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PROGRESSIVE RENAL VASCULAR PROLIFERATION AND INJURY IN OBESE ZUCKER RATS

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Abstract

Objective—Obesity, an independent risk factor for chronic kidney disease, may induce renal injury by promoting inflammation. Inflammatory cytokines can induce neovascularization in different organs, including the kidneys. However, whether obesity triggers renal neovascularization and, if so, its effect on renal function has never been investigated.

Methods—Blood pressure, proteinuria and glomerular-filtration-rate (GFR) were measured in-vivo. Renal microvascular (MV) architecture was studied by 3D micro-CT in lean and obese Zucker rats (LZR and OZR, n=7/group) at 12, 22, and 32 weeks of age. Renal inflammation was assessed by quantifying interleukin (IL)-6, tumor-necrosis-factor (TNF)-alpha, and ED-1 expression, as renal fibrosis in trichrome-stained cross-sections.

Results—Mild inflammation and lower GFR was only observed in younger OZR, without renal fibrosis or changes in MV density. Interestingly, renal MV density increased in OZR at 32 weeks of age, accompanied by pronounced increase in renal IL-6 and TNF-alpha, ED-1+ cells, proteinuria, decreased GFR, and fibrosis.

Conclusion—This study shows increased renal cortical vascularization in experimental obesity, suggesting neovascularization as an evolving process as obesity progresses. Increased renal vascularization, possibly triggered by inflammation, may reflect an initially compensatory mechanism in obesity. However, increased inflammation and inflammatory-induced neovascularization may further promote renal injury as obesity advances.

Keywords

obesity; kidney; microcirculation; inflammation; fibrosis

Introduction

The prevalence of obesity and overweight exceeds 60% among US adults¹ and constitutes a population that is exposed to numerous related co-morbidities², which represents a significant impact on the US health-care budget. Obesity is an independent risk factor for renal disease and is considered to represent the most preventable cause of renal failure. Obesity itself may increase the risk for chronic kidney disease (CKD) by increasing the metabolic demands on the kidney, which leads to higher glomerular capillary pressures and

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glomerular hypertrophy³. Notably, prevalence of CKD increases from 2.9% among non-obese to 4.5% among obese adults. CKD has increased about 16% in the US during the last decade⁴, paralleling the sustained increased in obesity. Furthermore, obesity is frequently associated with several cardiovascular risk factors such as hypertension, atherosclerosis, and diabetes that may in turn confound renal risk adding a burden to obese patients that can further promote renal damage^{3, 5}.

One of the pathways by which obesity can promote renal injury is by favoring the activation of pro-inflammatory factors (e.g. interleukins, tumor-necrosis factor- α) thereby promoting inflammation, a complex process initially activated as a defensive mechanism that is also capable to later induce tissue injury as increases. Increased and progressive inflammation in obesity may compromise the renal microcirculation by promoting intrinsic modifications of vascular hemodynamics, reactivity, and permeability^{5, 6}. There is indeed evidence that the kidney exposed to obesity presents significant microvascular (MV) dysfunction⁷. Furthermore, inflammatory cytokines can induce neovascularization⁸ and by formation of new vessels sustain the perfusion of the injured or ischemic tissues. We have previously shown that the kidney exposed to hypercholesterolemia, a condition associated with significant inflammation, shows augmented MV density^{9, 10}. Interestingly, this increased vascularity reflected a compensatory mechanism that serves to sustain renal perfusion under basal conditions, because when we inhibited vascular proliferation, renal function decreased⁹. Therefore, it is reasonable to hypothesize that obesity may promote vascular proliferation in the kidney. However, whether obesity triggers renal neovascularization and if so, whether neovascularization relates to renal injury as obesity progresses have never been investigated.

Methods

All protocols were reviewed and approved by the Institutional Animal Care and Use Committee at University of Mississippi Medical Center. The model of obesity we used in our study was the obese Zucker rat (OZR, fa/fa, n=21), which exhibits a missense mutation of the leptin receptor that resulted in many features of human obesity^{11, 12}. The OZR were studied and compared to lean counterparts (LZR, n=21). Rats were maintained on 12h/12h light/dark cycle and given ad libitum access to tap water and standard rat chow (Teklad, Harlan). Some animals were studied at 12 weeks (young LZR and young OZR, n=7 each) and others at 32 (old LZR and old OZR, n=7 each) weeks. Animals were placed in metabolic cages and 24 hours urine samples were collected to measure proteinuria using a BioRad kit.

For direct measurement of blood pressure, catheters were implanted in the femoral arteries of rats under isoflurane anesthesia, and exteriorized in the interscapular region. At least 3 days after the surgery, arterial pressure was measured for 4h in conscious, freely moving rats, via the indwelling arterial catheters in all groups, as previously described¹³. Glomerular filtration rate (GFR) was measured from 3H-inulin clearance, as previously described¹⁴. Briefly, the rats were anesthetized with Inactin (Sigma, MO) (100mg/kg BW), their body temperature was maintained at 37°C with a servo-controlled heating pad. The animals were instrumented with bilateral jugular vein catheters for infusions of artificial rat plasma (2.5% Bovine Serum Albumin and 2.5% Bovine Immunoglobulin at a rate of 12.5 mL/kg/h for the first 45 min and 1.5 mL/kg/min thereafter, for maintenance of euvoemia) and 3H-Inulin (5 μ Ci/mL; 1.2 mL/h). Urine was collected over two 20 min periods from a catheter inserted in the bladder and GFR was determined from the clearance of inulin averaged over the 2 collection periods, normalized by body weight, and expressed as mL/min/kg.

Furthermore, additional groups of LZR and OZR at 22 weeks of age (mid LZR and mid OZR, n=7 each) were also included to investigate whether changes in the MV architecture were progressive as obesity evolves, and whether these changes are associated with increases in pro-inflammatory cytokines.

All the rats were euthanized using an intra-peritoneal overdose of Inactin. Using a mid-abdominal incision, one of the kidneys of each animal was removed (previous ligation of the main renal artery and vein) and immersed in Krebs's solution containing heparin (10 units/ml) to prevent drying. One part of the kidney was preserved in 10% buffered formalin (Sigma), while the rest of the kidney shock-frozen in liquid nitrogen and stored at -80°C ^{10, 15, 16}. *In vitro* studies were performed in renal homogenates to assess the protein concentration of pro-inflammatory and angiogenic factors. By ELISA and immunohistochemistry, inflammatory cytokines and pro-angiogenic factors such as tumor-necrosis factor (TNF)- α , interleukin (IL)-6, vascular endothelial growth factor (VEGF) were investigated. Furthermore, inflammatory infiltration and MV and renal tissue remodeling were assessed in 5 μm mid-hilar renal paraffin-embedded slices stained against ED-1 (RDI, NJ, 1:50) positive cells and using trichrome, respectively.

Micro-CT

A saline-filled cannula was placed in the aorta, the aorta ligated below and above the renal arteries, and infusion of 0.9% saline (containing 10 units/ml heparin) was initiated under physiological perfusion pressure (Syringe Infusion Pump 22, Harvard Apparatus, Holliston, MA) at a rate of 2 ml/min. A small incision was performed in the inferior vena cava to allow the saline infusion to drain. After 10–15 minutes of saline infusion and when it drained freely from the vein, it was replaced with an infusion of intravascular contrast agent (2 ml/min), which was a freshly mixed radio-opaque silicone polymer containing lead chromate (Microfil MV122, Flow Tech, Inc., Carver, MA) until the polymer drained freely from the vein. Then, the polymer-filled kidneys were left at 4°C overnight, and then immersed in 10% buffered formalin for 72 hours before scanning. The kidney samples were scanned at 0.3° increments using a micro-CT scanner, (SkyScan 1076 system, Micro Photonics Inc, PA) and the X-ray transmission images were acquired in each angle of view at a resolution of 18 μm and digitized to 16 bits gray scale. 3D volume images were reconstructed using a filtered back-projection algorithm and displayed on a computer workstation by volume rendering for display and analysis of renal MV using the AnalyzeTM (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN) software package.

Immunohistochemistry

staining was performed in 5 μm frozen mid-hilar renal cross-sections to assess the expression of ED-1 (Chemicon International, CA, 1:80) in young and old LZR and OZR. The secondary antibody, IgG Envision Plus (Dako), was followed by staining with the Vector NovaRED substrate kit (Vector Laboratories, Burlingame, CA), following vendor's instructions.

Data analysis

Micro-CT analysis

Using the AnalyzeTM software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN), the three-dimensional tomographic images were oriented so that the z-axis was parallel to the radial vessels. Based on the number of cortical sections, the cortex was tomographically divided into 15 levels obtained at equal intervals, starting at the juxtamedullary cortex. For analysis, levels 1–5 were considered as inner third, levels 6–10 as middle third, and levels 11–15 as the outer third of the cortex. The spatial density,

average diameter, and vascular volume fraction (sum of cross-sectional areas of all vessel/area of the region of interest) of cortical microvessels (diameters <200µm) were calculated in each level. In addition, to further characterize the renal MV architecture of microvessels under 200µm, microvessels were then divided in those vessels between <80µm, 80–120µm, and 120–200µm, respectively. Tortuosity index factor was calculated by tomographically isolation of 3–5 arterioles in each sample, and then the 3D path distance (total length) and linear distance (shortest distance between endpoints) of the main branches were calculated, and the tortuosity index calculated by dividing path distance by linear distance.

Histology—Mid-hilar 5 µm trichrome-stained cross sections of each kidney (1 per animal) were examined to assess renal fibrosis and glomerulosclerosis, as previously described^{10, 15, 16}.

Statistical Analysis

Results are expressed as mean±SEM. Comparisons within groups were performed using paired student's t-test, and among groups using two-way ANOVA for the variables age and obesity, followed by one-way ANOVA with the Neumann-Keuls correction for multi-group comparisons. Statistical significance was accepted for $p \leq 0.05$.

Results

MV 3D architecture

Renal MV density, tortuosity, renal pro-inflammatory and pro-angiogenic factors were similar between young LZR and OZR, and between mid LZR and OZR. However, OZR at 32 weeks showed a significant increased MV density, evident throughout the cortex and mainly accentuated in those vessels <80 µm, compared to old LZR, suggesting MV proliferation as obesity evolves (Figure 1). The progressively increased vascularization ($p<0.05$ old vs. young OZR) was accompanied by a significant increase in renal TNF- α and IL-6 in OZR compared to LZR, which started at 22 weeks and was further elevated in old OZR, suggesting a progressive pro-inflammatory and pro-angiogenic milieu, as renal protein levels of VEGF were substantially reduced in the old OZR (Table 1).

Renal inflammation and morphology

inflammatory infiltrates were slightly elevated in young OZR compared to young LZR, mainly at the tubulo-interstitial compartment. Fibrosis was absent in young LZR and OZR, suggesting absence of damage at the early stage of obesity in this model (Figure 2). However, at 32 weeks, the old OZR showed a dramatic increased inflammation, showing ED-1+ cells infiltrating the glomerular, perivascular, and tubulo-interstitial compartments. The increased inflammation in old OZR was also accompanied by increased glomerulosclerosis and tubulo-interstitial fibrosis compared to old LZR (Figure 3).

Mean arterial pressure and renal function

Mean arterial pressure was similar between LZR and OZR in both young (114 ± 2.1 vs. 113.3 ± 2.1 mm/Hg, $p=NS$) and old (123.3 ± 3.2 vs. 125.2 ± 1.8 mm/Hg, $p=NS$) rats. However, proteinuria was markedly increased, accompanied by a significant and progressive decreased GFR in old OZR compared to lean and younger controls (Figure 4, $p<0.05$ old vs. young OZR, $p=NS$ old vs. young LZR), suggesting a progressive deterioration of the renal function.

Discussion

The current study shows for the first time increased renal cortical vascularization in experimental obesity, and demonstrates that renal vascular proliferation is an evolving process as obesity progresses. Increased renal vascularization in obesity, possibly triggered by inflammation, may reflect an initially compensatory mechanism that may sustain renal perfusion and contribute to preserve renal function at an early stage. However, the progressive increase in renal inflammation and neovascularization is accompanied by evolving renal functional and structural injury as obesity progresses, suggesting that neovascularization may promote renal damage as obesity advances.

The most recent data from the National Health and Nutrition Examination Survey (NHANES) showed that 16.8% of the U.S. population aged ≥ 20 years had CKD, showing an overall 16% increase in its prevalence during the last decade⁴. It is remarkable that despite the downward trend in the incidence of traditional cardiovascular risk factors such as cigarette smoking, hypertension, and hyperlipidemia, there is a rising prevalence of CKD paralleled by an increase in the incidence of obesity. Indeed, there is a strong correlation between body mass indices and the relative risk of progression of CKD¹⁷, underscoring the role of obesity as an independent risk factor for renal disease. However, despite the fact that the prevalence of CKD doubles in obese patients and obese individuals show an astonishing 68% increased odds of developing CKD¹⁸, the mechanisms involved in obesity-induced renal injury are still not elucidated.

Obesity is a progressive disease that may deteriorate the function and promote injury in practically every organ, either directly or by favoring the onset and progression of cardiovascular risk factors. In the kidney, obesity can alter renal function by physical compression of the organ as well as by promoting sodium retention, glomerular hyperfiltration, and hypertension, leading to proteinuria, glomerular and tubular injury¹⁹. Moreover, obesity has been shown to be associated with inflammation²⁰. Renal interstitial inflammation is a prominent feature in CKD and may determine the severity and progression of renal injury towards kidney failure^{21, 22}. The current study shows that renal inflammation is present in the kidneys of OZR at the earlier stages of obesity, without any evidence of overt renal injury other than a mild increase in proteinuria. Interestingly, inflammation significantly increases in old OZR at 32 weeks as obesity progresses, as suggested by a substantial increase in renal TNF- α and IL-6, accompanied by abundant ED-1 positive inflammatory cells, a marker for macrophages, at the tubulo-interstitial, perivascular, and glomerular compartments. Moreover, these findings showed that progression of obesity develops in parallel with growing renal inflammation and proteinuria and decreased GFR, underscoring the role of obesity as a risk factor and promoter for progressive renal injury.

The increased renal pro-inflammatory factors such as TNF- α and IL-6 can also promote vascular proliferation. IL-6 is a multifunctional cytokine capable of modulating new vessel growth and inflammatory cell influx²³. The inflammatory and angiogenic effects of IL-6 have been suggested to be independent and were demonstrated both *in vivo* and *in vitro*²³, and may help to support its role in the increased renal vascularization and injury in old OZR. Furthermore, TNF- α has been shown to be a potent pro-angiogenic protein as well²⁴. It could be released by cells such as macrophages, endothelial and mesangial cells, and is able to promote *de novo* angiogenesis in several tissues directly or by interaction with other mediators^{24, 25}. Therefore, increased vascularization in an inflammatory milieu such as the kidney in the obese old OZR may have been the response to or the reflection of evolving injury during progression of obesity, considering that the vascular density was unchanged in the younger controls and in the absence of significant renal damage. It is possible that these

new vessels are generated to somewhat sustain renal perfusion⁹ as obesity progresses, in a context of a progressive deterioration of the renal function in this model. It is well established that advanced stages of obesity with aging in OZR is accompanied by end-stage renal disease, the major cause of mortality in OZR fed ad libitum²⁶. Nevertheless, considering that inflammatory-induced angiogenesis may result in vessels with blind ends or loose endothelial cell junctions^{27, 28}, these new vessels may not be fully functional or even further contribute to tissue injury allowing the extravasation of further pro-inflammatory or pro-growth factors to the perivascular, tubulo-interstitial, and glomerular compartments, which could explain the significant increased inflammation and fibrosis that accompanied the increased MV density in old OZR.

Surprisingly, there was an unexpected progressive decrease in renal VEGF accompanying the increased cortical MV density in old OZR compared to lean controls. VEGF is a prominent angiogenic factor to sustain the MV networks in different organs, including the kidneys, by activating cell division, migration, and endothelial cell survival, that generate, repair, and maintain MV integrity²⁹. VEGF is also a prominent regulator of renal fibrosis²⁹ and glomerular and capillary repair³⁰, and is produced constitutively by several cell types in the adult kidney. The reasons for this decrease in VEGF might be speculated. One possibility to explain the reduction in renal VEGF is that the assay used in our study measured free VEGF, thereby not measuring a significant portion of VEGF that is bound to its receptors, which may result in a greater angiogenic stimulus that helps to support the increased MV density in old OZR. On the other hand, a different scenario that may explain the decreased renal VEGF is related to the increased oxidative stress in OZR kidneys shown in other studies³¹. Reactive oxygen species can activate redox-sensitive fibrogenic factors (e.g. transforming-growth factor- β , nuclear factor kappa-B) thereby favoring renal scarring, as observed in the old OZR animals, and consequently decreasing the sources of VEGF³² and blunting its production. The latter proposed mechanism would support the notion of a VEGF-independent pathway for vascular proliferation in the OZR kidneys. Furthermore, this decrease in VEGF may also in turn contribute to renal scarring as well²⁹, suggesting a vicious circle.

Although we have different time-points in our study, it is unlikely that the aging process *per se* played a role in mediating the increase in MV density and renal injury in our current study, considering that none of those changes were observed in the mid-age and older (22 and 32 weeks) LZR. However, we cannot rule out the possibility that at a later stage, aging combined with progressive obesity may contribute to increase renal injury by, for example, further increasing oxidative stress and thereby aggravating inflammation, fibrosis, and other injurious mechanisms such as apoptosis^{33, 34}. The current study was designed to investigate the effects of obesity on the renal MV architecture and the significance of vascular proliferation. These findings suggest that MV proliferation, possibly triggered initially as a compensatory mechanism, do not curtail the evolving renal injury during progression of obesity, since inflammation and renal fibrosis were dramatically increased in old OZR. Nevertheless, to prove this concept, we are aware that interventions to halt vascular proliferation would be necessary to further understand the role of these new vessels, which might have had a role in renal function or in promoting inflammation and fibrosis that was not elucidated in this study. Similarly, the role of inflammation as a promoter of neovascularization would be better characterized if anti-inflammatory interventions are performed, which will be pursued in future studies. On the other hand, a limitation of our study is that the resolution of the micro-CT does not allow quantification of capillaries, which are $<10\mu\text{m}$ in diameter. A reduction in capillary density may be expected in association with a reduction in renal VEGF, which is associated with capillary trophic support that could have consequently contributed to reduce GFR and increase fibrosis^{35, 36} in old OZR. Therefore, future studies are needed to further advance our understanding in the

pathophysiology of obesity-induced renal disease and its progression. However, our work may represent the first step, and describes a novel association between progression of obesity, inflammation, MV proliferation, and renal injury. Furthermore, this study deals with a novel concept of renal vascular proliferation in response to injury, which seems to differ depending on the renal insult ^{9, 10, 28}.

In summary, the current study shows for the first time the time-dependent changes in renal MV architecture and damage during progression of obesity. Renal microvessels proliferate as obesity evolves, likely triggered by a progressive increase in inflammatory mediators and inflammation. Inflammatory-induced vascular proliferation possibly reflects a contributory and previously unknown novel mechanism to renal injury, suggesting neovascularization as a target for interventions to preserve the kidney in obesity.

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Figure 1. Representative 3D tomographic images of the renal microcirculation and quantification of MV density per vessel diameter (0–80 μm, 80–120 μm, and 120–200 μm) from lean and obese Zucker rat kidneys after 12 (top row), 22 (middle row), and 32 (bottom row) weeks of age. Renal microvascular density significantly increased in obese Zucker rats after 32 weeks and longer exposure to obesity, compared to the lean animals. * $p < 0.05$ vs. LZR.

Figure 2. Representative renal trichrome and ED-1 staining (top and bottom, respectively, x40) and quantification of renal fibrosis and inflammation in lean and obese Zucker rat kidneys after 12 weeks of age. Only a mild increase in inflammation was observed in OZR kidneys.

Figure 3. Representative renal trichrome and ED-1 staining (top and bottom, respectively, x40) and quantification of renal fibrosis and inflammation in lean and obese Zucker rat kidneys after 32 weeks of age. Inflammation, renal tubulo-interstitial fibrosis and glomerulosclerosis significantly increased after 32 weeks in obese Zucker rats. *p<0.05 vs. LZR.

Figure 4. Proteinuria and GFR (normalized per body weight) at 12 (top row) and 32 (bottom row) weeks of age in lean and obese Zucker rats. Despite a mild increase in proteinuria, and decreased in GFR (when normalized by body weight) was observed at 12 weeks of age. However, these changes were significantly accentuated after 32 weeks, as obesity progressed. * $p < 0.05$ vs. LZR.

Table 1

Renal protein concentration (mean $\pm$ SEM) of pro-inflammatory and pro-angiogenic factors in LZR and OZR after 12, 22, and 32 weeks of age (n=7 in each group).

		Interleukin-6 (pg/mg protein)	Tumor necrosis factor- α (pg/mg protein)	Vascular endothelial growth factor (pg/mg protein)
12 weeks	LZR	11.0 $\pm$ 1.2	0.54 $\pm$ 0.1	30.6 $\pm$ 5.6
	OZR	17.4 $\pm$ 7.4	0.82 $\pm$ 0.4	44.6 $\pm$ 9.9
22 weeks	LZR	11.8 $\pm$ 0.8	1.09 $\pm$ 0.03	38.4 $\pm$ 1.3
	OZR	10.1 $\pm$ 1.0	1.20 $\pm$ 0.05 [#]	38.4 $\pm$ 2.8
32 weeks	LZR	11.2 $\pm$ 0.8	0.75 $\pm$ 0.08	36.8 $\pm$ 3.6
	OZR	19.2 $\pm$ 2.1 [*]	1.3 $\pm$ 0.08 [*]	14.3 $\pm$ 3.7 [*]

^{*} p<0.05 vs. LZR at 32 weeks;

[#] p=0.07 vs. LZR