Upregulation of Angiotensin II Type 1 Receptor, Inflammatory Mediators, and Enzymes of Arachidonate Metabolism in Obese Zucker Rat Kidney
Reversal by Angiotensin II Type 1 Receptor Blockade

Zhong-Gao Xu, MD, PhD; Linda Lanting, BS; Nosratola D. Vaziri, MD; Zhen Li, MD; Lili Sepassi, BA; Bernardo Rodriguez-Iturbe, MD; Rama Natarajan, PhD

Background—Severe obesity can result in proteinuria and progressive glomerulosclerosis in humans and experimental animals. The associated renal disease is ameliorated by weight reduction and/or blockade of the renin-angiotensin system. Various growth factors, cytokines, and lipid mediators are implicated in the pathogenesis of renal disease. To explore the possible involvement of these mediators in obesity-induced renal disease, we examined the expression of key enzymes of arachidonate metabolism and inflammatory genes in untreated and losartan-treated obese Zucker rats, a model of obesity, insulin resistance, and renal injury.

Methods and Results—Seven-week-old male obese Zucker rats were randomized to losartan-treated (100 mg/L drinking H₂O) and untreated groups, with lean Zucker rats as controls. After 4 months, RNA and protein were obtained from renal cortical tissue for relative reverse transcription–polymerase chain reaction, Western blots, and immunohistochemistry. Compared with the lean controls, obese Zucker rats showed significant glomerular matrix expansion and increased mRNA expression of the extracellular matrix protein fibronectin, inflammatory mediators interleukin-6 and monocyte chemoattractant protein-1, and 2 major enzymes of arachidonate metabolism, namely, 12/15-lipoxygenase and cyclooxygenase-2. This was associated with significant increases in p38 and extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinase activities and marked upregulation of angiotensin II type 1 receptor (AT1R) mRNA and protein expression. These abnormalities and the associated glomerulopathy and proteinuria were prevented by administration of the AT1R blocker losartan.

Conclusions—These findings indicate that obesity-induced glomerulopathy is associated with upregulation of key inflammatory mediators. These events are associated with and perhaps in part due to upregulation of AT1R, as evidenced by their reversal with AT1R blocker treatment. (Circulation. 2005;111:1962-1969.)

Key Words: obesity • angiotensin • inflammation • kidney

Massive obesity in humans can result in focal segmental glomerulosclerosis (FSGS), which presents with glomerular proteinuria.¹² In addition, by promoting insulin resistance and type 2 diabetes mellitus, obesity can lead to diabetic nephropathy (DN), which has emerged as the major cause of end-stage renal disease in the United States and many other countries.³ The associated proteinuria in patients with massive obesity can be ameliorated by weight reduction and ACE inhibition.¹ Obese Zucker rats (fa/fa rats) exhibit hyperphagia, obesity, peripheral insulin resistance, hyperinsulinemia, and hyperlipidemia.⁴ This is due to the autosomal recessive mutation of the gene that encodes the leptin receptor.⁸ With aging, obese Zucker rats develop proteinuria and FSGS, which eventually leads to advanced renal failure.⁵,⁷,⁹ The associated proteinuria and glomerulosclerosis in obese Zucker rats can be ameliorated by food restriction,⁷ ACE inhibitors,¹⁰ lipid-lowering agents,¹¹ and insulin sensitizers.¹²

The prominent features of glomerular lesions early in the course of renal disease in the obese Zucker rat consist of marked increases in glomerular monocyte/macrophage counts and evidence of podocyte injury marked by a significant de novo desmin expression.¹³,¹⁴ It is of note that micropuncture studies have revealed no significant differences in glomerular capillary pressure, single-nephron glomerular plasma flow, or glomerular filtration rates among 9- to 13-week-old obese and lean Zucker rats.¹⁵ These observations exclude glomerular capillary hypertension or hyperfiltration as a primary cause of glomeruloscle-
rosis in young prediabetic Zucker rats. Taken together, the above observations suggest that severe obesity and the associated metabolic consequences in prediabetic obese Zucker rats may contribute in part to the glomerulosclerosis process via an inflammatory pathway that involves recruitment of monocytes/macrophages. These events can lead to podocyte injury, proteinuria, mesangial expansion, glomerulosclerosis, and progressive renal disease. Moreover, the favorable effects of renin-angiotensin system blockade in retarding FSGS in this model must be mediated in part by such nonhemodynamic mechanisms as an antiinflammatory action.

Chemokines (particularly monocyte chemotactrant protein-1 [MCP-1]), cytokines, and products of the major enzymes of arachidonate metabolism play an important role in promoting cell proliferation, macrophage recruitment, and inflammation.16–20 In this regard, lipid mediators and oxidized lipids participate in the pathogenesis of various renal diseases, including DN. In particular, products of the cyclooxygenase (COX) and lipoxygenase (LO) pathways of arachidonate metabolism exert numerous physiological and pathological effects in the kidney. For instance, 12-LO activation can lead to the formation of oxidized lipids such as 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE].21 It is of note that the leukocyte-type 12-LO and 15-LO are classified as 12/15-LO because they have high structural homology and can form both 12(S)-HETE and 15(S)-HETE from arachidonic acid.21 12/15-LO is present in the kidney,22,23 and 12/15-LO mRNA and protein are increased in parallel with fibronectin in a type 1 diabetes mellitus model of experimental DN.23 Moreover, urinary excretion of 12(S)-HETE is increased in diabetic patients.24 Factors relevant to the pathogenesis of DN, such as high glucose and angiotensin II (Ang II), increase 12/15-LO activity and expression in rat mesangial cells.16,23 Furthermore, 12(S)-HETE directly stimulates cellular hypertrophy and extracellular matrix (ECM) protein (fibronectin) expression in rat mesangial cells. Likewise, it mediates Ang II-induced mesangial cell growth and ECM production.16 12(S)-HETE-induced effects are mediated, at least in part, by p38 mitogen-activated protein kinase (MAPK) and its target transcription factor, cAMP response-element binding protein (CREB).16,25 Furthermore, cellular growth, matrix production, oxidant stress, and activations of MAPK and transcription factor CREB are attenuated in 12/15-LO knockout mice.26 Taken together, the observations cited above illustrate the relevance of 12/15 LO pathway to the pathogenesis of DN and Ang II–induced renal injury. In addition, COX-2 can participate in the pathogenesis of DN and inflammation mainly through hemodynamic and pro-oxidant effects.17,18,27–29

In contrast to diabetes, very little is known regarding the role of the enzymes of the arachidonate pathway in the pathogenesis of kidney disease associated with obesity and insulin resistance. The present study was designed to examine the expression of 12/15-LO and COX enzymes of arachidonate metabolism, inflammatory genes such as MCP-1 and interleukin-6 (IL-6), and MAPK activities in the renal cortex of prediabetic obese Zucker rats relative to control lean Zucker rats. In addition, Ang II type 1 receptor (AT1R) expression and response to the AT1 receptor blocker (ARB) losartan were tested. The latter was justified by the fact that Ang II can activate the 12/15-LO and COX pathways, the products of which, in turn, can activate MAPKs and inflammatory genes.

Methods

Materials

AT1R antibody was from Santa Cruz Biotechnology; fibronectin (FN-Ec) antibody was from Chemicon; COX-2 antibody was from Cayman Chemical Company; and antibodies for phosphospecific and nonphospho-p38 and -ERK1/2 (extracellular signal-regulated kinase) MAPKs were from Cell Signaling. We also used a 12/15-LO peptide antibody as described previously.21 In addition, we used horseradish peroxidase–conjugated secondary antibodies from Cell Signaling; β-actin antibody from Sigma; Supersignal chemiluminescence reagent from Pierce; relative multiplex reverse transcription–polymerase chain reaction (RT-PCR) kits and primers for Quantum RNA 18S internal standards from Ambion Inc; and RNA-STAT60 reagent from Tel-Test. Losartan was obtained from Merck Co.

Animals and Experimental Design

All animal studies were conducted under a protocol approved by the Animal Care and Use Committee of the University of California, Irvine. Seven-week-old male lean Zucker rats (n = 6) and male obese Zucker rats (n = 12) were used in the present study. Obese animals were further randomized into 2 groups of 6 rats each. One group of obese Zucker rats was administered losartan in the drinking water (100 mg/L) for 4 months. Under general anesthesia, the rats were killed by exsanguination with cardiac puncture. Renal cortical tissues were removed and stored at −70°C for further study. Additional specimens were fixed in 10% formalin for histological evaluation. Body weight, tail arterial pressure, 24-hour urinary albumin, serum glucose, cholesterol, and triglyceride concentrations were determined by standard methods.

Relative and Competitive RT-PCR

Total RNA was isolated with RNA-STAT60 reagent according to the manufacturer’s instructions. cDNA was synthesized with 1 μg of RNA using murine leukemia virus reverse transcriptase and random hexamers. Primers for the 18S ribosomal RNA (489 or 324 bp) were reverse transcribed. The primers used are summarized in Table 1. Relative RT-PCRs were performed as described previously.16 For AT1R mRNA expression, we used a quantitative competitive RT-PCR method. The AT1R competitor cDNA (212 bp) used as internal standard was designed to contain the same base pair sequence as the target cDNA that would allow efficient priming, but it had a portion deleted so that the competitor PCR-generated fragment could be easily distinguished electrophoretically by size. Band densities were analyzed by laser densitometry (Bio-Rad Laboratories). The RT-PCR products were separated by electrophoresis, and the values were log transformed against the competitor concentration for each PCR tube. The quantity of cDNA in the test sample was defined as the amount at which the competitor and wild-type optical density bands were equal.23

Western Blot Analysis

Cortical tissue samples were lysed in SDS sample buffer (2% SDS, 10 mmol/L Tris-HCl, pH 6.8, 10% [vol/vol] glycerol). Lysates were centrifuged at 12 000 rpm for 15 minutes at 4°C and the supernatant stored at −70°C. Fifty micrograms of protein per lane was separated on 10% SDS-PAGE gels (Bio-Rad), transferred onto a nitrocellulose membrane, and immunoblotted with antibodies to AT1R (1:1000), 12/15-LO (1:500), COX-2 (1:500), phospho-p38 (1:500), and phospho-ERK1/2 (1:500). The blots were stripped and then reprobed with an antibody to β-actin (1:5000), total p38 MAPK (1:1000), or total ERK1/2 (1:1000). Immunoblots were scanned with a GS-800 den-
sitionometer and protein bands quantified with Quantitation One software (Bio-Rad).

**Histological Evaluation**

Renal cortical slices for routine light microscopy were placed into alcoholic Bouin’s solution processed in the standard fashion, and sections were stained with periodic acid-Schiff (PAS). Renal cortical slices for immunohistochemical staining were fixed in 10% neutral buffered formalin and paraffin embedded by standard techniques, slices for immunohistochemical staining were fixed in 10% neutral alcoholic Bouin’s solution processed in the standard fashion, and Renal cortical slices for routine light microscopy were placed into Histological Evaluation

<table>
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<td>Antisense 5′-CTCGAGCCCTGATC-3′</td>
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**Statistical Analyses**

Data are expressed as mean±SEM from multiple experiments. ANOVAs with Tukey post tests for multiple groups were used in statistical evaluation of the data with PRISM software (Graph Pad). Statistical significance was detected at the 0.05 level.

**Results**

**General Animal Data**

Untreated obese Zucker rats had significant increases in body weight, plasma cholesterol, and triglyceride concentrations and significant albuminuria compared with lean Zucker rats (Table 2); however, nonfasting serum glucose and creatinine concentrations and arterial pressure did not significantly differ between these groups. Losartan (ARB) administration significantly lowered blood pressure and normalized urinary albumin excretion without altering body weight or serum glucose concentration in treated obese Zucker rats (Table 2).

**Histological Data**

One of the most striking characteristics of FSGS and progression of renal disease is mesangial expansion, which results from accumulation of ECM proteins. The glomeruli in obese Zucker rats showed marked mesangial expansion characterized by increased PAS staining (Figure 1B) and heavy fibronectin staining (Figure 1E) relative to lean Zucker rats.

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These abnormalities were prevented by ARB administration (Figures 1C and 1F). The observed changes in immunodetectable fibronectin were accompanied by parallel changes in fibronectin mRNA in the 3 groups (Figure 2), which points to transcriptional regulation of this matrix protein. In addition to the glomerular changes cited above, significant tubulointerstitial abnormalities were found on histological examination of the kidneys in untreated obese Zucker rats. These included tubular dilation, tubular epithelial cell effacement, and peritubular cellular infiltrations that affected 25% to 50% of tubulointerstitial space. The observed tubulointerstitial abnormalities were mitigated by long-term ARB administration.

Expression of AT1R
To determine the changes of AT1R mRNA, we performed quantitative competitive RT-PCR with AT1R-specific primers. As internal standard, we used an AT1R deletion mutant that is coamplified with the endogenous gene. The use of the same primers for the mutant and for the endogenous gene ensures comparable amplification efficiencies. Competitive RT-PCR data showed a significant increase in AT1R mRNA expression in obese Zucker rat cortex, and this increment was reversed by ARB therapy (Figure 3A). Similarly, AT1R protein expression was increased significantly in obese Zucker rat kidney cortex and was normalized by ARB treatment (Figure 3B).

p38 and ERK1/2 MAPK Activities
The MAPK pathway has been implicated in the pathogenesis of renal disease. We therefore compared the activation of key growth- and stress-related MAPKs in cortical tissues from lean, obese, and obese-plus-ARB groups. The results showed increased activation of both p38 and ERK1/2 MAPKs, as assessed by phosphorylated kinase levels, in obese Zucker rat cortex compared with lean Zucker controls. Furthermore, these changes were significantly attenuated by ARB treatment (Figures 4A and 4B).

12/15-LO and COX-2 Expression
Several studies indicate that enzymes of arachidonate metabolism, such as 12/15-LO and COX-2, play important roles in the pathogenesis of renal disease via hemodynamic and nonhemodynamic actions and inflammatory responses. We observed that 12/15-LO and COX-2 protein and mRNA expressions were increased in the cortex of obese Zucker rats relative to the lean controls (Figures 5A and 5B, Figures 6B and 6C). Furthermore, ARB treatment significantly attenuated the rise in 12/15-LO and COX-2 expressions in obese Zucker rats. In contrast, COX-1 expression was unchanged (Figure 6A). These results suggest that obesity-induced glomerulopathy is associated with altered arachidonic acid enzymes and their oxidized lipid products that may contribute to renal damage. The results further suggest that renin-angiotensin system activation is involved in the upregulation of these enzymes in animals with obesity and insulin resistance.

Expression of Inflammatory Genes MCP-1 and IL-6
Inflammatory factors such as MCP-1 and IL-6 have been implicated in glomerular and tubulointerstitial injury. We found significant upregulation of MCP-1 and IL-6 mRNAs in
the kidney cortex of obese Zucker rats. Inflammation and fibrosis are pathological processes that are regulated in part by signaling through the MAPK pathway. Because MAPK activation can increase expression of inflammatory genes, we hypothesized that the ARB-induced reduction of MAPK activation in obese Zucker rats, whereas administration of ARB to obese Zucker rats significantly inhibited these parameters. Data are mean±SEM from 6 rats per group. *P<0.01 vs lean Zucker; +P<0.05 vs obese Zucker rat (by ANOVA).

Discussion

Morbid obesity can cause glomerulosclerosis in humans and experimental animals. In the present study, we evaluated the potential mediators of renal injury in the obese Zucker rat, a model of prediabetic obesity and insulin resistance. The male obese Zucker rats used in the present study exhibited significant kidney disease (as evidenced by the presence of albuminuria and histological abnormalities) in the absence of hypertension or frank diabetes. This was associated with significant upregulation of 12/15-LO, COX-2, and the inflammatory cytokines MCP-1 and IL-6 in the kidney cortex. Given the critical role of these proinflammatory cytokines and products of arachidonate metabolism in the pathogenesis of renal disease, the observed association is of considerable interest. The upregulation of MCP-1 and IL-6 in the obese rats was accompanied by activation of the p38 and ERK1/2 MAPK pathways. Because MAPK activation can promote the expression of proinflammatory/profibrotic cytokines, the observed activation of MAPKs in the kidney cortex of the untreated obese Zucker rats may have contributed to the augmented expression of MCP-1 and IL-6.

It is well known that Ang II increases blood pressure, induces renal cell hypertrophy, and promotes synthesis of ECM proteins, processes linked to the progression of renal disease.16,30–32 The effects of Ang II are mediated by 2 plasma membrane receptors, referred to as the AT1 and AT2 subtypes.33,34 Most of the known effects of Ang II in adult tissues are attributable to AT1R.34 In the present study, the untreated obese Zucker rats exhibited marked upregulation of AT1R mRNA and protein expressions. AT1R mediates not only the hemodynamic but also the nonhemodynamic actions of Ang II, including those that lead to oxidative stress, cellular hypertrophy, proliferation, and tissue remodeling. Consequently, the observed upregulation of AT1R expression in the obese Zucker rat kidney may have contributed to the
associated glomerulopathy by augmenting the susceptibility of this organ to the available circulating and locally produced Ang II. This supposition is supported by the favorable response to ARB administration in treated obese Zucker rats in the present study. In both clinical and experimental studies, ACE inhibitors and AT1R antagonists have been shown to exert renoprotective effects that cannot be explained entirely by their hemodynamic actions. In the present study, chronic ARB administration prevented glomerular injury and reversed upregulation of AT1R and several key inflammatory mediators in obese Zucker rats.

AT1R overexpression is one potential molecular mechanism that links a variety of exogenous risk factors to cellular events in several renal diseases. According to a recent study, transgenic rats overexpressing human AT1R in podocytes develop FSGS. The major new finding of the present study is the upregulation of AT1R mRNA and protein expression in untreated obese Zucker rat cortex and its normalization by ARB treatment. Several studies have suggested that the AT1R gene expression is influenced by various cytokines and growth factors. In addition, insulin could upregulate AT1R in mesangial cells in vitro and in vivo, and this upregulation of AT1R by insulin was suggested to be responsible for the additive effects of insulin and Ang II in mesangial cells. Because hyperinsulinemia is a known feature of metabolic syndrome in obese Zucker rats, the observed upregulation of AT1R in the kidney cortex of obese Zucker rats in the present study in part may be due to hyperinsulinemia. Upregulation of AT1R in obese Zucker rats was reversed by ARB administration. The precise mechanism responsible for this phenomenon is not clear; however, it may be due to the compensatory rise in renin activity and Ang II production leading to a simultaneous activation of AT-2 receptor (AT2R) and downregulation of AT1R. It is also possible that AT1 antagonists may cause hyperstimulation of the AT2 subtype. Because AT2R may counteract the effects of AT1R, concomitant stimulation of AT2R and blockade of AT1R may contribute to the beneficial effects of AT1R antagonists.

The 12/15-LO and COX-2 pathways have been implicated in the pathogenesis of inflammation and DN. Products of these pathways have potent inflammatory, vasoactive, growth, and matrix-inducing properties. We recently demonstrated that the 12/15-LO pathway was enhanced in mesangial cells cultured in high glucose and in rats with DN. The changes in 12/15 LO correlated with expression of the...
matrix protein, fibronectin,\textsuperscript{23,26} and 12/15-LO could mediate Ang II–induced effects.\textsuperscript{16} COX-2 metabolites have also been implicated in the pathogenesis of functional and structural abnormalities associated with certain glomerular and tubulo-interstitial inflammatory disorders. Moreover, COX inhibitors can ameliorate proteinuria and/or structural injury.\textsuperscript{27,28,42} COX-2 has been implicated in the early stages of DN\textsuperscript{28}; however, its role in development of the overt nephropathy is not entirely clear. In the present study, we showed for the first time that 12/15-LO and COX-2 levels are increased in the renal cortex of prediabetic obese Zucker rats. We also demonstrated that an ARB can reduce 12/15-LO and COX-2 expression, thereby supporting the role of the Ang II–AT1R pathway in regulating these enzymes. Moreover, these data reveal an additional mechanism for renoprotective effects of ARBs in obese Zucker rat cortex.

The chemokine MCP-1 is produced mainly by tubular epithelial cells in kidney and contributes to inflammation and fibrosis.\textsuperscript{43,44} Recently, urinary MCP-1 excretion was shown to increase in proportion to the degree of albuminuria in patients with type 2 diabetes mellitus.\textsuperscript{45,46} Blockade of the renin-angiotensin system patients with type 2 diabetes mellitus with DN reduces urinary MCP-1 levels and preserves renal function.\textsuperscript{46,47} Because MCP-1 induces monocyte/macrophage infiltration, which promotes matrix accumulation and tubulo-interstitial fibrosis, pharmacological interventions to inhibit AT1R expression or action may exert their beneficial effects in part by downregulating renal MCP-1. The present data demonstrate that MCP-1 expression and IL-6 were increased in obese Zucker rat kidney and were significantly lowered by ARB treatment. These findings implicate the Ang II–AT1R pathway in the regulation of proinflammatory cytokines in the kidney of animals with obesity and insulin resistance.

Proinflammatory cytokines and enzymes of arachidonate metabolism may represent the effectors and downstream targets of well-known signal transduction pathways such as protein kinase C and MAPKs in mediating renal damage. MAPKs have been implicated in both glomerular matrix accumulation and tubulo-interstitial fibrosis.\textsuperscript{48–50} They are also activated by Ang II, LO, and COX products. In the present studies, p38 and ERK1/2 MAPKs were significantly activated in cortex of obese Zucker rats and were normalized by chronic ARB treatment. Taken together, the present data suggest that expressions of enzymes of arachidonate metabolism, key inflammatory genes, and AT1R and activity of MAPKs are elevated in obese Zucker rat kidneys and are most likely involved in the pathogenesis of nephropathy in this model. Moreover, these changes are associated with and mediated, at least in part, by Ang II action, as evidenced by their reversal with ARB treatment. This reversal with ARB was accompanied by significant amelioration of proteinuria and histological abnormalities of nephropathy in the obese Zucker rats. Therefore, the present results have provided additional rationale for the use of ARBs in the treatment and prevention of the obesity-associated FSGS.

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