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Chronic Losartan Therapy in OLETF Rats Has a Differential Effect on Adipose and Vascular PAI-1

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Abstracts

Angiotensinogen (AGT) and plasminogen activator inhibitor-1 (PAI-1) are expressed in both vascular and adipose tissues. Angiotensin II (AG II) has an adipogenic effect and increases PAI-1 expression. To evaluate the chronic effects of AG II type 1 receptor (AT_1R) antagonism on adipose mass and PAI-1 expression in vascular and adipose tissues, losartan (30 mg/kg/day) was administered to Otsuka Long Evans Tokushima Fatty (OLETF) rats, a model of type 2 diabetes, for 20 weeks. Adipose mass and regional fat distribution in the abdomen did not change after chronic AT_1R antagonism in OLETF rats. AGT and PAI-1 mRNA expressions in adipose tissue of OLETF rats were significantly increased compared with Long-Evans Tokushima Otsuka (LETO) rats, the normal control. Chronic losartan therapy further increased the level of adipose AGT in OLETF rats, but did not affect the level of adipose PAI-1 mRNA. In contrast, aortic PAI-1 protein expression, assessed by immunofluorescent staining, was attenuated by chronic losartan therapy. Our results have three implications. First, chronic AT_1R antagonism with losartan increases AGT expression in adipose tissue; increased AGT may sustain PAI-1 expression in adipose tissue despite the AT_1R blockade. Second, chronic losartan therapy does not affect adipose mass. Third, chronic AT_1R antagonism with losartan has a differential effect on vascular and adipose PAI-1 expression.

Key words : Plasminogen activator inhibitor-1, Angiotensinogen, OLETF rats, Type 2 diabetes; Losartan

Introduction

In addition to the classical pathway of AG II synthesis, several components of the renin-angiotensin system (RAS) have been identified in a variety of tissues including adipose tissue (1, 2), implying that these tissues have the ability to synthesize AG II independent of the systemic RAS (3, 4). RAS in adipose tissue could be an important link between obesity and hypertension. A positive correlation was found between the expression of AGT in subcutaneous and visceral adipose tissue and human obesity (5, 6).

The effects of AG II on adipocyte growth need to be further clarified. Several reports suggest that AG II may

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play a role in adipocyte growth and differentiation (7-9). AG II type-2 receptor (AT_2R) - mediated formation of prostacyclin by mature adipocytes can induce preadipose cells to differentiate into mature cells (7). However, an elegant study showed that targeted overexpression of AGT in adipose tissue of mice causes hypertension accompanied by an increase in fat mass and in the level of circulating AGT (10). In the study, the fat mass was shown to be increased through adipocyte hypertrophy and hypoplasia rather than through hyperplasia with differentiation (10).

The *in vivo* effects of AT_1R blockers on adipose tissue are complex. Although irbesartan and telmisartan were reported to induce adipocyte differentiation and improve insulin resistance (11-13), these two AT_1R blockers also behave like partial agonists for peroxisome proliferator-activated receptor-γ (PPARγ). In addition, there may be some species-related differences in the adipose response to AT_1R blockers. The AT_2R subtype rather than the AT_1R subtype contributes to mediating the action of AG II in mouse adipose cells (7, 14), whereas AT_1R is predominant in rat adipocytes (15), human preadipocytes, and human adipocytes (2, 4, 15). In mice fed a high fat diet, various metabolic abnormalities and increases in adipose PAI-1 are attenuated after chronic treatment with an AT_1R antagonist for 12 weeks (16). To date, however, there are only a few conclusive rat and human studies that have been conducted over several weeks or months that show the chronic salutary effect of AT_1R antagonism on adipose development or adipokine expression (17). Further *in vivo* studies about the effects of a chronic AT_1R blockade on adipose physiology are required.

PAI-1 is the primary physiological inhibitor of tissue-type plasminogen activator and urokinase-like plasminogen activator; it inhibits both fibrinolysis and proteolysis (18, 19). A previous study demonstrated that in murine tissues, next to the aorta, adipose tissue has the highest level of PAI-1 mRNA (20). Adipose PAI-1 expression is enhanced by AG II, insulin, and TNF-α (19, 21, 22). Circulating PAI-1 levels in humans are increased in obesity and the insulin resistance syndrome and correlate with the degree of insulinemia (23). Because adipose tissue itself is an important source not only of PAI-1, but also AGT, RAS activation and hyperinsulinemic states of obesity may further up-regulate PAI-1 in adipose tissue (14, 24).

Although AT_1R antagonist decrease PAI-1 antigen acutely (25), such an effect cannot be maintained over several weeks (26). On the contrary, a slight elevation in plasma PAI-1 after chronic losartan therapy was observed (27). Enhanced secretion from various organs may explain the rebound elevation after the chronic AT_1R blockade (28). However, a chronic candesartan treatment was shown to reduce cardiac and aortic PAI-1 expression levels (17). An evaluation of the adipose PAI-1 response to chronic AT_1R antagonism should be meaningful; therefore, we decided to use a rat model of type 2 diabetes to evaluate the long-term therapeutic effect of a well-known AT_1R antagonist, losartan, on adipose mass, AGT gene expression, and adipose and vascular PAI-1 expression levels.

Materials and Method

1. Animal preparation

All experimental procedures were conducted in accordance with the Guide for Animal Experimentation (College of Medicine, Cheju National University, Republic of Korea). Four-week-old male OLETF rats, a rat model of type 2 diabetes (29, 30), were provided by the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima,

Japan) and were maintained in an animal facility with ventilation, controlled temperature (22 ± 2 ℃), and a 12-hour light/dark cycle (lighting beginning at 8:00 h). All animals were housed in plastic cages (n = 3 per cage) and were fed ad libitum with a standard chow diet and tap water.

At the age of 17 weeks, OLETF rats were randomly assigned to the following 2 groups; an OLETF control group (n=6) and a losartan-treated (30 mg/kg/day in drinking water) OLETF group (Losartan-OLETF) (n=6). Body weight and fasting blood glucose levels were the same between the groups at the beginning of the experiment. A non-diabetic rat strain (LETO) was used as an age-matched control.

At midnight, on the final day of the 20-week losartan treatment, the food and losartan were withdrawn from the cages. Ten hours later, the rats were euthanized by decapitation and the trunk blood was collected in pre-chilled EDTA tubes (Vacutainer; BD Biosciences Franklin Lakes, NJ). Mesenteric, perirenal, and epididymal fat pads were rapidly removed, weighed, and snap-frozen with liquid nitrogen. Frozen samples were kept at -80℃ until analyzed. The distal thoracic aorta was also taken and fixed in 10% buffered formalin.

2. Systolic blood pressure measurement

Systolic blood pressure (SBP) was measured every four weeks using the tail-cuff method (Narco Biosystems Electro-Sphygnomanometer, Houston, TX). SBP was measured under conscious conditions, and the average of three pressure readings was recorded for each measurement.

3. Blood/Plasma analyses

After decapitation, collected whole blood was centrifuged at 3000 rpm (Sorvall RT 6000 D; Sorvall, Newton, CT) at 4℃ for 20 min to separate plasma. Plasma glucose levels were determined with a Beckman Glucose Analyzer II (Beckman-Coulter, Fullerton, CA). Plasma insulin levels were assayed using a commercial radioimmunoassay kit (Linco, St. Charles, MO).

4. Semi-quantitative analysis of mRNA expression

Total RNA was extracted from 300 mg of mesenteric adipose tissue, using TRIZOL (Invitrogen, Carlsbad, CA). Extracts of RNA were kept at -80℃ until reverse transcription polymerase chain reaction (RT-PCR) analyses were performed. A RT-PCR kit (Qiagen, Valencia, CA) was used for semi-quantitative analyses of AGT, TNF-α, PAI-1, and β-actin mRNA expression. The sense and antisense primers used were 5'-GCT TCT CCC AGC TGA CTG GG-3' and 5'-GGT TGG TGT CAC CCA TCT TGC C-3' for AGT (31), 5'-CACGCTCTT CTGTCTACTGA-3' and 5'-GGACTCCGTGATGTCTAA GT-3' for TNF-α (32), 5'-GACAATGGAAGAGCAACA TG-3', and 5'-ACCTCGATCTTGACCTTT TG-3' for PAI-1 (33), and 5'-CAGATCATGTTTGAGA CCTT-3' and 5'-CGGATGTCMACGTCACACTT-3' for β-actin (34), respectively. The amplified fragment sizes were 404-, 546-, 205-, and 509-bp for AGT, TNF-α, PAI-1, and β-actin cDNA, respectively. The relative RT-PCR was performed according to the manufacturer's recommendations, using 50 ng total RNA as a template, 0.6 μM primers for the target transcript, and 0.3 μM primers for the internal control transcript (β-actin). Following the reverse transcription reaction at 50 ℃ for 30 min, an initial denaturation at 95 ℃ for 15 min, 25-28 cycles of denaturation at 94 ℃ for 0.5 min, annealing at 57 ℃ for 0.5 min, elongation at 72 ℃ for 1 min, and a final extension reaction at 72 ℃ for 10 min proceeded. PCR products were electrophoresed on a

1.5% agarose gel and stained with ethidium bromide. Densitometry was performed using a GENE GENIUS image analyzer (Synoptics, Cambridge, UK). The reliability of the semi-quantitative RT-PCR technique for measuring relative gene expression was verified using the amounts of template RNA (from 25 to 150 ng) and number of amplification cycles (from 15 to 35 cycles). Results were expressed as a relative value after normalization to the β -actin expression.

5. Histological analysis and PAI-1 immunofluorescence microscopy

Thoracic aortas were fixed in 10% buffered formalin and embedded in paraffin. Serial sections (5 μm thick) were cut from each specimen. Immunohistochemistry was performed using the free-floating method, as previously described (35). Rabbit anti-PAI antibody (American Diagnostica Inc. Stamford, CT. at a dilution ratio of 1:3000) was used as primary antibody. All sections were selected and surveyed at the same interval in each area of rat aorta (n =3) using an unbiased sampling method. Slides were observed with an optical microscope. Fluorescence photomicrographs were taken from an identical field. The obtained photographs were assessed by densitometry using the NIH image program (Scion Image, Frederick, MD) to determine the staining intensities. Automated image analysis allowed quantification of the fluorescence intensity of the samples. The sums of the fluorescent values of all pixels in the selected region were divided by the number of pixels within the selection. And, the mean fluorescence intensity in slides from each group was expressed using arbitrary fluorescence units (AFU).

6. Statistical analysis

The results are expressed as means ± S.E. Statistical differences between the mean values were evaluated by the Student's t test or analysis of variance (ANOVA) when appropriate, with $p < 0.05$ considered statistically significant for all tests.

Results

1. Physiological data

Using OLETF rats, a rat model of type 2 diabetes (29, 30), the effects of an AT_1R antagonist, losartan, on glucose homeostasis and the development of adipose mass were examined. The mean body weight, amount of visceral fat, plasma glucose, and plasma insulin of each group at 36 weeks of age are shown in Table 1. When fed with normal chow, OLETF rats and Losartan-OLETF rats gained more weight than LETO rats, but there was no difference between OLETF and Losartan-OLETF rats (Table 1). All three fat depots taken from OLETF and Losartan-OLETF rats were larger than those from LETO rats. Chronic losartan therapy for 20 weeks in OLETF rats did not affect regional fat distribution and adipose mass (Table 1). Neither plasma glucose nor plasma insulin levels were significantly affected by chronic losartan treatment in OLETF rats, although plasma insulin tended to increase after chronic losartan treatment (p=0.062, OLETF vs. Losartan-OLETF rats).

SBP of OLETF rats at the age of 16 weeks was already higher than that of non-diabetic rats. Losartan treatment significantly decreased the blood pressure of OLETF rats throughout the experimental period (Figure 1).

2. Expression of AGT, PAI-1, and TNF-α in mesenteric adipose tissue

The level of AGT mRNA expression in mesenteric

Table 1. Body weight and biochemical data of the experimental groups

	Experimental groups		
Parameters	LETO	OLETF	Losartan-OLETF
Body weight (g)	466.7 ± 12.3	567.6 ± 6.7*	560.3 ± 8.7*
Plasma glucose (mg/dL)	114.7 ± 3.0	144.2 ± 6.2*	137.2 ± 5.3*
Plasma insulin (μU/mL)	20.5 ± 5.4	200.7 ± 18.9**	255.1 ± 3.1**
Mesenteric fat (g)	2.8 ± 0.2	8.1 ± 0.4**	8.3 ± 1.3*
Both perinephric fat (g)	5.3 ± 0.5	16.9 ± 0.6**	19.4 ± 3.2*
Both epididymal fat (g)	6.8 ± 0.4	10.8 ± 0.5**	10.6 ± 0.6*

Values are means ± S.E. (n=6, each group). *, $p<0.05$; **, $p<0.01$ vs. LETO group. #, $p<0.05$; ## $p<0.01$ vs. OLETF group. Losartan-OLETF; OLETF rats given losartan in drinking water (30mg/Kg of body weight /day) for 20 weeks.

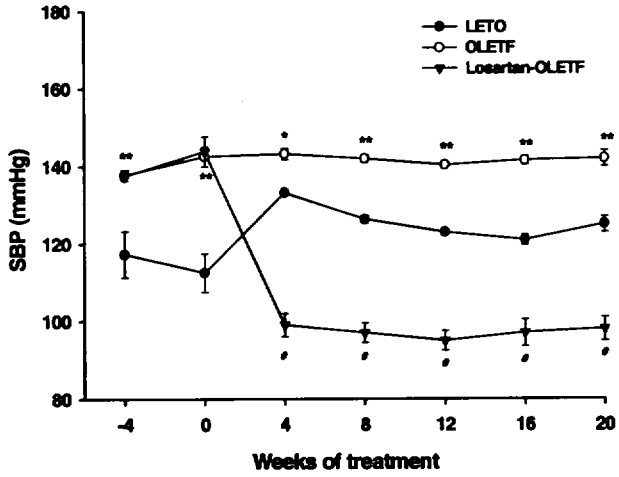

Fig. 1. Effect of chronic losartan treatment on the time course of SBP. Experimental groups: Normal control rats (LETO, n=6), untreated OLETF (OLETF, n=6), Losartan-treated OLETF (Losartan-OLETF, n=6). Values are expressed as means ± S.E. *, $p<0.05$; **, $p<0.01$ vs. LETO group. #, $p<0.01$ vs. OLETF group.

adipose tissue of OLETF rats was higher than that in LETO rats. Chronic losartan treatment of OLETF rats caused a further significant increase in the level of AGT mRNA in mesenteric fat compared with untreated OLETF rats ($p < 0.05$, 587.4 ± 14.6% vs. 416.0 ± 34.6% of the abundance in LETO) (Fig. 2. A., B.). The level of PAI-1 mRNA in mesenteric adipose tissue was higher in OLETF rats compared with LETO rats (401.6 ± 47.7% vs. 101.4 ± 19.8%, $p < 0.05$). Chronic losartan treatment in OLETF rats tended to increase adipose PAI-1 mRNA (510.7 ± 17.8% of the abundance in LETO rats, $p=0.1$ vs. OLETF) (Fig. 2. A., C.). TNF-α mRNA was similarly expressed in LETO and OLETF rats. Losartan therapy of OLETF rats did not influence the abundance of TNF-α mRNA in mesenteric adipose tissue (Fig. 2. A., D.).

3. PAI-1-specific immunofluorescent staining of the thoracic aorta

To investigate the long-term effect of losartan on PAI-1 expression in the vascular wall, we performed PAI-1-specific fluorescence immunostaining of the distal thoracic aortas from LETO, OLETF, and Losartan-OLETF rats. As shown in figure 3, compared with LETO and Losartan-OLETF rats, the section of thoracic aorta from untreated OLETF rats exhibited a stronger PAI-1 immunofluorescence in the *intima* and *media layers* of the aortic wall. In contrast, in the *tunica adventitia* of aorta from each group, the immunofluorescence staining was relatively weak and there were no significant difference in the intensity of PAI-1 immunofluorescence between the groups. Automated image analysis allowed quantification of the fluorescence intensity of the samples (Table 2): mean fluorescence intensity in slides

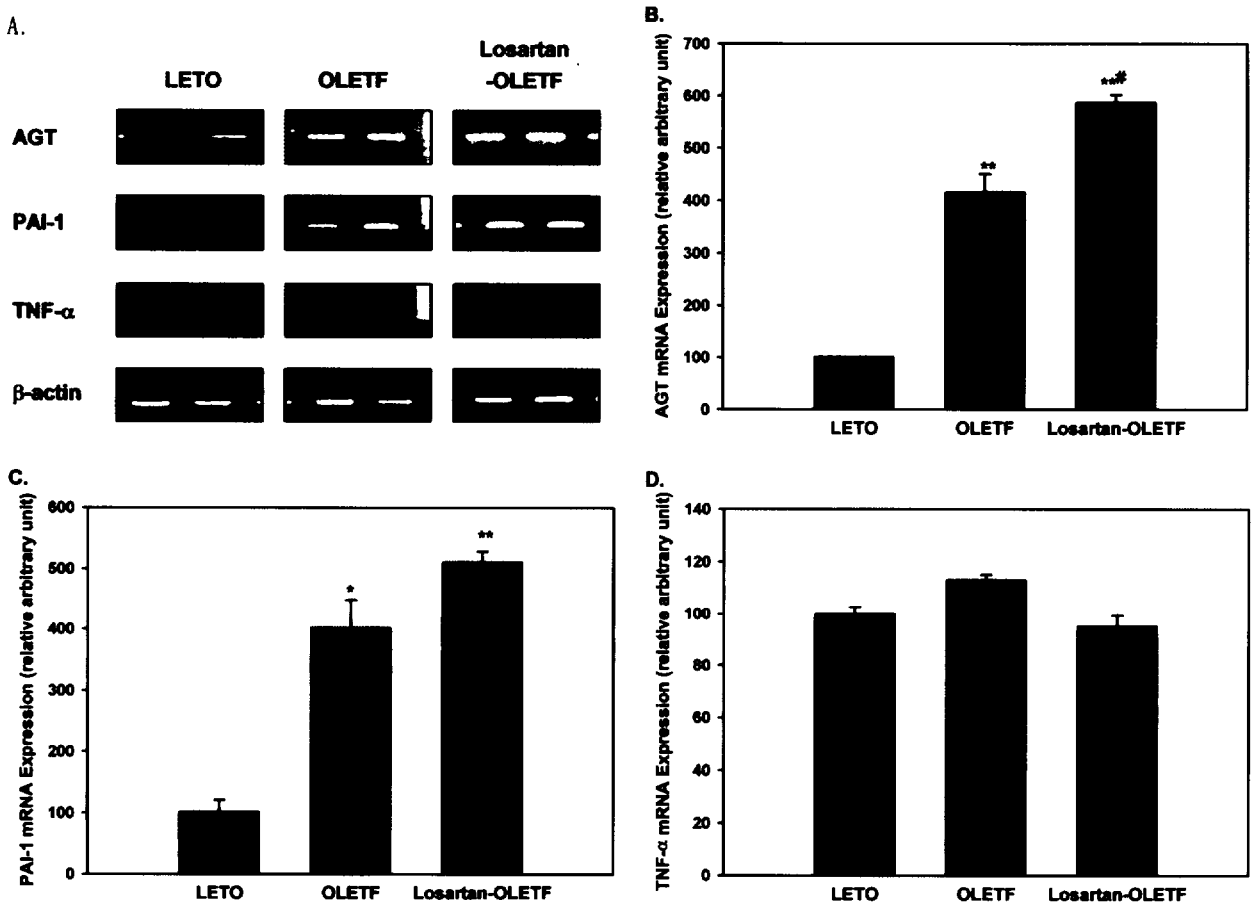

Fig. 2. Effect of chronic losartan treatment on mRNA expressions of AGT, PAI-1, and TNF-α in mesenteric white adipose tissue. Representative RT-PCR products are shown (A). Abundance of mRNAs was determined by densitometric analysis. Data were normalized using βactin mRNA and expressed as a percentage of the value obtained from control chow-treated LETO rats. Each bar represents means ± S.E. (n = 6). *, $p<0.05$; **, $p<0.01$ vs. LETO group. #, $p<0.01$ vs. OLETF group (B,C, D).

Table 2. The measured mean arbitrary fluorescence unit (AFU) for PAI-1 and the wall thickness of thoracic aorta sections from the experimental groups

	Experimental groups		
	LETO	OLETF	Losartan-OLETF
AFU	59.5 ± 3.1	130.3 ± 5.8**	62.5 ± 5.2##
Thickness (μm)	76.5 ± 6.5	101.7 ± 10.7**	91.4 ± 6.9##

Values are means ± S.E. (n=3, each group). **,$p<0.01$ vs. LETO group. ##, $p<0.01$ vs. OLETF group. Losartan-OLETF; OLETF rats given losartan in drinking water (30mg/Kg of body weight /day) for 20 weeks.

from OLETF rats was 130.3 ± 5.8 AFU. This was almost 2 fold larger than the fluorescence intensity in samples from LETO rats (59.5 ± 3.1 AFU, $P<0.001$). However, in Losartan-OLETF rats, both the thickness of the aortic wall and PAI-1 expression (62.5 ± 5.2 AFU, $P<0.01$) were significantly decreased compared with untreated OLETF rats (Table 2).

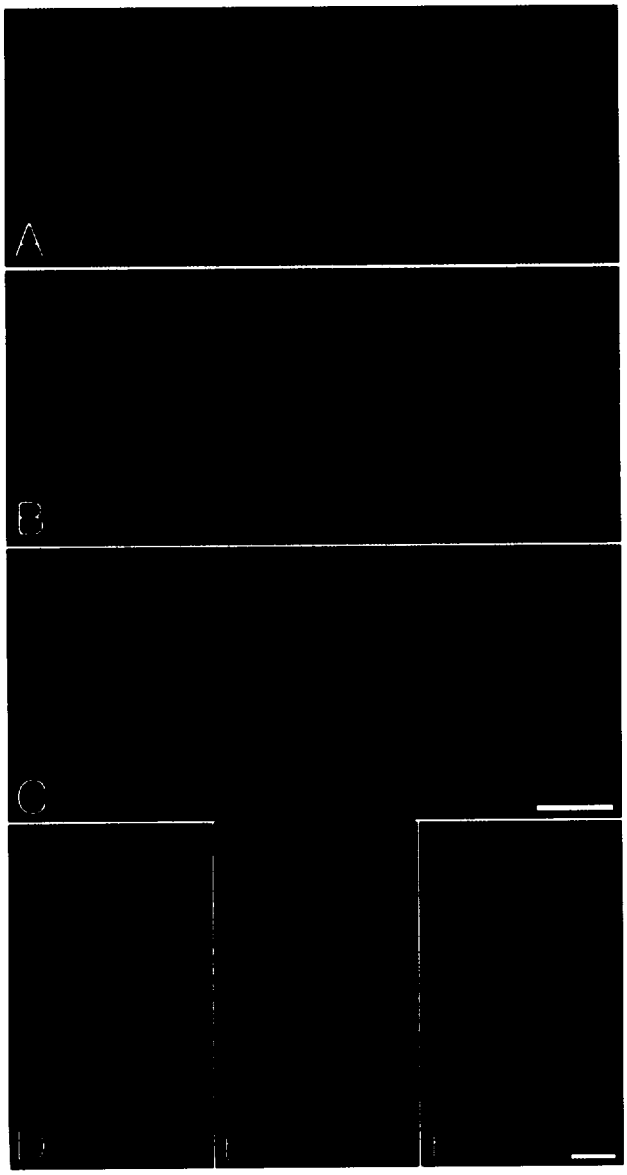

Fig. 3. Immunofluorescent staining for PAI-1 in the distal thoracic aorta of LETO (A, D), untreated OLETF (B, E), and Losartan-OLETF (C, F) rats. Representative images of the bottom row (D, E, F) show the relative thickness of the vascular wall. Homogenous PAI-1 immunofluorescence was distinctively exhibited in the *tunica media* of the arterial wall. Scale bars = 50 μm. For quantitative data, refer to Table 2.

Discussion

In the present study, we evaluated the chronic *in vivo* effects of AT_1R blockade using a high dose of losartan that has little effect on PPARγ . Our results show that in OLETF rats neither the development of three intra-abdominal fat depots nor the adipose expression of PAI-1 mRNA was affected by treatment with losartan for 20 weeks. In contrast, in OLETF rats, aortic PAI-1 expression as well as aortic wall thickness was decreased by chronic losartan treatment. The present study is the first to evaluate the chronic *in vivo* effects of losartan on adipose development and on both aortic and adipose PAI-1 expression in a rat model of type 2 diabetes.

Although previous studies suggest a trophic effect of AG II on adipose tissue, blockade of AG II action via AT_1R did not change the weight of intra-abdominal fat depots of OLETF rats in the present study. And, there was no difference in the size of epididymal adipocytes under light microscopy between the treated and un-treated OLETF rats (data not shown). In contrast, chronic AT_1R antagonism using losartan was reported to attenuate the development of obesity and insulin resistance in mice induced by an HF diet (16). And, there are a few reports that irbesartan and telmisartan could improve insulin sensitivity and change body weight even in rats (36). These discrepancies may be due to species' differences of the experimental animals and the partial PPARγ agonistic action of irbesartan and telmisartan.

In line with previous data about obese rodents and human subjects with metabolic syndrome (37, 38), the level of AGT mRNA expression in mesenteric adipose tissue of OLETF rats was higher than that of LETO rats. Chronic treatment with AT_1R antagonist further induced AGT mRNA expression in OLETF rats. Though there are a substantial number of reports showing that chronic AT1 antagonism in rodents and humans increases plasma AG II (39, 40), the effects of chronic

AT_1 antagonism on adipose AGT remain unevaluated. Our results suggest that increased adipose AGT may contribute to the elevation of plasma AG II during chronic AT_1R antagonism.

TNF-α induces important metabolic changes in glucose transport and lipid metabolism, mimicking insulin resistance and the metabolic changes found in patients with type II diabetes (41). But, in the present study, TNF-α mRNA expression in adipose tissue was not different between the experimental groups. Previous studies showed that TNF-α mRNA expression in white adipose tissue of high fat diet-induced obese rats was increased compared with control rats (42). Further studies including other fat depots may be helpful.

Many studies suggest that chronic AT_1R antagonism does not suppress circulating PAI-1 antigen concentrations in humans (43, 44). But, a few studies have reported that chronic AT_1R blockade could decrease cardiovascular or renal PAI-1 expression (17, 33). Considering that adipose tissue and the aorta are significant sources of PAI-1 in obesity, both adipose and aortic PAI-1 expressions were evaluated in the present study. Our results show that in OLETF rats, chronic AT_1R antagonism with losartan for 20 weeks decreases aortic PAI-1expression and aortic wall thickening. Adipose expression of PAI-1 mRNA was significantly enhanced in OLETF rats compared with LETO rats in the present study. However, even after chronic AT_1R antagonism with losartan, the mesenteric adipose tissue was shown to maintain the enhanced expression of PAI-1.

Taken together, these results suggest that, especially in the case of AT_1R antagonists that have no activity on PPARγ, differential effects of chronic AT_1R antagonism on vascular and adipose tissues may be present. Further *in vivo* studies about the effects of various AT_1 blockers in adipose tissue are required to find a reasonable blockade of RAS in both adipose and cardiovascular tissues to manage metabolic syndrome.

One possible mechanism for the lack of effect of chronic losartan on adipose PAI-1 may also involve the loss of feedback inhibition of AG II synthesis and the subsequent interactive effects of AG II and aldosterone on PAI-1 synthesis. In accordance with this view, treating OLETF rats with losartan further increased the expression of adipose AGT. A recent study supports this hypothesis and indicates that a combined therapy with AT_1 and aldosterone receptor antagonists is required to attenuate the effect of diuretic induced activation of the RAS on circulating PAI-1 antigen (40). Another possibility is that AT_1R antagonists with no PPARγ activity such as losartan, candesartan, and eprosartan may have little effect on adipocytes.

In conclusions, the present study shows that in OLETF rats, chronic AT_1R antagonism with losartan increases AGT expression in adipose tissue, but does not influence the size of adipose mass and adipose PAI-I expression. In addition, chronic losartan treatment was shown to decrease PAI-1 expression and aortic wall thickness. Therefore, chronic AT_1R antagonism with losartan may cause differential effects on vascular and adipose tissues.

Acknowledgments

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