Metabolic Syndrome

Telmisartan Ameliorates Nephropathy in Metabolic Syndrome by Reducing Leptin Release From Perirenal Adipose Tissue

Hao Li, Min Li, Ping Liu, YaPing Wang, Heng Zhang, HongBin Li, ShiFeng Yang, Yan Song, YanRong Yin, Lan Gao, Si Cheng, Jun Cai, Gang Tian

Abstract—Metabolic syndrome (MetS) is associated with nephropathy. Along with common risk factors such as hypertension and hyperglycemia, adipokines released from perirenal adipose tissue (PRAT) are implicated in the pathogenesis of MetS nephropathy. The study was designed to elucidate the adverse effects of PRAT-derived leptin on nephropathy and to determine whether the angiotensin II type 1 receptor antagonist telmisartan exerts a renoprotective effect by decreasing the PRAT-derived leptin level in the high-fat diet–induced MetS rat. In MetS rats, PRAT-derived leptin expression increased concomitant with dysfunction of adipogenesis, and the activities of the angiotensin II–angiotensin II type 1 receptor and the angiotensin-converting enzyme 2–angiotensin (1–7)–Mas receptor axes were imbalanced in PRAT. PRAT-derived leptin from MetS rats promoted proliferation of rat glomerular endothelial cells (GERs) by activating the p38 MAPK (mitogen-activated protein kinase) pathway, thereby contributing to the development of nephropathy. Long-term telmisartan treatment improved metabolic parameters and renal function, decreased the amount of PRAT, prevented adipogenesis, increased the expression of angiotensin-converting enzyme 2, restored balanced activities of the angiotensin II–AT1R and angiotensin-converting enzyme 2–angiotensin (1–7)–Mas axes, and exerted an indirect renoprotective effect on MetS rats by decreasing PRAT-derived leptin release. Our results demonstrate a novel link between nephropathy and PRAT in MetS and show that telmisartan confers an underlying protective effect on visceral adipose tissue and the kidney, suggesting that it has potential as a therapeutic agent for the treatment of MetS-associated nephropathy. (Hypertension. 2016;68:478-490. DOI: 10.1161/HYPERTENSIONAHA.116.07008.) ● Online Data Supplement

Key Words: adipocytes ■ angiotensin-converting enzyme 2 ■ leptin ■ obesity ■ telmisartan

Metabolic syndrome (MetS) is a strong risk factor for nephropathy, and multiple etiologic factors involved in renal pathogenesis, including insulin resistance, obesity, and hypertension, are components of MetS. MetS is often accompanied by visceral adipose tissue (VAT) accumulation and dysfunction, along with elevated production of VAT-derived adipokines, and adipokines derived from perivascular adipose tissue have been implicated in the vascular lesions associated with MetS. Thus, in addition to common risk factors, such as insulin resistance and hypertension, adipokines released from perirenal adipose tissue (PRAT) may be involved in pathogenesis of MetS nephropathy.

Among these adipocytokines, leptin plays a key role in the development of nephropathy. Previous studies showed that the kidney expresses abundant leptin receptors and that obese patients with high concentrations of leptin are more prone to develop nephropathy. Furthermore, leptin is a renal growth factor that can stimulate proliferation of rat glomerular endothelial cells (GERs) in vitro. In a previous study, we found that leptin derived from perivascular adipose tissue exacerbates vascular remodeling in MetS rats; on this basis, we hypothesized that PRAT-derived leptin from MetS animal may exert an adverse effect on kidney and sought to identify the underlying mechanism.

All components of the renin–angiotensin system (RAS), including angiotensin II (Ang II) and angiotensin 1–7...
(Ang(1–7)) and their receptors, are expressed locally in VAT. Ang(1–7) is generated from Ang II by angiotensin-converting enzyme 2 (ACE2) and exerts its effects through the Mas receptor. In pathological states such as insulin resistance and obesity, activity of local RAS components in adipose tissue may change, disrupting adipocyte function and ultimately resulting in VAT accumulation and dysfunction. Furthermore, Ang II affects the secretory function of adipocytes and influences leptin release through its interaction with Ang II type 1 receptor (AT1R). The ACE2–Ang(1–7)–Mas receptor axis opposes the pressor, proliferative, anti-adipogenesis, profibrotic, and prothrombotic activities mediated by the Ang II–AT1R axis.

The drug telmisartan induces a remarkable increase in myocardial ACE2 and Mas receptor expression in a rat model of autoimmune myocarditis, and a previous study by our group also showed that telmisartan significantly decreases VAT weight and contributes to improvements in VAT function. These observations led to the novel insight that telmisartan, an Ang II receptor blocker, may influence VAT-derived leptin release and improve VAT function by regulating the local activity of the ACE2–Ang(1–7)–Mas receptor and Ang II–AT1R axes in MetS. Therefore, we hypothesized that, in MetS rats, (1) the ACE2–Ang(1–7)–Mas receptor and Ang II–AT1R axes in PRAT are locally dysregulated, resulting in an increase in PRAT-derived leptin and ultimately exacerbating MetS nephropathy and (2) telmisartan would decrease the release of PRAT-derived leptin by regulating the activities of both of the aforementioned signaling axes, thus exerting a renoprotective effect.

**Methods**

**Animal Care**

Male Wistar rats were purchased from Vital River Laboratory Animal Technology Co, Ltd (Beijing, China). The study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Rats were randomly divided into 4 groups. Rats in the control (Con, n=10) and control-telmisartan (Con-Tel, n=10) groups were fed a normal diet consisting of 66.5% carbohydrate, 23.3% protein, and 10.2% fat. Rats in the MetS (n=11) and MetS-telmisartan (MetS-Tel, n=11) groups were fed a high-fat diet (HFD) containing 37% carbohydrate, 7.0% protein, and 56% fat. Starting after 12 weeks of normal diet or HFD feeding, rats in the Con-Tel and MetS-Tel groups received telmisartan (8 mg/kg/d) by gastric lavage, and rats in the Con and MetS groups received vehicle alone, for 20 consecutive weeks. Telmisartan was obtained from DongRui (Suzhou, Jiangsu, China). The duration of the experimental period was 32 weeks for all groups. At the end of the study period, all animals were euthanized.

**Statistical Methods**

Statistical analysis was performed using SPSS version 16.0 (SPSS, Chicago, IL). Data are presented as means±SEM. Independent t test was used to compare means of 2 groups with normal distribution. Multiple comparisons of >2 groups with normal distributions were performed by analysis of variance, followed by Scheffe (Equal variance assumed) multiple comparison post hoc test or Tamhane’s T2 (Equal variance not assumed) test. For non-normally distributed data, differences between 2 groups were analyzed using the Mann–Whitney U test, and differences among multiple groups were analyzed using the Kruskal–Wallis test followed by Dunn multiple comparison post hoc test. P<0.05 was considered statistically significant.

**Results**

**Telmisartan Ameliorates Metabolic Disorders in HFD-Fed Rats**

At the beginning of the experiment, no metabolic parameters exhibited a significant difference among the 4 groups (Table S1 in the online-only Data Supplement). At the end of 12 weeks, HFD-fed rats in the MetS and MetS-Tel groups had significantly higher body weights and fast blood glucose and insulin levels, an elevated homeostasis model assessment index, and higher systolic blood pressure, total cholesterol, triglyceride, and low-density lipoprotein cholesterol than normal diet-fed rats in the Con group (P<0.01, Table S2). The rats in the MetS and MetS-Tel groups exhibited profound obesity, insulin resistance, hypertension, hyperglycemia, and hyperlipidemia. Together, these findings demonstrate that HFD-fed rats developed MetS. After telmisartan treatment for 20 weeks, metabolic parameters were significantly lower in the MetS-Tel group than in the MetS group (P<0.05, Table S3), whereas metabolic parameters did not differ significantly between the Con and Con-Tel groups. Thus, long-term telmisartan treatment can ameliorate metabolic disorders in HFD-induced MetS rats.

**Telmisartan Decreases the Amount of PRAT and Restores Adipocyte Phenotypes in MetS Rats**

As shown in Figure S1, the amounts of PRAT and epidydimal and perivascular adipose tissue were significantly higher in MetS rats than in Con rats, and telmisartan treatment decreased the relative amounts of PRAT, epidymidal, and perivascular adipose tissue in MetS rats (P<0.05). Furthermore, MetS rats had fewer adipocytes, and the PRAT cells were arranged in a disorderly manner and showed signs of hypertrophy (P<0.01; Figure 1A through 1C). After telmisartan treatment, the number of adipocytes increased significantly, concomitant with a marked amelioration of cell hypertrophy (P<0.01; Figure 1A through 1C). There were no differences in the PRAT amount or adipocyte phenotypes between the Con and the Con-Tel group.

**Telmisartan Promotes Adipogenesis and Decreases Inflammatory Cytokine Expression in PRAT of MetS Rats**

Adipogenesis is a complex process by which immature or undifferentiated preadipocytes differentiate into mature or differentiated adipocytes. The differentiation of preadipo-cytes is regulated by various adipogenic transcription factors, including peroxisome proliferator–activated receptor γ (PPAR-γ) and CCAAT/enhancer-binding proteins α, which are regarded as markers of adipogenesis. On the contrary, lipogenesis plays a crucial role in adipogenesis; consequently, fatty acid synthase and fatty acid–binding protein 4, which play important roles in lipid metabolism, are also regarded as adipogenesis markers. Adiponectin, an adipocyte-specific adipocytokine that is critical for improving insulin sensitivity, is also present. Consistent with the morphological changes in adipocytes, expression levels of adipogenesis markers (PPAR-γ, CCAAT/enhancer-binding proteins α, fatty acid synthase, and fatty acid–binding protein 4) and adiponectin...
in PRAT were remarkably reduced in MetS rats (P<0.05; Figure 1D through 1I). After telmisartan treatment, local expression of adipogenesis markers and adiponectin was significantly elevated (P<0.05; Figure 1D through 1I). On the contrary, mRNA levels of the inflammatory cytokines interleukin-6 and monocyte chemoattractant protein-1 in PRAT were significantly elevated in MetS rats, and telmisartan treatment remarkably decreased inflammatory cytokine expression in PRAT (P<0.05; Figure 1J and 1K). Neither adipogenesis nor inflammatory cytokine expression in PRAT differed significantly between the Con and Con-Tel groups. These results demonstrate that long-term telmisartan treatment can promote adipogenesis and decrease inflammatory cytokine expression in PRAT in MetS rats.

**Effect of Telmisartan on Ang II and Ang(1–7) levels**

As expected, Ang II level in adipose tissue and plasma was significantly higher in the MetS group than in the Con group (P<0.01; Figure 2A and 2B). However, after telmisartan treatment, changes in Ang II level in adipose tissue and plasma were inconsistent: plasma Ang II level increased, whereas tissue Ang II level decreased. On the contrary, Ang(1–7) level in local adipose tissue was significantly lower in the MetS group than in the Con group. Telmisartan treatment increased local Ang(1–7) level relative to the MetS group (P<0.01; Figure 2D). The plasma Ang(1–7) level in the MetS group was slightly higher than that in the Con group, but the difference was not statistically significant. After telmisartan treatment, plasma Ang(1–7) level increased (P<0.01; Figure 2C).

**The Activities of the Ang II–AT1R and ACE–Ang(1–7)–Mas Axes Are Locally Imbalanced in PRAT of MetS Rats**

As shown in Figure 2E, the Ang II–AT1R–extracellular signal-regulated kinase (ERK) pathway was locally activated in local adipose tissue in MetS rats, but its activity was suppressed after telmisartan treatment (MetS-Tel group). However, the activity change in the ACE2–Ang(1–7)–Mas receptor–phosphoinositide 3-kinase (PI3K)–Akt pathway was inconsistent with that of the Ang II–AT1R–ERK pathway. Telmisartan treatment significantly increased the expression of ACE2 and the Ang(1–7) level, and the ACE2–Ang(1–7)–Mas receptor–PI3K–Akt pathway was activated in the MetS-Tel group. These results indicate that activity of the Ang II–AT1R and ACE–Ang(1–7)–Mas receptor axes is locally imbalanced in the PRAT of MetS rats and that long-term telmisartan treatment can ameliorate this imbalance by downregulating the Ang II–AT1R axis and upregulating the ACE–Ang(1–7)–Mas receptor axis.
The Renoprotective Effect of Telmisartan on MetS Rats

As shown in Table S4, 24-hour urinary protein was significantly higher in the MetS and MetS-Tel groups than in the Con group after HFD feeding for 12 weeks ($P<0.01$), but neither blood urea nitrogen nor serum creatinine differed among the 4 groups at that time. However, as shown in Table S5, after HFD feeding for 32 weeks, blood urea nitrogen, serum creatinine, and 24-hour urinary protein levels in the MetS group were significantly higher than those in the Con group, and telmisartan treatment significantly decreased serum creatinine ($61.11\pm6.72$ versus $88.55\pm7.66$ μmol/L; $P<0.05$), blood urea nitrogen ($11.02\pm2.77$ versus $15.12\pm3.31$ mmol/L; $P<0.05$), and 24-hour urinary protein ($104.91\pm18.25$ versus $300.88\pm13.75$ mg/24 hours; $P<0.05$) relative to the MetS group. Figure 3A shows representative renal pathology in all 4 groups at the end of the experimental period. The glomerulosclerosis index was higher in the MetS group than in the Con group ($P<0.01$; Figure 3B), but consistent with the significant attenuation of albuminuria, this index was improved in the MetS-Tel group. These results indicate that long-term telmisartan treatment exerts a renoprotective effect in MetS rats.

ObRa mRNA Expression Increases in Renal Cortical Tissue of the MetS Rats

As expected, long-form leptin receptor ObRb (ObRa) mRNA levels did not differ significantly among the 4 groups (Figure 3D). In renal cortical tissue, expression of the short-form leptin receptor ObRa (ObRa) mRNA was 2.34-fold higher in MetS rats than in Con rats ($P<0.01$, Figure 3C). Telmisartan treatment significantly decreased ObRa mRNA expression ($P<0.01$; Figure 3C). These results were consistent with previous studies.5

Higher Proliferating Cell Nuclear Antigen Expression Is Associated With Activation of the p38 MAPK Pathway in the Renal Cortical Tissue of the MetS Rats

As shown in Figure 3E through 3G, the renal cortical tissue of the MetS rat exhibited higher proliferating cell nuclear antigen (PCNA) expression, and the upregulation of PCNA was associated with activation of the p38 mitogen-activated protein kinase (MAPK) pathway. Telmisartan treatment markedly decreased PCNA expression and inhibited the p38 MAPK pathway. Because proliferation of GERs may be an important prerequisite for MetS kidney damage, we speculate that GER proliferation involved in such pathogenesis requires the p38 MAPK pathway. Further experiments are necessary to identify the specific signaling pathway responsible for these effects.

Telmisartan Treatment Decreases Leptin Expression in PRAT of the MetS Rats

PRAT-derived leptin expression was significantly higher in MetS rats than in Con rats ($P<0.01$, Figure 4A and 4B), and telmisartan treatment remarkably decreased PRAT-derived leptin expression ($P<0.01$, Figure 4A and 4B).
Leptin Levels in Plasma and PRAT-Conditioned Medium

As expected, the plasma leptin level was higher (3.77-fold) in MetS rats than in Con rats \((P<0.01; \text{Figure 4C})\). Telmisartan treatment significantly decreased the plasma leptin level, from 8.51±0.88 ng/mL (MetS group) to 3.00±0.23 ng/mL (MetS-Tel group; \(P<0.01; \text{Figure 4C}\)). As shown in Figure 4D, the leptin level in PRAT-conditioned medium (PRAT-CM) was significantly higher in MetS rats than in Con rats (3.49±0.41–1.10±0.22 ng/mL; \(P<0.05\)), whereas after telmisartan treatment, the leptin level in MetS-Tel rat PRAT-CM was remarkably reduced. These results were consistent with those obtained in vivo. Together, these findings suggested that PRAT-CM is an appropriate in vitro model for the PRAT microenvironment. Therefore, we incubated GERs with PRAT-CM to investigate the effects of PRAT-derived leptin on GERs proliferation and explore the underlying mechanisms.

PRAT-Derived Leptin From MetS Rats Stimulates GERs Proliferation by Activating the p38 MAPK Signaling Pathway

After incubation with MetS rat PRAT-CM, PCNA expression was significantly higher in GERs from the MetS group than those from the Con group, and PCNA upregulation was accompanied by activation of the p38 MAPK pathway \((P<0.05; \text{Figure 4E through 4G})\). Conversely, PCNA expression in GERs of the MetS-leptin tA group was significantly lower than that in the MetS group, and the downregulation of PCNA was accompanied by inhibition of the p38 MAPK pathway \((P<0.05)\). In addition, leptin increased PCNA expression in GERs in a concentration-dependent manner (Figure 4H); the minimal and maximal effective concentrations of leptin were 40 and 100 ng/mL, respectively. As shown in Figure 4I and 4J, leptin-induced GERs proliferation was accompanied by activation of the p38 MAPK pathway, and pretreatment with the p38 inhibitor SB 203580 markedly inhibited the effect of leptin on GERs proliferation. These results showed that PRAT-derived leptin in MetS rats stimulates GER proliferation by activating the p38 MAPK pathway.

After incubation with MetS-Tel rats PRAT-CM, PCNA expression was significantly lower in GERs from the MetS-Tel group than in those from the MetS group, and PCNA downregulation was associated with inhibition of the p38 MAPK pathway \((P<0.05; \text{Figure 4E through 4G})\). The result is consistent with those obtained in vivo (Figure 3E through 3G). Because PRAT-derived leptin is involved in the development of nephropathy in MetS rats, it is reasonable to conclude that the indirect renoprotective effect of telmisartan is because of the decrease in PRAT-derived leptin. To explore the effect of telmisartan on the regulation of leptin release from adipocytes, we incubated mature visceral adipocytes (VAs) in the presence of Ang II and/or telmisartan.
Ang II Promotes Leptin Release From VAs by Activating the AT1R-ERK1/2 Pathway

Cells isolated from the rat PRAT differentiated into mature VAs at day 9 (Figure S2). The results showed that Ang II stimulated leptin release from VAs in a concentration-dependent manner, accompanied by activation of the AT1R-ERK1/2 pathway (Figure S3). The minimal and maximal effective concentrations of Ang II were 0.1 and 10 μmol/L, respectively. At an Ang II concentration of 10 μmol/L, the leptin concentration was 4.74-fold higher than control levels (Figure S3A; *P<0.01). Moreover, Ang II decreased ACE2 expression in a dose-dependent manner, accompanied by inhibition of the Mas receptor–PI3K–Akt pathway. Expression of neither AT1R nor the Mas receptor was affected by stimulation with Ang II.

As expected, telmisartan treatment caused a concentration-dependent reduction in Ang II-induced leptin release into the culture medium (Figure S4). Figure 5 shows that Ang II stimulated leptin release from VAs via activation of the AT1R-ERK1/2 pathway, and this was accompanied by inhibition of the ACE2–Mas receptor–PI3K–Akt pathway. Telmisartan treatment suppressed Ang II-induced leptin release by inhibiting the AT1R-ERK1/2 pathway, concomitant with activation of the ACE2–Mas receptor–PI3K–Akt pathway.

Ang(1–7) Inhibits Leptin Release From VAs by Activating the Mas Receptor–PI3K–Akt Pathway

As expected, Ang(1–7) inhibited leptin release from VAs in a dose-dependent manner, accompanied by activation of the Mas receptor–PI3K–Akt pathway (Figure S5). The minimal and maximal effective concentrations of Ang(1–7) were 0.1 and 10 μmol/L, respectively. At an Ang(1–7) concentration of 10 μmol/L, the leptin concentration was decreased by 50% relative to the Con group (Figure S5A; *P<0.01). Ang(1–7) had no effect on the expression of AT1R, ERK1/2, ACE2, or Mas receptor. Figure 6 shows that Ang(1–7) inhibited leptin release by activating the Mas receptor–PI3K–Akt pathway. Combined with our results showing that Ang II stimulates leptin release from VAs by activating the AT1R–ERK1/2 pathway, these observations suggest that Ang(1–7) antagonizes the effect of the Ang II–AT1R axis via activation of the Mas receptor–PI3K–Akt pathway. ACE2 cleaves Ang II to yield Ang(1–7) and is the rate-limiting enzyme in this conversion. We speculate that ACE2 is the key component that negatively regulates
leptin release; further experiments are necessary to confirm this hypothesis. Because our animal experiments were based on MetS rats, and the basis of MetS is insulin resistance, we sought to explore the effect of changes in ACE2 expression on leptin level by establishing an insulin-resistant (IR) VA model and using ACE2 siRNA and lentiviral-ACE2 (lenti-ACE2) to silence and overexpress ACE2, respectively.

Establishment of an IR VA Model

Incubation with dexamethasone decreased basal and insulin-stimulated 2-deoxyglucose (2-DG) uptake in VAs in a dose-dependent manner (Figure S6). The maximal inhibition of 2-DG uptake occurred at 1 μmol/L dexamethasone, which decreased basal and insulin-stimulated 2-DG uptake by 40% and 50%, respectively (Figure S6; P < 0.01). These results demonstrated that the IR VA model had been successfully established. Next, we incubated IR VAs in the presence or absence of lenti-ACE2, ACE2 siRNA, and telmisartan.

Telmisartan Treatment Decreases Leptin Release From IR VAs by Restoring Balanced Activities of the Ang II–AT1R–ERK1/2/Ace2–Ang (1–7)–Mas Receptor Axes

Cell viability was similar among the experimental groups (Figure S7). ACE2 expression was significantly lower in the IR group than in the Con group; after telmisartan treatment, ACE2 expression increased by 2.47-fold relative to that in the IR group (P < 0.01; Figure 7A; Figure S8A). This upregulation of ACE2 was accompanied by inhibition of the AT1–ERK1/2 pathway and activation of the Mas receptor–PI3K–Akt pathway. ACE2 was successfully overexpressed using lentiviral vectors: expression of ACE2 in the IR-lentiACE2 group was 3.22-fold higher than that in the IR group (P < 0.05; Figure 7A; Figure S8A). Conversely, expression of ACE2 in the IR-ACE2 siRNA group was only 5.4% of that in the IR group (P < 0.05; Figure 7A; Figure S8A).

Telmisartan treatment further increased the expression of ACE2 in ACE2-overexpressing cells: the ACE2 level was 1.99-fold higher in the IR-lentiACE2-Tel group than in the IR-lentiACE2 group (P < 0.01; Figure 7A; Figure S8A). Furthermore, telmisartan treatment rescued ACE2 siRNA–induced downregulation of ACE2 expression: the ACE2 level was 10.88-fold higher in the IR-ACE2 siRNA-Tel group than in the IR-ACE2 siRNA group (P < 0.01; Figure 7A; Figure S8A). On the contrary, as shown in Figure 7A to 7C, leptin and Ang II levels were significantly higher in the IR group than in the Con group. Telmisartan treatment significantly decreased leptin (1.57±0.14–3.12±0.31%; P < 0.01) and Ang II levels (1.42±0.12–2.83±0.26%; P < 0.01) and increased the Ang(1–7) level (0.98±0.11–0.61±0.03%; P < 0.05), relative to those of the IR group. After transfection with lentiACE2, leptin and Ang II levels were significantly reduced (leptin: 2.27±0.15% versus 3.12±0.31%; P < 0.01 and Ang II: 1.92±0.15% versus 2.83±0.26%; P < 0.05), and the Ang(1–7) level was remarkably elevated (1.10±0.14% versus 0.61±0.03%; P < 0.05), relative to those in the IR group. On the contrary, after transfection with ACE2 siRNA, leptin and Ang II levels were
significantly elevated (leptin: 3.92±0.20 versus 3.12±0.31%; \(P<0.01\) and Ang II: 3.99±0.17 versus 2.83±0.26%; \(P<0.01\)), and the Ang(1–7) level was remarkably reduced (0.29±0.04 versus 0.61±0.03%; \(P<0.05\)), relative to those in the IR group. Furthermore, telmisartan treatment and lentiACE2 transfection worked together to lower the leptin and Ang II levels and increase the level of Ang(1–7) relative to those in the IR-lentiACE2 group (\(P<0.01\); Figure 7A through 7C). Telmisartan treatment ameliorated the ACE2 siRNA–induced increase in leptin and Ang II levels, accompanied by an increase in Ang(1–7) levels relative to those in the IR-ACE2 siRNA group (\(P<0.01\); Figure 7A through 7C). These results indicate that ACE2 is a key component in the negative regulation of the leptin level and that telmisartan treatment increases ACE2 expression and decreases the leptin level in IR VAs by activating the Ang(1–7)–Mas receptor pathway and inhibiting the Ang II–AT1R signaling pathway. These results are consistent with the in vivo observations described above. In other words, the activity of Ang II–AT1R–ERK1/2 and the ACE2–Ang (1–7)–Mas receptor pathway are imbalanced in IR VAs, and telmisartan treatment re-establishes the normal balance by increasing expression of ACE2, suppressing the Ang II–AT1R axis, activating the ACE2–Ang(1–7)–Mas receptor pathway, and ultimately decreasing the leptin level.

**Telmisartan Treatment Increases 2-DG Uptake in IR VAs by Upregulating ACE2 Expression**

Consistent with the in vivo results, telmisartan treatment ameliorated insulin resistance in VAs of the IR-telmisartan group. As shown in Figure 7D and 7E, lentiACE2-induced ACE2 overexpression resulted in an increase in insulin sensitivity, whereas ACE2 siRNA–induced silencing of ACE2 expression resulted in a decrease in insulin sensitivity. These results indicate that ACE2 is also a potent factor that can improve insulin resistance and that telmisartan treatment ameliorates insulin resistance by upregulating ACE2 expression.

**Telmisartan Treatment Does Not Affect Adipogenesis of IR VAs**

Viability of mature VAs was similar among the various experimental groups (Figure S9B). Telmisartan and GW9662 had no effect on lipid accumulation or expression of the transcription factors PPAR-\(\gamma\) and CCAAT/enhancer-binding proteins \(\alpha\) in IR VAs (Figure S9A and S9C through S9F). These results indicate that telmisartan treatment does not affect adipogenesis of IR VAs.

**Telmisartan Treatment Decreases Leptin Release From IR VAs Independently of the PPAR-\(\gamma\) Signaling Pathway**

As expected, leptin release was higher from IR VAs, and the higher level of leptin release was accompanied by activation of the Ang II–AT1R–ERK1/2 axis and inhibition of the Ang(1–7)–Mas receptor–PI3K–Akt axis. Telmisartan treatment decreased the leptin level by restoring balanced activities of the 2 signaling axes (Figure S10). The PPAR-\(\gamma\) antagonist GW9662 did not abolish telmisartan-induced reduction in leptin release in IR VAs or the positive effect of telmisartan in equilibrating the activities of the 2 axes. Neither telmisartan nor GW9662 exerted an effect on the expression of AT1R and
Mas receptor in IR VAs. Likewise, the PPAR-γ agonist rosiglitazone did not affect AT1R expression in IR VAs (Figure S11). These results indicate that telmisartan treatment decreases leptin release from IR VAs by restoring balanced activities of the Ang II–AT1R–ERK1/2 and ACE2–Ang (1–7)–Mas receptor axes, independently of the PPAR-γ signaling pathway.

Telmisartan Treatment Decreases Production of Inflammatory Cytokines and Improves 2-DG Uptake in IR VAs Partly Through Activation of the PPAR-γ Signaling Pathway

Telmisartan treatment improved basal and insulin-stimulated 2-DG uptake in IR VAs, and the PPAR-γ antagonist GW9662 blocked the beneficial effect of telmisartan on basal and insulin-stimulated 2-DG uptake (basal: 88.46±2.06% versus 68.6±4.93%; P<0.01; insulin-stimulated: 93.00±1.58% versus 65.60±3.29%; P<0.01; Figure S12A and S12B). As expected, although the PPAR-γ signaling pathway was blocked by GW9662, telmisartan treatment also promoted insulin sensitivity in IR VAs (P<0.01; Figure S12A and S12B). Consistent with the changes in 2-DG uptake, after blockade of the PPAR-γ signaling pathway, telmisartan treatment also decreased expression of inflammatory cytokines and increased mRNA expression of adiponectin (P<0.01; Figure S12C through S12E). These results indicate that telmisartan treatment decreases production of inflammatory cytokines and improves 2-DG uptake in IR VAs partly through activation of the PPAR-γ signaling pathway.

Discussion

The main findings of this study are as follows: (1) In MetS rat PRAT, local leptin expression increases along with adipocyte hypertrophy, accompanied by imbalanced expression of the Ang II–AT1R and ACE2–Ang(1–7)–Mas receptor axes. (2) PRAT-derived leptin from MetS rats promotes GERs proliferation by activating the p38 MAPK pathway. (3) Long-term telmisartan treatment can increase the expression of ACE2, restore balanced expression of the Ang II–AT1R and ACE2–Ang(1–7)–Mas receptor axes, and ultimately exert an indirect renoprotective effect by decreasing the level of PRAT-derived leptin. Our main findings are illustrated in Figure 8.
Telmisartan is an antihypertensive drug that can effectively improve metabolic parameters in diabetic subjects. Our findings reveal that telmisartan has an underlying protective effect on VAT of the MetS rat and may therefore be an effective drug for treatment of MetS-associated PRAT dysfunction and nephropathy. In addition, our results showed that ACE2 is a potent factor in the regulation of insulin sensitivity in adipocytes. A previous study showed that the absence of ACE2 resulted in exaggerated glucose intolerance with insulin resistance in mice fed a HFD and that ACE2 acts as a regulator of glucose homeostasis after caloric overload. Taken together with these observations, present results lead us to speculate that the suppression of ACE2 expression in local adipose tissue contributes to the reduction of insulin sensitivity in PRAT of the MetS rat and that telmisartan treatment can increase the expression of ACE2 to ameliorate local insulin resistance, and finally improve metabolic disorders throughout the body. Furthermore, because ACE2 is the rate-limiting enzyme in the cleavage of Ang II to yield Ang (1–7) and is involved in the regulation of the Ang(1–7)–Mas receptor axis, we speculate that ACE2 exerts its effect on glucose uptake by activating the Ang(1–7)–Mas–PI3K–Akt signaling pathway. A previous study also showed that Ang(1–7) reduces insulin resistance in rat skeletal muscle by activating the Mas receptor–Akt signaling pathway. These observations suggest the existence of cross talk between the insulin signaling pathway and the local RAS signaling pathway in adipose tissue and imply that telmisartan could affect both pathways by increasing the expression of ACE2.

The regulatory mechanism underlying the effect of telmisartan on Ang II remains unclear. As shown in Figure 2A, after telmisartan treatment, the changes in Ang II concentration in adipose tissue and plasma were inconsistent. We speculate that the plasma Ang II level was elevated in the MetS-Tel group because of the antagonistic effect of telmisartan on AT1R. However, complex cell–cell communication mediated by adipocytokines occurs in local adipose tissue, and the local tissue level of Ang II and Ang(1–7) are determined by their rates of production and degradation, indicating that RAS in local adipose tissue is regulated independently of RAS in circulation; however, the exact regulatory mechanism remains unclear, and further study is needed to explore this issue.

In addition, a previous study from Rubio-Ruiz et al showed that (1) Mas receptor expression in abdominal white adipose tissue does not differ significantly between MetS and control rats and (2) local Ang(1–7) concentrations in white adipose tissue are higher in MetS rats than in age-matched control rats. These results contradict those reported here. This apparent discrepancy could be resolved in 2 ways. First, the system used in the previous study was abdominal white adipose tissue, but the authors did not define clearly either the location or the characteristics of this tissue. Abdominal white adipose tissue contains PRAT, mesenteric adipose tissue, and perivascular adipose tissue; thus adipose tissue in different locations may express different levels of RAS components. Second, our study period was 32 weeks, whereas that of the study by Rubio-Ruiz et al was 72 weeks, and multiple dramatic declines and increases in tissue Ang II and Ang(1–7) concentrations occurred during the longer study period.

We demonstrated that the regulation of leptin release from mature VAs is dependent on the Ang II–AT1R and ACE–Ang(1–7)–Mas receptor axes signaling pathways (Figures 5 and 6). A previous study demonstrated that Ang II can promote leptin release from human and mouse 3T3-L1 adipocytes by activating the AT1R-ERK1/2 pathway, illustrating a positive regulatory mechanism of leptin release that is consistent with our results. However, no report to date has focused on the negative regulation of leptin release from mature VAs, and, to our knowledge, our work is the first to demonstrate a negative effect of Ang(1–7) on leptin

Figure 8. Schematic illustration of the mechanism. A, Perirenal adipose tissue (PRAT)–derived leptin from metabolic syndrome (MetS) rats promotes rat glomerular endothelial cells (GERs) proliferation by activating the p38 MAPK pathway. B, Illustration of the positive and negative regulatory mechanism of leptin release from mature insulin-resistant (IR) adipocytes, and the regulatory effect of telmisartan on leptin release. ACE2 indicates angiotensin-converting enzyme 2; Ang II, angiotensin II; Ang(1–7), angiotensin 1–7; AT1R, angiotensin II type 1 receptor; p38 MAPK, p38 mitogen-activated protein kinase.

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release from mature VAs. On the contrary, in addition to AT1R, another Ang II receptor subtype (AT2R) expressed in adipocytes can mediate Ang II signaling to decrease insulin sensitivity, increase adipocyte mass, and mediate the deleterious effects of a HFD.28 On the basis of this knowledge, we speculate that other signaling pathways may be involved in the regulation of leptin release in differentiated VAs, and complex cross talk between multiple signaling pathways may occur in adipocytes.

As shown by our findings, PRAT-derived leptin has a detrimental effect on the kidney in MetS rats. This result reveals a novel link between nephropathy and PRAT in MetS and suggests that leptin in local adipose tissue represents a new therapeutic target. In this study, the minimum concentration of leptin that induced proliferation of GERs in vitro was 40 ng/mL, remarkably higher than the serum leptin level in MetS rats (8.51±0.88 ng/mL; Figure 4C) or MetS rat PRAT-CM (3.49±0.41 ng/mL; Figure 4D). Vital organs are surrounded by a microenvironment that contains VAT and VAT-derived adipocytokines, which can directly affect the function of adjacent organs via paracrine mechanisms, even at low concentrations.29 On the contrary, in MetS rat renal cortical tissue, ObRa mRNA expression also increased by 2.34-fold, concomitant with a change in the local level of adipose tissue-derived leptin, which activated the leptin signaling pathway (Figures 3C and 4A). In addition, a previous study by our group also showed that VAT-derived leptin from MetS rats stimulates phenotypic switching in vascular smooth muscle cells, even at a low concentration.7 Therefore, it is reasonable to assume that a low local concentration of leptin can exacerbate nephropathy via a paracrine mechanism. Moreover, in the context of MetS, PRAT is dysfunctional and produces multiple detrimental adipocytokines, such as resistin.2,29,30 Given that local leptin may induce nephropathy in collaboration with other detrimental adipocytokines, further study is required to identify such adipocytokines and explore their effect on GER proliferation.

Furthermore, we found that telmisartan treatment exerts an indirect renoprotective effect by decreasing the PRAT-derived leptin level in MetS rats. As shown in Figure 4E through 4G, after the incubation of GERs in CM prepared from the PRAT of Met-Tel rats, PCNA expression in GERs was significantly reduced. Because we used an in vitro system to eliminate interference from whole-body conditions such as hypertension, it is reasonable to suggest that telmisartan exerts its indirect renoprotective effect in MetS rats independently of its antihypertensive effect. On the contrary, another line of evidence suggests that long-term telmisartan treatment can improve insulin resistance and metabolic parameters and decrease blood pressure, all of which are beneficial to the kidney.20,31 Therefore, it is difficult to precisely assess how much the indirect renoprotective effect of telmisartan contributes to amelioration of kidney damage in MetS rats. Future studies should address this issue.

Here, we demonstrated that telmisartan treatment can decrease both serum leptin and the PRAT-derived leptin level (Figure 4A and 4C). However, previous studies by Wohl et al32 and Usui et al13 showed that telmisartan treatment increases the circulating leptin level in patients with MetS. The duration of the telmisartan intervention in the study by Wohl et al was as short as 3 weeks, and in the study by Usui et al, the serum leptin only increased 12 weeks after telmisartan treatment and returned to the baseline level at 24 weeks. On the contrary, multiple studies demonstrated that telmisartan treatment can decrease serum leptin in MetS and hypertensive obese patients; the intervention times of telmisartan in these studies were between 12 and 48 weeks.19,34 Furthermore, Fernández-Galaz et al35 showed that telmisartan treatment can prevent leptin resistance in diet-induced obese rats. Based on these observations, we speculate that hyperleptinemia and leptin resistance are features of MetS and obesity and that long-term telmisartan treatment may decrease the leptin level by improving leptin resistance in both human and rodents.

Telmisartan is a partial agonist of the PPAR-γ, and PPAR-γ activation plays crucial roles in promoting adipogenesis, improving insulin resistance, increasing adiponectin gene expression, decreasing inflammation, and deposition of visceral adipose.36-41 Consistent with these studies, our results also demonstrated that long-term telmisartan treatment decreases adipocyte area, promotes adipogenesis, reduces inflammatory cytokines, and improves tissue adiponectin mRNA expression in PRAT of MetS rats. Moreover, telmisartan treatment decreased inflammatory cytokine production and improved 2-DG uptake in IR VAs partly through activation of the PPAR-γ signaling pathway. These data indicate that telmisartan may improve VAT function partly through activation of the PPAR-γ signaling pathway. On the contrary, Boccara et al42 showed that telmisartan treatment can prevent protease inhibitor–induced AT1R overexpression and adipocyte dedifferentiation, in part through the activation of a PPAR-γ–dependent signaling pathway. In this study, telmisartan treatment decreased AT1R and Mas receptor expression in local adipose tissue and promoted adipogenesis in MetS rats but had no effect on the expression of AT1R or Mas receptor in IR VAs, and the PPAR-γ agonist rosiglitazone also had no effect on AT1R expression in IR VAs. In addition, telmisartan treatment did not affect adipogenesis in IR VAs. In the study by Boccara et al, adipocytes were incubated with 2 combined protease inhibitors and telmisartan for 5 days, which was longer than the interference time in our study. Furthermore, in this study, after telmisartan treatment, AT1R mRNA expression decreased in IR VAs, and GW9662 attenuated this decrease (Figure S11), although the difference was not statistically significant. On the basis of these observations, we speculate that the telmisartan-induced change in AT1R expression and adipogenesis may take a long time, potentially explaining why we observed no change after addition of telmisartan in vitro.

**Perspectives**

Long-term telmisartan treatment increases the expression of ACE2, restores balanced activities of the Ang II–AT1R and ACE2-Ang (1–7)-Mas axes, and ultimately exerts an indirect renoprotective effect by decreasing the PRAT-derived leptin level. These results reveal the mechanism underlying the renoprotective effect of telmisartan. However, because complex
cross talk may occur between local RAS signaling and insulin signaling, future studies are necessary to elucidate in detail the downstream regulatory mechanisms that control leptin release.

Acknowledgments
We gratefully acknowledge the technical assistance of Yue Wu, Jin Zeng, ChaoFeng Sun, Weiqi Xu, Tianyu Meng, Lei Liu, Xiawei Dang, and YaNa Li.

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Disclosures
None.

References

What Is New?
• In perirenal adipose tissue (PRAT) of rats with metabolic syndrome (MetS), local leptin expression increases in association with imbalanced activities of the angiotensin (Ang) II-AT1R and ACE2-Ang (1–7)-Mas axes. PRAT-derived leptin from MetS rats promotes glomerular endothelial cell proliferation by activating the p38 MAPK pathway. Long-term telmisartan treatment of MetS rats increases the expression of ACE2, restores balanced activities of the Ang II-AT1R and ACE 2-Ang (1–7)-Mas axes in local adipose tissue and ultimately exerts an indirect renoprotective effect by decreasing the PRAT-derived leptin level.

What Is Relevant?
• Telmisartan, an antihypertensive drug, can exert an indirect renoprotective effect on MetS rats by decreasing PRAT-derived leptin.

Novelty and Significance

PRAT-derived leptin from MetS rats promotes proliferation of rat glomerular endothelial cells by activating the p38 MAPK pathway, thereby contributing to the development of nephropathy. Long-term telmisartan treatment exerts an indirect renoprotective effect on MetS rats by decreasing PRAT-derived leptin release. Our results demonstrate a novel link between nephropathy and PRAT in MetS and show that telmisartan exerts a protective effect on visceral adipose tissue and kidney, suggesting that it could be used as a therapeutic agent for the treatment of MetS-associated nephropathy.

Summary

What Is New?
• In perirenal adipose tissue (PRAT) of rats with metabolic syndrome (MetS), local leptin expression increases in association with imbalanced activities of the angiotensin (Ang) II-AT1R and ACE2-Ang (1–7)-Mas axes. PRAT-derived leptin from MetS rats promotes glomerular endothelial cell proliferation by activating the p38 MAPK pathway. Long-term telmisartan treatment of MetS rats increases the expression of ACE2, restores balanced activities of the Ang II-AT1R and ACE 2-Ang (1–7)-Mas axes in local adipose tissue and ultimately exerts an indirect renoprotective effect by decreasing the PRAT-derived leptin level.

What Is Relevant?
• Telmisartan, an antihypertensive drug, can exert an indirect renoprotective effect on MetS rats by decreasing PRAT-derived leptin.
Telmisartan Ameliorates Nephropathy in Metabolic Syndrome by Reducing Leptin Release From Perirenal Adipose Tissue
Hao Li, Min Li, Ping Liu, YaPing Wang, Heng Zhang, HongBin Li, ShiFeng Yang, Yan Song, YanRong Yin, Lan Gao, Si Cheng, Jun Cai and Gang Tian

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TELMISARTAN AMELIORATES NEPHROPATHY IN METABOLIC SYNDROME BY REDUCING LEPTIN RELEASE FROM PERIRENAL ADIPOSE TISSUE

Hao Li¹,8, Min Li², Ping Liu³, YaPing Wang⁴, Heng Zhang², HongBin Li², ShiFeng Yang⁵, Yan Song⁶, YanRong Yin², Lan Gao¹, Si Cheng², Jun Cai⁷, Gang Tian²,8

From the ¹Department of Critical Care Medicine, ²Department of Cardiology, ⁵Department of Nephrology, ⁶Department of Ultrasound Medicine, the First Affiliated Hospital, Xi’an Jiaotong University, 277 Yanta West Road, Xi’an, Shaanxi, 710061, P. R. China; ³Department of Endocrinology, the Affiliated Xi’an Central Hospital of Xi’an Jiaotong University College of Medicine, Xi’an, Shaanxi, 710003, P.R. China; ⁴Department of Geriatric Cardiology, Shaanxi Provincial People’s Hospital, Xi’an, Shaanxi, 710068, P. R. China; ⁷Hypertension Center, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences & Peking Union Medical College, ⁸Key Laboratory of Shaanxi Province on Molecular Cardiology, and Key Laboratory of Ministry of Education of People’s Republic of China on Environment and Genes Related to Diseases, 277 Yanta West Road, Xi’an, Shaanxi, 710061, P. R. China.

Short title: Renoprotective Effect of Telmisartan

Correspondence to Gang Tian or Jun Cai.

Gang Tian, Department of Cardiology, the First Affiliated Hospital, Xi’an Jiaotong University, 277 Yanta West Road, Xi’an, Shaanxi, 710061, P. R. China; Tel: 86-29-85323715; Fax: 86-29-85323715; E-mail: tiangang@xjtu.edu.cn.
Jun Cai, Hypertension Center, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences & Peking Union Medical College, 167 Beilishi Road, Xicheng District, Beijing 100037, P. R. China; Tel: 86-10-88322161; Fax: 86-10-88322161; E-mail: caijun@fuwaihospital.org.
Supplementary Methods

Metabolic Parameters and Evaluation of Renal Function Evaluation
Body weights were measured once a week. In the 12th and 32nd weeks of the experiment, 24 hour urine samples were collected by using metabolism cages. Plasma triglyceride, total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol and renal function parameters (blood urea nitrogen, serum creatinine, and 24 hour urinary protein) were measured using a Hitachi 7600-010 Automatic Biochemical Analyzer (Hitachi High Technologies, Tokyo, Japan). Fast blood glucose was measured by the glucose oxidase method. Fast insulin was determined by radioimmunoassay (RIA), and insulin resistance was assessed by homeostasis model assessment, as described previously. Systolic blood pressure was measured noninvasively by the tail-cuff method (BP-98A, Softron Beijing Biotechnology, Beijing, China).

Pathological Evaluation of Kidney and PRAT
At the end of the experiment, rats were sacrificed, and both kidneys, PART, epididymal adipose tissue, and perivascular adipose tissue were carefully dissected and removed. After careful removal of the vessels and connective tissue of adipose tissue, the remaining adipose tissue was weighed on an electronic balance. The isolated kidneys were fixed with 10% formalin solution, embedded in paraffin, and sectioned for hematoxylin–eosin (H&E) and Periodic Acid–Schiff (PAS) staining. Glomerulosclerosis index (GSI) was measured as described previously. Briefly, the severity of sclerosis for each glomerulus was graded from 0-4+ as follows: 0, no lesion; 1+, sclerosis of <25% of the glomerulus; 2+, sclerosis of 25-50%; 3+, sclerosis of 50-75%; 4+, sclerosis of >75%. GSI was calculated as GSI = [(1×N1) + (2×N2) + (3×N3) + (4×N4)]/(N0 + N1 + N2 + N3 + N4), where Nx is the number of glomeruli with each given score for a given section. Portions of adipose tissue were dissected from PRAT to count adipocyte number, measure tissue Ang II and Ang 1–7 levels, and prepare PRAT-conditioned medium (PRAT-CM). Adipocyte number was determined based on the method of Fine and DiGirolamo. Another portion of PRAT was fixed in 10% paraformaldehyde, dehydrated, embedded in paraffin, and cut into four μm slices for H&E staining. Adipocyte size was determined by capturing bright field images and measuring 200 cells from each rat.

Quantitative Real-time RT-PCR
The levels of mRNA encoding the renal cortical tissue leptin receptor, AT1R, adiponectin, and inflammatory cytokines in PRAT or adipocytes were determined by quantitative real-time RT-PCR. The leptin receptor has six isoforms, including ObRa-f. In hypothalamus, leptin combines with long-form receptor ObRb, the main signaling receptor, to exert its biological effects via the JAK-STAT pathway. However, in peripheral tissues, especially kidney, ObRa (a short-form leptin receptor) is expressed more abundantly than other leptin receptors; ObRa can also transduce a signal after
binding to leptin. Moreover, the exact functions of other isoforms, including ObRe-f, remain unclear. Therefore, we measured the expression of ObRa and ObRb mRNA. Total RNA was extracted from tissues or cells using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Primers used for PCR, based on a previous study, were as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ObRa</td>
<td>TGATATCGCCAAACAGCAAA</td>
<td>AGTTTGCGCTCTCTTTTGGGA</td>
</tr>
<tr>
<td>ObRb</td>
<td>TGACCACCTCAGATTCCACA</td>
<td>CCACGTGGTCAGGCTGCTG</td>
</tr>
<tr>
<td>MCP-1</td>
<td>ATGCAGGTCTCTGTACG</td>
<td>CTTGGTCTCTGTACACT</td>
</tr>
<tr>
<td>IL-6</td>
<td>TCTACACCCAATTCCATGTCTC</td>
<td>TTGGAGTTGGTCTGGATCTCTG</td>
</tr>
<tr>
<td>AT1R</td>
<td>ACTTTTCTCCCGCCCTCC</td>
<td>TTAGCCAATGGTCTCTG</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>GGAATTTGTGCAGGTGGATG</td>
<td>GGTTACCCTTAGGACCAAGAA</td>
</tr>
<tr>
<td>β-actin</td>
<td>CTATCGGCAATGACGCTTCC</td>
<td>TGTGTGGGATAGGTATTGCTC</td>
</tr>
</tbody>
</table>

Quantitative gene expression was measured by real-time PCR using SYBR Premix ExTaq II (TaKaRa Biotechnology, Tokyo, Japan). Relative quantities of cDNA were calculated from cycle thresholds (Ct) and normalized to the corresponding levels of the housekeeping gene β-actin using the 2^−ΔΔCt method.

Preparation of PRAT-CM
At the end of the experiment, PRAT was used to prepare PRAT-CM as described previously, and leptin levels in plasma and PRAT-CM were determined using the Rat Leptin ELISA kit (CUSABIO, Wuhan, China). In brief, 400 mg of PRAT was collected and conditioned at 37°C for 24 hours in 1 mL of serum-free Dulbecco’s Modified Eagle’s Medium/Ham’s F-12 Medium (DMEM/F12) containing 0.2% BSA. The PRAT-CM was centrifuged, frozen, and stored at -80°C until use. Cells were incubated with PRAT-CM to evaluate the effects of PRAT-derived leptin on GER proliferation.

Materials
Telmisartan, GW9662, rosiglitazone, Ang II, and Ang 1–7 were purchased from Sigma-Aldrich Biotechnology (St. Louis, MO, USA). The p38 MAPK inhibitor SB 203580, ERK1/2 inhibitor PD 98059, and PI3K inhibitor LY 294002 were obtained from Cell Signaling Technology (Beverly, MA, USA). The Mas receptor inhibitor A779 (ab142820) was from Abcam (Cambridge, MA, USA). The leptin antagonist triple mutant (leptin tA), a mutant leptin analogue that functions as a competitive inhibitor and that was previously employed as a leptin receptor antagonist, was purchased from Protein Laboratories (Rehovot, Israel). Recombinant rat leptin was purchased from PeproTech (Rocky Hill, NJ, USA). Opti-MEM was obtained from Life Technologies (Carlsbad, CA, USA).

Cell Culture
GERs were obtained from Chi Scientific Inc. (Jiangyin, Jiangsu, China). To investigate the proliferative effects of PRAT-derived leptin on GERs and the protective effects of telmisartan, GERs were cultured in PRAT-CM with or without leptin tA and the p38 MAPK inhibitor SB 203580. Next, GERs were divided into five groups: Con (incubated with control rat PRAT-CM for 48 hours), MetS (incubated with MetS rat PRAT-CM for 48 hours), MetS-Tel (incubated with MetS-Tel rat PRAT-CM for 48 hours), Leptin tA
(pretreated with 10 μM leptin for 24 hours, and then incubated with MetS rat PRAT-CM for 48 hours), and SB 203580 (pretreated with 10 μM SB203580 for 24 hours, and then incubated with MetS rat PRAT-CM for 48 hours). Further proof-of-principle studies were performed to determine the concentration-dependent response of GERs to leptin administration with or without p38 MAPK inhibitor, as indicated in the Results section.

**Culture of VAs**

VAs were cultured based on the method of previous studies. Briefly, rats were killed and PRAT was removed. The adipose tissue was minced and added to DMEM containing 0.2% collagenase and 1% bovine serum albumin, and then incubated for 40 min. The digested adipose tissue suspension was filtered and centrifuged. The sediment was collected and seeded in a 96-well plate at a density of 1 × 10⁶ cells per well; the date of seeding was considered day 0. The cells were incubated with DMEM/F12 supplemented with 100 μM ascorbic acid, 100 units/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL insulin, 50 nM triiodothyronine, 17 μM pantothenic acid, 1 μM octanoic acid, 33 μM biotin, and 10% newborn calf serum. The culture media were changed every 2 days. The cells expressed the adipocyte phenotype and became mature VAs on days 7–9. Mature VAs were identified by Oil Red O staining. After staining, the dye was extracted from cells using isopropyl alcohol, and absorbance was measured at 510 nm to quantitate intracellular lipids. To explore the effect of telmisartan on the regulatory mechanism of leptin release from adipocytes, mature VAs were incubated with various concentrations of Ang II and Ang 1–7, with or without telmisartan, ERK inhibitor (PD 98059), PI3K inhibitor (LY 294002), or Mas receptor inhibitor (A779).

**ACE2 Transfection to Mature VAs**

Lentiviral-ACE2 and lentiviral-GFP were constructed by Shanghai Genechem Co., Ltd. Mature VAs were incubated with lentiviral-GFP or lentiviral-ACE2 for virus transfection. After 48 hours of transfection, the medium was changed, and cells were incubated with dexamethasone to generate insulin-resistant VAs as described below.

**ACE2 siRNA Silencing**

Silencing of ACE2 expression in VAs was achieved using a ACE2 siRNA (Sigma). Transfection was performed as previously described. Mature VAs were transfected for 48 hours with ACE2 siRNA using Lipofectamine 3000 (Invitrogen Co., Carlsbad, CA, USA). After transfection, the medium was changed, and VAs were incubated with dexamethasone to generate insulin-resistant VAs as described below.

**Insulin-Resistant VAs**

To investigate the protective effect of telmisartan on insulin-resistant adipocytes, mature rat VAs were treated with various concentrations of dexamethasone for 24 hours to induce formation of insulin-resistant VAs in vitro. After establishment of an insulin-resistant VA model, cells were incubated with or without 10 μM telmisartan for 24 hours, in the presence or absence of the PPAR-γ antagonist GW9662 (10 μM), to investigate the effect of telmisartan as a PPAR-γ agonist on IR adipocytes.

**Measurement of Glucose Uptake in VAs**

Briefly, mature VAs were serum-starved for 3 hours, and then basal and insulin-stimulated 2-deoxy-D-[3H] glucose (2-DG) uptake was measured.
Cell Viability Assays
The viability of mature VAs was assessed using the Cell Counting Kit-8 (CCK-8, Boster Biological Technology Co., Ltd, Wuhan, China).

Determination of Ang II, Ang(1–7), IL-6, and MCP-1 Concentrations
Ang II and Ang 1–7 concentrations in local adipose tissue, serum, and cell culture medium were determined using the Rat Ang(1–7) ELISA kit (MyBiosource, San Diego, CA, USA) and Rat Ang II ELISA kit (CUSABIO, Wuhan, China), respectively. Concentrations of the inflammatory cytokines interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) in cell culture medium were determined using the Rat IL-6 ELISA kit (CUSABIO) and the Rat MCP-1 ELISA kit (CUSABIO), respectively.

Western Blot Analysis
Western blotting was used to detect PCNA, p-p38 MAPK, and p38 MAPK in renal cortical tissue, and PPAR-γ, C/EBPα, FAS, FABP4, leptin protein, AT1R, ACE2, Mas receptor, p-ERK1/2, ERK1/2, PI3K, p-Akt, and Akt in PRAT. To further explore the underlying protective mechanism of telmisartan in insulin-resistant VAs, the expression of key components of the AT1R–ERK1/2 and ACE2–Mas receptor–PI3K-Akt pathways, as well as PPAR-γ and C/EBPα, was analyzed by western blotting. The following primary antibodies were used: anti-PCNA (Epitomics, Burlingame, CA, USA), anti-p38 MAPK (Cell Signaling Technology, Beverly, MA, USA), anti-p-p38 MAPK (Thr180/Tyr182, Cell Signaling Technology), anti-leptin (Abcam, Cambridge, MA, USA), anti-PPAR-γ (Abcam), anti-C/EBPα (Abcam, Cambridge, MA, USA), anti-FAS (Abcam), anti-FABP4 (Abcam), anti-ACE2 (sc20998, Santa Cruz Biotechnology, Dallas, TX, USA), anti-Ang II-AT1R (Abcam), anti-Ang(1–7)–Mas receptor (Alomone Labs, Jerusalem, Israel), anti-p-ERK1/2 (Thr202/Tyr204, Cell Signaling Technology), anti-ERK1/2 (Cell Signaling Technology), anti-PI3K (Cell Signaling Technology), anti-p-Akt (Ser473, Cell Signaling Technology), and anti-Akt (Cell Signaling Technology). Protein concentration was determined by the bicinchoninic acid (BCA) method. Equivalent amounts of sample were loaded onto 10% acrylamide gels for electrophoresis and blotting. Immunoreactivity was visualized using an enhanced chemiluminescence (ECL) detection system and quantitated by scanning densitometry (Bio-Rad Quantity One 1-D Analysis Software). Relative protein quantities were normalized against the corresponding level of β-actin.

Supplementary References


Table S1. Baseline Metabolic Parameters of Four experimental Groups

<table>
<thead>
<tr>
<th>Metabolic parameters</th>
<th>Con</th>
<th>Con-Tel</th>
<th>MetS</th>
<th>MetS-Tel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>460.54±8.28</td>
<td>455.80±22.07</td>
<td>465.73±20.46</td>
<td>467.82±22.70</td>
</tr>
<tr>
<td>Fast blood glucose, mmol/L</td>
<td>4.77±0.50</td>
<td>5.05±0.47</td>
<td>5.13±0.53</td>
<td>5.25±0.56</td>
</tr>
<tr>
<td>Fast insulin, μIU/mL</td>
<td>11.00±1.73</td>
<td>9.60±1.76</td>
<td>10.49±1.60</td>
<td>11.06±2.44</td>
</tr>
<tr>
<td>HOMA index</td>
<td>2.62±0.24</td>
<td>2.55±0.28</td>
<td>2.75±0.12</td>
<td>2.56±0.24</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>1.90±0.26</td>
<td>1.83±0.22</td>
<td>1.96±0.25</td>
<td>1.92±0.33</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>1.72±0.24</td>
<td>1.66±0.41</td>
<td>1.59±0.31</td>
<td>1.70±0.20</td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>0.70±0.20</td>
<td>0.82±0.18</td>
<td>0.87±0.29</td>
<td>0.72±0.12</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.30±0.20</td>
<td>1.20±0.30</td>
<td>1.23±0.34</td>
<td>1.38±0.28</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>122.90±4.48</td>
<td>128.30±6.55</td>
<td>128.45±3.86</td>
<td>123.36±3.80</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. Con group (control rat, fed normal diet, received vehicle alone for 20 consecutive weeks, n=10); Con-Tel group (control rat, fed normal diet, received telmisartan 8 mg/kg per day for 20 consecutive weeks, n=10); MetS group (metabolic syndrome rat, fed high fat diet, received vehicle alone for 20 consecutive weeks, n=11); MetS-Tel group (MetS rat, fed high fat diet, received telmisartan 8 mg/kg per day for 20 consecutive weeks, n=11). HOMA: homeostasis model assessment; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; SBP: systolic blood pressure.
Table S2. Metabolic Parameters of Four Experimental Groups After Diet Treatment for 12 Weeks

<table>
<thead>
<tr>
<th>Metabolic parameters</th>
<th>Con</th>
<th>Con-Tel</th>
<th>MetS</th>
<th>MetS-Tel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>544.20±25.76</td>
<td>548.80±28.09</td>
<td>607.27±20.07*</td>
<td>611.27±29.74*</td>
</tr>
<tr>
<td>Fast blood glucose, mmol/L</td>
<td>4.48±0.50</td>
<td>4.98±0.39</td>
<td>7.58±1.03*</td>
<td>6.79±0.50*</td>
</tr>
<tr>
<td>Fast insulin, μIU/mL</td>
<td>10.50±1.51</td>
<td>12.57±2.15</td>
<td>23.26±4.12*</td>
<td>20.07±5.49*</td>
</tr>
<tr>
<td>HOMA index</td>
<td>2.46±0.28</td>
<td>2.75±0.25</td>
<td>7.36±1.76*</td>
<td>7.28±1.12*</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>1.92±0.19</td>
<td>1.77±0.36</td>
<td>2.86±0.21*</td>
<td>2.73±0.20*</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>1.17±0.21</td>
<td>1.00±0.21</td>
<td>1.95±0.32*</td>
<td>2.01±0.26*</td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>0.77±0.14</td>
<td>0.87±0.16</td>
<td>1.37±0.21*</td>
<td>1.29±0.20*</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.45±0.16</td>
<td>1.56±0.18</td>
<td>1.02±0.18*</td>
<td>0.98±0.22*</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>124.90±5.51</td>
<td>125.40±8.59</td>
<td>148.09±9.78*</td>
<td>144.36±11.35*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD; *P<0.01 vs Con group. HOMA: homeostasis model assessment; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; SBP: systolic blood pressure.
Table S3. Effects of Long-term Telmisartan Treatment (20 weeks) on Metabolic Parameters in Experimental Animals

<table>
<thead>
<tr>
<th>Metabolic parameters</th>
<th>Con</th>
<th>Con-Tel</th>
<th>MetS</th>
<th>MetS-Tel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>654.70±25.95</td>
<td>655.02±26.60</td>
<td>780.17±19.92*</td>
<td>711.19±22.21†</td>
</tr>
<tr>
<td>Fast blood glucose, mmol/L</td>
<td>5.59±0.27</td>
<td>5.94±0.31</td>
<td>13.37±1.84*</td>
<td>6.32±1.08†</td>
</tr>
<tr>
<td>Fast insulin, μIU/mL</td>
<td>20.43±3.30</td>
<td>19.15±2.78</td>
<td>80.57±4.08*</td>
<td>28.42±3.05†</td>
</tr>
<tr>
<td>HOMA index</td>
<td>4.15±0.33</td>
<td>4.57±0.32</td>
<td>24.19±4.78*</td>
<td>7.82±2.06†</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>2.05±0.25</td>
<td>1.92±0.19</td>
<td>3.48±0.36*</td>
<td>2.41±0.29†</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>1.15±0.25</td>
<td>1.07±0.23</td>
<td>2.17±0.28*</td>
<td>1.83±0.18†</td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>0.97±0.34</td>
<td>1.01±0.18</td>
<td>2.12±0.24*</td>
<td>1.49±0.29†</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.25±0.11</td>
<td>1.27±0.18</td>
<td>0.89±0.24*</td>
<td>1.16±0.24†</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>127.30±12.59</td>
<td>128.50±11.58</td>
<td>151.55±6.62*</td>
<td>130.00±11.87†</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD; *P<0.01 vs Con group; †P<0.05 vs MetS group. HOMA: homeostasis model assessment; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; SBP: systolic blood pressure.
Table S4. Renal Function in Four Experimental Groups after Diet Treatment for 12 Weeks

<table>
<thead>
<tr>
<th>Renal function</th>
<th>Con</th>
<th>Con-Tel</th>
<th>MetS</th>
<th>MetS-Tel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood urea nitrogen, mmol/L</td>
<td>5.08±0.31</td>
<td>5.01±0.55</td>
<td>4.91±0.29</td>
<td>4.91±0.37</td>
</tr>
<tr>
<td>Serum creatinine, μmol/L</td>
<td>49.21±5.10</td>
<td>52.00±8.73</td>
<td>53.36±8.29</td>
<td>55.36±5.20</td>
</tr>
<tr>
<td>Proteinuria, mg/24 hours</td>
<td>21.50±4.06</td>
<td>23.10±6.89</td>
<td>89.18±7.49*</td>
<td>83.73±7.55*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. *P<0.01 vs Con group.
Table S5. Protective Effect of Long-term Telmisartan Treatment (20 Weeks) on Renal Function in MetS Rats

<table>
<thead>
<tr>
<th>Renal function</th>
<th>Con</th>
<th>Con-Tel</th>
<th>MetS</th>
<th>MetS-Tel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood urea nitrogen, mmol/L</td>
<td>5.29±0.47</td>
<td>5.09±0.52</td>
<td>15.12±3.32*</td>
<td>11.02±2.77†</td>
</tr>
<tr>
<td>Serum creatinine, μmol/L</td>
<td>56.50±6.77</td>
<td>55.00±12.43</td>
<td>88.55±7.66*</td>
<td>61.11±6.72†</td>
</tr>
<tr>
<td>Proteinuria, mg/24 hours</td>
<td>25.86±5.61</td>
<td>30.60±5.97</td>
<td>300.88±13.75*</td>
<td>104.91±18.25†</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. *P<0.01 vs Con group; †P<0.05 vs MetS group.
S1. Telmisartan decreases the amount of PRAT in MetS rats. A, Images of the right kidney, with PRAT. B, C and D, Amount of PRAT and epididymal and perivascular adipose tissue in four experimental groups. Data are mean ± SEM. Con group (control rat, fed normal diet, received vehicle alone for 20 consecutive weeks, n=10); Con-Tel group (control rat, fed normal diet, received 8 mg/kg telmisartan per day for 20 consecutive weeks, n=10); MetS group (metabolic syndrome rat, fed high fat diet, received vehicle alone for 20 consecutive weeks, n=11); MetS-Tel group (MetS rat, fed high fat diet, received 8 mg/kg telmisartan per day for 20 consecutive weeks, n=11). *P <0.01 vs Con group; #P<0.05 vs MetS group.
S2. Cultured rat visceral adipocytes (VAs) at different stages. A, Rat VAs on day 0 of culture. B, Proliferation of VAs at day 2. C, Small lipid droplets formed at day 4. D, Accumulation of lipid droplets appeared in the cytoplasm at day 5. E, Large droplets of lipid in the cytoplasm at day 7. F, Mature VAs at day 9, stained with Oil Red O. Original magnification, ×200.
S3. Ang II promotes leptin release from mature VAs in a concentration-dependent manner, accompanied by activation of the AT1R-ERK1/2 pathway. A, Leptin level of rat VAs exposed to various concentrations (0.001–10 μM) of Ang II for 24 hours. Data are expressed as percentages of the value in the control group. Expression of key components of the AT1R–ERK1/2 and ACE2–Mas receptor–PI3K–Akt signaling pathways were analyzed by western blotting. B, C, D, E, F, and G, Densitometric analyses of immunoblots. Data are means ± SEM. *P<0.01 vs control group.
S4. Telmisartan treatment resulted in a concentration-dependent reduction in Ang II–induced leptin release from mature VAs. VAs were exposed to 10 μM Ang II and various concentrations (1–100 μM) of telmisartan for 24 hours. The maximal effective concentration of telmisartan was 100 μM, and the effects of telmisartan at 10 μM and 100 μM did not differ significantly. Because we planned to incubate mature VAs with 10 μM Ang II in the next experiment, we choose 10 μM as the dose of telmisartan for subsequent research. Data are expressed as percentages of the value in the control group, and are presented as means ± SEM; *P<0.01 vs control group; **P<0.01 vs Ang II 10 μM.
S5. Ang I–7 inhibits leptin release from mature VAs in a concentration-dependent manner, accompanied by activation of the Mas receptor–PI3K–Akt pathway. A, Leptin level of mature VAs. Cells were incubated with various concentrations of Ang 1–7 (0.001–10 μM) for 24 hours. Data are expressed as percentages of the value in the control group. Expression of key components of the AT1R–ERK1/2 and ACE2–Mas receptor-Pi3K–Akt pathways were analyzed by western blotting. B, C, D, E, F, and G, Densitometric analyses of immunoblots. Data are means ± SEM. *P<0.01 vs control group.
S6. Establishment of an insulin-resistant VAs model *in vitro*. A, Effect of various concentrations (0.01–1 μM) dexamethasone (Dex) on basal 2-DG uptake in VAs. B, Cells were incubated with 1 μM insulin for 15 min to test insulin-stimulated 2-DG uptake. Data are expressed as percentages of the value in the Dex 0 μM group. *P< 0.01 vs Dex 0 μM group.
S7. Cell viability assays. Cell viability was similar among the twelve groups. Data are expressed as percentages of the value in the control group.
S8. Densitometric analyses of immunoblots of key components of the AT1R–
ERK1/2 and ACE2–Mas receptor–PI3K–Akt pathways. Data are means ± SEM. *P
<0.01 vs Con group; †P<0.01 vs IR group; ‡P<0.01 vs IR-Tel group.
S9. Telmisartan treatment has no effect on adipogenesis of insulin-resistant adipocytes. Control mature VAs (Con adipocytes) or insulin-resistant mature VAs (IR adipocytes) were incubated with or without 10 μM telmisartan for 24 hours in the presence or absence of the PPAR-γ antagonist GW9662 (10 μM). A, Oil Red O staining of cultured VAs. Original magnification, ×200. B, Cell viability was similar among groups. C, Intracellular lipid accumulation, and data are expressed as percentages of the value in the control group. D, Expression of transcription factors (PPAR-γ and C/EBPα) in cultured VAs was analyzed by western blotting. E and F, Densitometric analyses of immunoblots. Data are means ± SEM.
S10. Telmisartan treatment decreases leptin release from insulin-resistant VAs independently of the PPAR-γ signaling pathway. A, Leptin level in cell culture medium. Expression of key components of the AT1R–ERK1/2 and ACE2–Mas receptor–PI3K-Akt signaling pathways were analyzed by western blotting. B, C, D, E, F, and G, Densitometric analyses of immunoblots. H, and I, Ang II and Ang 1–7 levels in culture medium. Data are expressed as a percentage of the value in the control group. Data are means ± SEM. *$P<0.01$ vs control group; ‡$P<0.05$ vs IR group.
S11. The PPAR-γ agonist rosiglitazone has no effect on AT1R expression in insulin-resistant VAs. Con or IR adipocytes were incubated with or without 10 μM rosiglitazone or 10 μM telmisartan for 24 hours, in the presence or absence of 10 μM PPAR-γ antagonist GW9662. A, Expression of AT1R was analyzed by western blot analysis. B, AT1R mRNA expression was analyzed by RT-PCR, and data are expressed as percentages of the value in the control group. Data are means ± SEM.
S12. Telmisartan treatment decreases inflammatory cytokine production and improves 2-DG uptake in insulin-resistant VAs partly through activation of the PPAR-γ signaling pathway. A and B, Basal and insulin-stimulated 2-DG uptake in cultured VAs. C, Adiponectin mRNA expression in cultured cells. D and E, Levels of inflammatory cytokines IL-6 and MCP-1 in the culture medium. Data are expressed as percentages of the value in the control group. Data are means ± SEM; *P<0.01 vs control group; #P<0.05 vs IR group; △P<0.01 vs IR-Tel group.