbD Serotec, Raleigh, NC) was applied to the kidney sections at 1:50 dilution and rabbit anti-von Willebrand Factor (DAKO, Carpinteria, CA) was applied at 1:200 dilution. The sections were then incubated for 30 min at 37°C after treatment with 0.2% Triton X-100 for 10 min, washed three times in PBS, and incubated with the appropriate secondary antibody (anti-sheep Alexa Fluor 546 and anti-rabbit Alexa Fluor 488 were from Molecular Probes, both used at 1:250 dilution) again for 30 min at 37°C. The stained sections were washed and stored in PBS containing 2% DABCO (Sigma, St. Louis, MO). They were imaged using a Zeiss LSM-510 UV confocal microscope as described above.

Thinner sections (3-μm thickness) of paraffin-embedded kidneys were also stained with Mason’s trichrome. The sections were stained with the Artisan staining instrument, according to the manufacturer’s instructions (DAKO). The sections were visualized with a Nikon Eclipse TS100 inverted microscope, and images were captured with a Spot Insight2 digital camera (Diagnostic Instruments, Sterling Heights, MI).

**Statistical analysis.** The results are expressed as means ± SE, and any differences between the two groups were evaluated by two-tailed Student’s t-test.

**RESULTS**

**Metabolic phenotype.** Obese and lean ZS rats were studied at 21 wk of age, a point in time when the metabolic syndrome phenotype had been established for several weeks in obese rats (7). The most relevant features of the obese and lean rat phenotypes are summarized in Table 1. Body weights, kidney weights, liver weights, and heart weights were all much higher in obese rats than in lean litter mates. Serum levels of glucose, cholesterol, and triglycerides, normal in lean rats, were also higher in obese rats. Moreover, urinary albumin normalized to

**Table 1. Morphometric analysis**

<table>
<thead>
<tr>
<th>Group</th>
<th>Lean</th>
<th>Obese</th>
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<tbody>
<tr>
<td>Body wt, g</td>
<td>573 ± 22</td>
<td>703 ± 12*</td>
</tr>
<tr>
<td>Kidney wt, g</td>
<td>2.1 ± 0.1</td>
<td>4.1 ± 0.1*</td>
</tr>
<tr>
<td>Liver wt, g</td>
<td>17.8 ± 1.1</td>
<td>46.8 ± 0.2*</td>
</tr>
<tr>
<td>Heart wt, g</td>
<td>1.8 ± 0.1</td>
<td>2.4 ± 0.3*</td>
</tr>
<tr>
<td>Fasting blood glucose, mM</td>
<td>4.02 ± 0.36</td>
<td>9.78 ± 0.75*</td>
</tr>
<tr>
<td>Cholesterol, mM</td>
<td>2.64 ± 0.02</td>
<td>17.46 ± 0.69*</td>
</tr>
<tr>
<td>Triglycerides, mM</td>
<td>1.58 ± 0.47</td>
<td>37.2 ± 1.03*</td>
</tr>
<tr>
<td>Urine albumin/creatinine, μg/mg</td>
<td>10.2 ± 6.3</td>
<td>88.8 ± 12.6*</td>
</tr>
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</table>

Values are means ± SE. *Significantly higher than lean controls, P < 0.05. wt, Weight.
creatinine excretion was eightfold higher in obese rats compared with lean rats, consistent with significant nephropathy and glomerular capillary leak.

Two-photon intravitral images of lean and obese kidneys are shown in Figs. 2 and 3. The representative time sequence (0–25 min) in a lean rat is compiled in Fig. 2. In these experiments, colors of fluorescent labels were reversed: green fluorescence was from FITC-conjugated 10,000 molecular weight dextran, and red fluorescence from rhodamine-conjugated albumin. The appearance in the microscopic field of nuclear blue dye marked the initial frame at time 0. The subsequent images demonstrate that labeled albumin was largely within lumens of peritubular capillaries, while labeled dextran reached all proximal tubule lumens at the earliest time point of 4 min, and then during the entire sequence. In distal tubules, the luminal dye had a yellowish tinge, indicating either a more concentrated dextran dye in lumens of distal tubules, or admixture of dextran and far more concentrated luminal albumin.

The representative time sequences from one obese rat are in Fig. 3. The images are remarkable at several levels; tubules were dilated, and luminal flow rate was very slow in many tubules, as indicated by lack of intraluminal fluorescence in earlier images. Moreover, those tubules missing labeled ultrafiltrate frequently lacked discernable cells. The luminal fluorescence was yellow in proximal tubules, consistent with early...

Fig. 2. Sequential images of the peritubular microcirculation in a lean rat. The cortical landmarks were outlined in the initial frame by injected Hoechst 33342 (blue). Dextran (green) and albumin (red) were then injected, and the renal cortex was imaged for 25 min. The peritubular capillary network (arrows) was extensive and generally impermeant. Dextran in the ultrafiltrate reached all tubular lumens in proximal (pt) and distal tubules (dt).
occurrence of intraluminal dextran and albumin admixture. The peritubular capillaries were frequently surrounded by generous pools of yellow fluorescence, indicating large amounts of albumin and dextran leaking from peritubular capillaries throughout the entire time sequence. Specific fluorescence intensity was analyzed with MetaMorph, and data from one representative set of lean and obese rats are shown in Fig. 4. The intensity of interstitial rhodamine-albumin was indistinguishable from background in lean rats and clearly elevated in obese rats (Fig. 4). These results are consistent with leakage from peritubular capillaries, an assumption verified by multiple sampling of rhodamine-albumin intensities from vessels and the interstitium in their immediate vicinity (Table 2). The data indicate that during steady-state conditions, reached at 12 min postinjection, capillary albumin leakage averaged $50 \pm 1\%$ in obese rat kidneys. The extensive capillary leaks were associated with apparent pruning of the peritubular vasculature (Fig. 5).

One predictable corollary of albumin leakage is its presence in postmortem sections. The localization of leaked albumin was independently verified by confocal microscopy using an anti-albumin antibody to label the kidney sections (Fig. 6). We also used an antibody to von Willebrand factor (vWF or factor VIII-related antigen) in an attempt to colo-

Fig. 3. Sequential images of the peritubular microcirculation in an obese rat. The cortical landmarks were outlined in the initial frame by injected Hoechst 33342 (blue). Dextran (green) and albumin (red) were then injected, and the renal cortex was imaged for 25 min. The peritubular capillary network (arrows) was sparse and permeable throughout the period of observation. Dextran in the ultrafiltrate failed to reach most tubular lumens in initial frames, and when present, yellow fluorescence indicated admixture of albumin and dextran in pt and dt.
calize the endothelial marker vWF and leaked albumin. In lean rats, renal albumin was generally present as discrete peritubular delineation. In contrast, renal albumin in obese rats was heavily accentuated in peritubular and luminal surfaces of tubules, as well as inside tubules. Interstitial regions also contained abundant albumin residue. In lean rats, vWF was linearly distributed around tubules, consistent with endothelial labeling, or in a luminal location, consistent with urinary vWF fragments (33). Colocalization of both markers was mainly seen in interstitial and luminal spaces of obese rats.

The notion that peritubular capillary damage is linked to interstitial fibrosis predicts extracellular matrix expansion of interstitial space in obesity. Accordingly, trichrome stains were performed to visualize areas of fibrosis in renal sections of all rats. The low-power vistas in Fig. 7 revealed normal tubules without fibrosis in lean rats. In contrast, the virtual peritubular space in lean rats was greatly expanded by scar tissue in obesity, which seemed to enclose dilated tubules.

**Table 2. Interstitial albumin leak at 12 min**

<table>
<thead>
<tr>
<th>Area</th>
<th>Intensity</th>
<th>Capillary Leak</th>
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<tbody>
<tr>
<td>Lean vessels</td>
<td>1,142±94</td>
<td>135±3</td>
</tr>
<tr>
<td>Lean interstitium</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Obese vessels</td>
<td>1,900±1388</td>
<td>107±2§</td>
</tr>
<tr>
<td>Obese interstitium</td>
<td>897±34</td>
<td>108±2</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Average number of pixels interrogated in 33 separate fields of lean rat kidneys and 70 separate fields of obese rat kidneys, n = 3 rats per group. †Average intensity per pixel for red fluorescence by MetaMorph analysis. ‡Average capillary leak calculated from pixel intensity of each corresponding field: (obese interstitium/obese interstitium + obese vessels) × 100. ND, none detected. §Significantly different than lean rats, P < 0.01.

Fig. 4. A: data from Figs. 2 and 3, showing specific fluorescent intensity of albumin in the interstitium of lean (○) and obese (□) and in capillaries of lean (■) and obese (●) rats. B: mean and SE for interstitial albumin intensity in 38 determinations of lean and 36 determinations of obese rats at the 8-min peak of albumin leak. The peritubular albumin leak was 50 ± 1% of total capillary albumin in obese rats, at a time point (12 min) that approximated steady-state flow (see Table 2), n = 3.

Fig. 5. RECA antibody labeling of peritubular capillaries (red) in 50-µm renal sections obtained postmortem. A and B: images from a lean rat kidney. A, inset: amplified in B. The arrows point to abundant representation of peritubular endothelium. C and D: images from an obese rat kidney. C, inset: amplified in D. The arrows emphasize the relative sparseness of the peritubular endothelium. The orange coloration of tubules and yellow coloration of red blood cells clumps were from autofluorescence.
DISCUSSION

The original formulation of the Steno hypothesis envisioned widespread vascular damage to explain distant and concurrent complications of type 1 diabetes (6). This idea subsequently received experimental support in type 2 diabetes complicated by nephropathy (27). The overarching microvascular damage hypothesis is consistent with our current findings of loss of renal vascular barrier function, and those of others regarding defective renal nitric oxide synthesis (14), which presumably amplifies renal vasculopathy and resulting nephropathy (18).

In this work, we demonstrated a pervasive renal peritubular capillary leak in rats with the metabolic syndrome. While we were not able to visualize glomeruli due to their greater cortical depth in ZS rats, we can safely assume their capillaries also lost barrier function, as indicated by the existing proteinuria. Nevertheless, the images of the peritubular circulation conclusively showed albumin spilling into the space surrounding peritubular capillaries of the obese rats. The presence and distribution of renal albumin were subsequently verified in postmortem tissue. Similar conclusions were reached in earlier studies (28). However, subsequent investigators concluded that fluid collections neighboring renal tubules in diabetes (12), and other nephropathies (20), came from glomerular leakage occurring alongside the proximal nephron (12, 20). This alternative theory fits in with the glomerular view of diabetic nephropathy (12), but it is hampered by its reliance on postmortem studies, which cannot reveal the peritubular capillary circulation.

In our experiments with young rats, vasculopathy was manifested by dramatic peritubular capillary leakage and subtle attenuation of peritubular capillaries. We suggest that these two relatively early events in nephropathy of the metabolic syndrome have significant consequences for renal tubules, and ultimately for kidney survival. Thus we were able to show that peritubular vasculopathy shared similar regions with interstitial fibrosis in the nephropathy of the metabolic syndrome. These findings resemble those elicited by hypoxic nephropathy caused by loss of peritubular capillaries (23). Furthermore, tubular decay and peritubular (interstitial) fibrosis constitute a common pathway for progression to renal death in diabetes (13), and in interstitial nephropathies (3). Several potential factors may link peritubular vasculopathy and subsequent tubulointerstitial disease. For example, disruption of the extensive peritubular capillary network potentially affects most of renal blood flow, with impending renal hypoxia (10). In addition, we may hypothesize that proportional scaling of nutrient delivery required to sustain metabolic functions in a larger kidney (36) cannot be expected to occur with extensive vasculopathy, potentially leading to energy uncoupling between

Fig. 6. Renal albumin. Immunofluorescence stains of albumin and Von Willebrand’s Factor (FVIII) in 3 lean (left) and 3 obese (right) ZS rat kidneys. The rabbit polyclonal FVIII antibody was labeled with anti-rabbit 488 and appears green in endothelial cells and in some proximal tubules. The tubular label was found mainly in obese rats. The sheep polyclonal anti-albumin was visualized with anti-sheep-546 and it was found in some perivascular cells in both lean and obese rats. Interstitial and tubular albumin (red) is far more pronounced in obese rats than in lean rats. The images were made using a ×40 water immersion objective.
larger requirements and limited delivery from leaky peritubular capillaries (32). Furthermore, we propose that loss of peritubular capillary barrier function may allow direct epithelial contact with plasma rich in toxic lipid peroxides (7), perhaps leading to chronic inflammation within the interstitial microdomain (32).

Our studies revealed large scale extravasations of plasma fluid from peritubular capillaries of obese rats, but did not show what caused capillary leaks and attenuation. Potential mechanisms for capillary damage include intrinsic modifications of vascular permeability induced by advanced glycosylation end-products (17, 34) or oxidized lipids (34), and those secondary to barrier modifications affected by the inflammatory response within the peritubular space (7, 32). These pathogenic mechanisms may in fact have a common origin, as advanced glycosylation end-products (25) and oxidized lipids (22) also promote endothelial leukocyte adherence and migration. Hence, we suggest that renal protection in diabetes can be achieved by metabolic control, and failing that, by tactics that block the translation of metabolic abnormalities into destructive microvascular injury (4, 26).

GRANTS

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REFERENCES


