Angiotensin receptor blockers improve insulin resistance in type 2 diabetic rats by modulating adipose tissue

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Adipose tissue is recognized as a pivotal organ in the development of insulin resistance. This study seeks to determine the effect of angiotensin receptor blockade (ARB) on insulin resistance of adipocytes in culture and in a rat model of type 2 diabetes. Treatment of Otsuka Long-Evans Tokushima Fatty rats with the ARB L158809 for six months significantly lowered fasting plasma glucose, cholesterol and triglyceride levels but led to higher plasma adiponectin levels. Insulin resistance, measured by an intraperitoneal glucose tolerance test, of the treated rats was significantly improved along with an increase in the number of small differentiated adipocytes; however, epididymal fat mass decreased. Treatment significantly lowered lipid peroxidation and MCP-1 expression while increasing adiponectin production by the adipose tissue. ARB treatment significantly improved insulin sensitivity and markedly suppressed AT2-induced oxidative stress, PAI-1 and MCP-1 levels and NF-κB activation of adipocytes in culture. Treatment increased adiponectin and PPARγ expression along with intracellular triglyceride levels reflecting differentiation of the cultured adipocytes. Our study suggests that ARB treatment improves insulin resistance by modification of adipose tissue thereby blunting the development of diabetes.


KEYWORDS: angiotensin receptor antagonist; diabetes mellitus; adipose tissue; adipocytokine; insulin resistance; nuclear factor-κB

Recent hypertension trials have reported a lower incidence of diabetes mellitus among patients treated with renin-angiotensin system (RAS) inhibitor when compared with other classes of antihypertensive medications.¹⁻⁴ Although these studies were mostly performed with patients with hypertension or congestive heart failure, a 22% relative risk reduction for new-onset diabetes mellitus was demonstrated in a meta-analysis of randomized controlled trials, including 75,677 nondiabetic subjects after a mean follow-up period of 4–5 years.⁵ Furthermore, the activity of RAS is also linked with the metabolic syndrome.⁶ Taken together, these findings suggest that RAS may have an important role in the development of diabetes mellitus.

Adipocytes have recently attracted attention as dynamic endocrine cells that produce and secrete various bioactive molecules that are collectively referred to as adipocytokines, some of which affect the glucose homeostasis and insulin resistance of other tissues.⁷⁻⁹ Adipose tissue has now been targeted as a pivotal organ for the development of insulin resistance, rather than for its energy storage function. Among the many adipocytokines, tumor necrosis factor-α (TNF-α), leptin, plasminogen activator inhibitor-1 (PAI-1), interleukin-6 (IL-6), resistin, visfatin, monocyte chemoattractant peptide-1 (MCP-1), and adiponectin have been implicated as active molecules in the development of insulin resistance.⁷⁻¹¹

Interestingly, mature adipocytes express all components of the renin-angiotensin system, including angiotensinogen, angiotensin-converting enzyme, as well as angiotensin type 1 (AT1) and angiotensin type 2 (AT2) receptors.¹² For these reasons, it can be hypothesized that the antidiabetic effect of RAS inhibition may derive from the effect of these drugs on adipocyte function, including the modulation of adipocytokines.

The primary objective of this study was to investigate the effect of an AT1 receptor antagonist, L158809, on insulin resistance under the hypothesis that angiotensin receptor blockade (ARB) may improve insulin resistance through the modulation of adipose tissues in Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Additionally, to define the direct molecular mechanism of ARB, the effects of angiotensin
RESULTS

Biochemical parameters in experimental animals

Table 1 compares various biochemical parameters among three experimental groups of rats. The fasting plasma glucose level was significantly higher in OLETF rats compared with that in Long-Evans Tokushima Fatty (LETO) rats. Treatment of OLETF rats with L158809 for 6 months induced a significantly decreased fasting plasma glucose level. Body weight and kidney/body weight were also higher in OLETF rats than in LETO rats. L158809 treatment did not induce a significant change in body weight or kidney/body weight. Plasma insulin and c-peptide levels were significantly higher in OLETF rats than in LETO rats, although the L158809-treated group did not show a significant difference compared with the OLETF rats. Although serum creatinine levels and urine volumes were not different among the three groups, urinary albumin excretion in the OLETF rats was significantly higher than in the LETO rats. L158809 treatment significantly reduced urinary albumin excretion. Systolic blood pressure was greater in OLETF rats than in LETO rats, and L158809 treatment induced a significant decrease in systolic blood pressure.

Effect of L158809 on insulin resistance and metabolic parameters in experimental animals

As shown in Figure 1, improved glucose intolerance after treatment with L158809 in OLETF rats was confirmed by an i.p. glucose tolerance test (GTT). Although the basal levels of fasting blood glucose were slightly lower in the L158809-treated group, GTT clearly demonstrated improved glucose intolerance in L158809-treated rats vs OLETF rats. In accordance with improved glucose intolerance, L158809 treatment significantly decreased plasma total cholesterol and triglyceride levels (cholesterol, LETO; 113 ± 7.9, OLETF; 147 ± 23, OLETF + L158809; 100 ± 14, triglyceride, LETO; 39 ± 11, OLETF; 167 ± 19, OLETF + L158809; 69 ± 21, P < 0.05) (Figure 2a). In addition, plasma adiponectin levels were decreased in OLETF rats compared with control LETO rats, and L158809 treatment induced a significant increase in plasma adiponectin levels (LETO; 11 ± 1.2, OLETF; 8.1 ± 0.2, OLETF + L158809; 9.7 ± 0.36, P < 0.05) (Figure 2b). However, the homeostasis model assessment indices (HOMA-IRs) did not show a significant difference among the three groups (LETO; 0.65 ± 0.12, OLETF; 0.77 ± 0.11, OLETF + L158809; 0.69 ± 0.21) (Figure 2c).

Effect of L158809 on histological changes of kidney and adipose tissue in experimental animals

Figure 3 shows the representative renal pathology in the experimental groups at the end of the study period. Diabetic OLETF rats showed more severe glomerulosclerosis when compared with the LETO rats. Consistent with the marked attenuation of albuminuria, the glomerulosclerotic index was significantly improved in the L158809-treatment group. Next, this study assessed whether the differences in body weight were related to the alteration in adiposity and to the L158809-induced phenotypic changes in adipose tissue. As shown in Figure 4, adipose tissue obtained from epididymal fat revealed that OLETF rats had larger adipocytes than LETO rats, and L158809 treatment restored their phenotype to small differentiated adipocytes (Figure 4a–c). In addition to these phenotypic changes, epididymal fat mass in OLETF rats was also significantly higher than in LETO rats, and the L158809-treated group showed a significant decrease over OLETF rats (LETO; 0.54 ± 0.05, OLETF; 1.10 ± 0.15, OLETF + L158809; 0.71 ± 0.10, P < 0.05) (Figure 4d).

Effect of L158809 on renal lipid metabolism in experimental animals

As L158809 improves albuminuria and glomerulosclerosis associated with improvement in systemic metabolic abnormalities,
we next examined whether improvement of renal function was derived from correction of alteration of renal lipid metabolism. We measured the mRNA expression levels of the molecules that are involved in lipid synthesis and cholesterol efflux. As shown in Figure 5, mRNA expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, sterol-regulatory element-binding protein-1c, and acetyl-coenzyme A carboxylase were significantly increased in diabetic OLETF rats compared with LETO rats, however ATP-binding cassette transporter-1 that modulates cholesterol efflux was decreased in OLETF rats. Interestingly, AT1 receptor antagonist significantly decreased the gene expression of lipid synthesis and increased the gene expression that modulates cholesterol efflux. In accordance with these changes, renal cholesterol and triglyceride contents were significantly decreased in L158809-treated groups (Table 1). Furthermore, urinary excretion of 8-isoprostane, which is an oxidative stress marker, was significantly decreased in AT1 receptor antagonist treatment group (Table 1).

**Effect of L158809 on lipid peroxidation and adipocytokine gene expression from adipose tissue in experimental animals**

The insulin resistance state and phenotypic change of adipocytes in OLETF rats was associated with a 3.5-fold increase in MCP-1 mRNA expression compared with LETO rats. L158809 treatment significantly decreased MCP-1 gene expression (LETO; 1.00 ± 0.24, OLETF; 3.45 ± 0.45, OLETF + L158809; 2.21 ± 0.21, P < 0.05) (Figure 6a). In addition, adiponectin gene expression was also significantly upregulated by
L158809 treatment in the adipose tissues (Figure 6b), which is in agreement with significantly increased plasma levels of adiponectin (LETO; 1.00 ± 0.20, OLETF; 0.55 ± 0.14, OLETF + L158809; 1.20 ± 0.21, *P < 0.05) (Figure 2b). Furthermore, lipid peroxidation of adipose tissue, which is an oxidative stress marker, was significantly increased in diabetic OLETF rats compared with LETO rats, and L158809 treatment showed significantly decreased levels of lipid peroxidation (Table 1).

**Effect of AT2 and angiotensin receptor antagonist on adipocyte differentiation**

Before the in vitro experiment, the duration of incubation time was determined with stimulation by the addition of a hormonal cocktail to reach the maximal differentiation state. The degree of differentiation was evaluated by oil red O staining and measurement of intracytoplasmic triglyceride concentration. Full maturation of adipocytes was obtained after 15 days of incubation. As a result, all in vitro experiments were performed using adipocytes that had undergone 15 days of differentiation. High glucose stimuli induced a slightly decreased adipocyte differentiation, whereas AT2 resulted in a dramatic inhibition of adipocyte differentiation despite concomitant stimulation with the hormonal cocktail (Figure 7a–f). In contrast with stimulation with AT2, AT1 receptor blockade and AT2 receptor blockade restored the adipocytes to the differentiated form containing increased cytoplasmic lipid accumulation (Figure 7a–f). As expected, pioglitazone treatment significantly increased the differentiated adipocytes. In accordance with the lipid accumulation,
intracytoplasmic triglyceride levels were significantly decreased after AT2 stimulation, whereas AT1 and AT2 receptor blockades restored their level to control levels (control, 100 ± 10; high glucose, 92.3 ± 12.3; AT2, 80.8 ± 9.2; AT2 + AT1 blocker, 93.2 ± 10.2; AT2 + AT2 blocker, 97.4 ± 8.2; pioglitazone, 110.5 ± 14.3) (Figure 7g).

Effect of AT2 on 2-DOG uptake in cultured adipocytes
To evaluate the direct effect of AT2 on insulin sensitivity, glucose uptake occurring in adipocytes was determined using a 2-deoxy-D-glucose (2-DOG) uptake assay. First, the time course of 2-DOG uptake in adipocytes treated with 100 nM of AT2 was examined. As shown in Figure 8a, AT2 treatment prevented insulin-induced 2-DOG uptake in adipocytes by approximately twofold, with a maximal inhibition at 20 min. Interestingly, previous treatment with L158809 significantly restored insulin-induced glucose uptake. Furthermore, the inhibitory effect of 2-DOG uptake by AT2 showed a dose-dependent fashion from 1 nM and reached a maximal inhibition at 100 nM (Figure 8b).

Figure 7 | Effects of high glucose, AT2, and AT receptor antagonist on adipocyte differentiation in cultured adipocytes.
Oil red O stain: (a) control; (b) high glucose medium; (c) AT2; (d) AT2 with AT1 receptor antagonist; (e) AT2 with AT2 receptor antagonist; (f) pioglitazone. (g) The relative lipid contents in each condition. Differentiated adipocytes were treated for 24 h with 30 μM of glucose, 100 nM of AT2, 10 μM of L158809 (AT1R blocker), 10 μM of PD-1233319 (AT2R blocker). As a positive control, pioglitazone and TNF-α were administered at final concentrations of 10 μM and 10 ng/ml, respectively, to the culture media. The level of lipid accumulation in each condition was corrected by that in the control cells without stimulation. Data are shown as the mean ± s.e.m. AT1B, AT1 receptor blockade; AT2B, AT2 receptor blockade; *P < 0.05; ***P < 0.001 vs controls; ##P < 0.01, ###P < 0.001 vs AT2.

Figure 8 | Effect of AT2 and AT1 antagonist on 2-DOG uptake in adipocytes. (a) 2-DOG uptake in differentiated adipocytes was measured after stimulation with 100 nM of insulin in the presence or absence of 100 nM of AT2 at the indicated time intervals. In some wells, 10 μM of L158809 was pretreated for 30 min before AT2 treatment. The radioactivity was normalized for total protein concentration in each condition and 2-DOG uptake was expressed as % change over insulin-treated controls. (b) Dose-dependent effect of AT2 on 2-DOG uptake. As peak inhibition of glucose uptake occurred at 20 min, different concentrations of AT2 at final concentrations of 1, 10, and 100 nM were treated for 20 min, and glucose uptake was measured. Data are shown mean ± s.e.m.; AT1RB, AT1 receptor blockade; *P < 0.05 vs control; **P < 0.01 vs control; #P < 0.05 vs AT2.
insulin sensitivity, was decreased after AT2 stimulation. Although AT2 receptor blockade had no effect on adiponectin expression, AT1 receptor blockade significantly increased its expression (Figure 9b). Interestingly, MCP-1 expression was increased under high glucose stimulation compared with the controls. AT2 stimulation resulted in a more increase in MCP-1 expression, whereas AT1 and AT2 receptor blockades significantly decreased AT2-induced MCP-1 overexpression (Figure 9c). PAI-1 expression also showed a similar change to that of MCP-1, becoming increased after AT2 stimulation and completely suppressed with its receptor blockade (Figure 9d).

Effect of AT2 and angiotensin receptor antagonist on the transcriptional activity of NF-κB, 8-isoprostane, and MCP-1 protein secretion in cultured adipocytes

As high glucose and AT2 stimulation upregulate MCP-1 gene expression, we next evaluated whether MCP-1 protein secretion is also increased with high glucose and AT2 stimulation and whether MCP-1 production is accompanied with activation of the nuclear factor-κB (NF-κB) pathway. Similar to gene expression, MCP-1 protein secretion was significantly increased after high glucose and AT2 treatment. Again, AT1 and AT2 receptor antagonists significantly inhibited AT2-induced MCP-1 secretion (Figure 10a). Interestingly, endogenous NF-κB transcriptional activity in adipocytes was also increased to 25% after high glucose stimulation and 28% after AT2 treatment, and the AT1 receptor antagonist significantly suppressed AT2-induced NF-κB transcriptional activity (Figure 10b). To further confirm the role of NF-κB activation by AT2 stimulation, p65 western blotting was performed using extracted nuclear proteins. As shown in Figure 10c, AT2 treatment increased nuclear p65 protein expression, and the AT receptor antagonist abolished its expression. In addition, to further evaluate whether AT2-induced adipocytokine synthesis is associated with oxidative stress, we next examined whether AT2 increased oxidative stress in cultured adipocytes. As shown in Figure 10d, both high glucose and AT2 significantly increased 8-isoprostane production, and L158809 significantly suppressed its production.

DISCUSSION

This study first demonstrated that the AT receptor antagonist improves insulin resistance through the modulation of adipose tissue, including differentiation of adipocytes,
leading to an improvement in adipocytokine synthesis in type 2 diabetic rats. More importantly, evidence was found to show that AT2 upregulates the proinflammatory molecules in adipose tissue such as PAI-1, MCP-1, and NF-κB activation, which was prevented by the AT receptor antagonist.

There is increasing evidence indicating that insulin resistance is associated with systemic inflammation.13–16 Increased levels of inflammatory markers such as C-reactive protein, IL-6, and PAI-1 relate to the incidence of type 2 diabetes, and multifactorial intervention in diabetic patients reduces inflammatory markers.17 As one of the important sources of systemic inflammation, adipose tissue has emerged as an important player in this process, as adipocytes can secrete a large number of cytokines and chemokines such as TNF-α, IL-6, MCP-1, and adipocytokines, including leptin, adiponectin, and resistin, and all of these molecules can exert negative or positive effects on insulin resistance.18–20

In this study, it was found that plasma levels of adiponectin and insulin were significantly decreased and increased, respectively, in type 2 diabetic rats, and that these changes were accompanied by lipid abnormalities. The glucose intolerance state in diabetic rats was clearly demonstrated by the glucose tolerance test; glucose levels after an i.p. glucose injection showed markedly elevated levels after two hours, compared with control rats. It is of interest that the epididymal fat mass was significantly increased and that the adipocytes in diabetic rats were larger, which are more insulin-resistant phenotypes.21,22 In line with this phenotypic change, MCP-1 gene expression was significantly increased, and adiponectin expression was decreased in diabetic rats. In this study, L158809 treatment significantly decreased blood pressure, and it may be possible that blood pressure-lowering effect may affect the synthesis of MCP-1 and adiponectin. However, our in vitro data clearly showed that L158809 directly affects the synthesis of these cytokines rather than the blood pressure-lowering effect.

In this study, L158809 treatment improved glycemic control, increased plasma adiponectin levels, and resulted in much better lipid profiles, including decreases in cholesterol and triglyceride levels. However, there were no significant changes in plasma insulin levels and HOMA-IR in these overtly diabetic insulin-resistant rats. In this study, we
also evaluated the effect of AT2 receptor antagonist in OLETF rats using PD123319, but we could not find any significant effect in lipid profiles and metabolic parameters (data not shown).

Another important finding in this study was that phenotypic changes were observed in adipocytes after treatment with L158809. Epididymal fat mass was significantly decreased and L158809 treatment increased the number of small, newly differentiated adipocytes, which are known as a more insulin-sensitive phenotype than older, large adipocytes. Further, it was noted that these phenotypic changes were accompanied by an improvement in adipocytokine expression, such as downregulation of MCP-1 expression and upregulation of adiponectin expression. Although macrophage infiltration was not observed in adipose tissue, increased MCP-1 expression in adipose tissue has recently been suggested as an important mediator in the development of insulin resistance. On the other hand, adiponectin has been considered as an anti-inflammatory molecule, as adiponectin reduces the production of IL-6, inhibits NF-κB activation, and inhibits the production of endothelial adhesion molecules.

This study further investigated the effect of the AT1 blockade on adipocyte function in cultured adipocytes to define the molecular mechanism and direct the effects of RAS inhibition in adipocytes. Interestingly, AT2 stimulation inhibited the differentiation of adipocytes as measured by oil red O staining and intracytoplasmic lipid concentration measurements. In addition, the AT receptor antagonist augments lipid accumulation and the differentiation of adipocytes. Furthermore, adipocytokines such as peroxisome proliferator-activated receptor-γ and adiponectin, known to increase insulin sensitivity, were significantly downregulated by AT2, and the ARB inhibited these effects. On the contrary, diabetogenic adipocytokines including MCP-1 and PAI-1 were induced by AT2 treatment, and again the AT receptor antagonist also completely inhibited AT2-induced MCP-1 and PAI-1 expression. More importantly, our in vitro 2-TDG uptake results clearly showed that AT2 significantly inhibited insulin sensitivity and AT1 receptor antagonist restored insulin sensitivity. Interestingly, high glucose stimuli also directly upregulated MCP-1 and PAI-1 expression, although there was no effect on antidiabetogenic adipocytokines. These findings are in agreement with a previous report that high glucose and advanced glycosylation end products enhance the expression of PAI-1 in cultured adipocytes. As expected, the most dramatic effect on MCP-1 and PAI-1 expression was mediated by TNF-α stimulation, which has been known as an inflammatory cytokine that mediates insulin resistance.

Recently, several studies have suggested potential mechanisms for the activation of inflammation in adipose tissue. Dietary fat and obesity increase the lipid content of adipocytes, initiating a state of cellular stress, which is accompanied by the activation of c-Jun N-terminal kinase and NF-κB, leading to increased production of proinflammatory cytokines such as TNF-α, IL-6, MCP-1, and PAI-1. In this study, we could not observe any significant activation of c-Jun N-terminal kinase (data not shown), and next we performed another in vitro experiment to further confirm the role of AT2 and AT receptor antagonist in the development of insulin resistance by observing the direct effect of AT2 on NF-κB activation in adipocytes. Interestingly, the NF-κB reporter assays clearly showed that high glucose and AT2 activated endogenous NF-κB transcriptional activity in these cells. The AT1 receptor antagonist significantly suppressed AT2-induced NF-κB activation. In support of the role of AT2 in the activation of NF-κB, Tsuchiya et al. and Skurk et al. recently reported that AT2 induces MCP-1 and IL-6 production by way of an NF-κB-dependent pathway in cultured adipocytes. Taken together, the in vitro findings in this study suggest that the AT1 receptor antagonist contributes to the observed in vivo effects in insulin-resistant diabetic rats by means of modulation of adipose tissue.

In this study, we also found that decreased albuminuria and improvement in glomerulosclerosis was accompanied by improvement in renal lipid metabolism by AT1 receptor antagonist. In diabetic OLETF rats, mRNA expression levels of elements involved in lipid synthesis such as 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and sterol-regulatory element binding protein-1c were significantly increased, whereas ATP binding cassette transporter 1 that modulates cholesterol efflux was decreased. Interestingly, AT1 receptor antagonist induced improvement in alterations of renal lipid metabolism and subsequently reduced renal cholesterol and triglyceride contents. These results suggest that AT1 receptor antagonist improves renal function via improvement in renal lipid metabolic abnormalities.

The limitation in this study is that we used type 2 diabetic rat model, which is already in a diabetic state associated with insulin resistance instead of classical animal model of insulin resistance such as high fat diet-induced model. More in vivo studies using classical model of insulin resistance are necessary to further define the protective role of AT receptor antagonist in the development of diabetes.

In conclusion, this study suggests that ARB treatment improves the differentiation of adipocytes and the activation of the inflammatory process in adipose tissue, accompanied by an improvement in adipocytokine synthesis. The study data suggest that ARB treatment improves insulin resistance through the modulation of adipose tissues, leading to a protective effect for the prevention of diabetes mellitus.

**MATERIALS AND METHODS**

**Animal studies**

Otsuka Long-Evans Tokushima Fatty rats supplied by the Tokushima Research Institute (Otsuka Pharmaceutical Co., Tokyo, Japan) were used as type 2 diabetic models. Age-matched male LETO rats served as the genetic control for the OLETF rats. The rats at 20 weeks of age were divided into three groups (n = 6 per group). Group 1 consisted of LETO control rats. Group 2 animals were OLETF type 2 diabetes rats, and group 3 consisted of OLETF rats...
treated with 1.5 mg/kg of L158809 (Merck & Co. Inc, Rahway, NJ, USA) mixed in drinking water for 6 months. Rats had free access to rat food and tap water and were caged individually under a controlled temperature (23 ± 2°C) and humidity (55 ± 5%) environment with an artificial light cycle. Daily amounts of water intake were checked at regular intervals to affirm the dose of the administered drug. At the end of the study period, systolic blood pressure was measured using tail-cuff plethysmography (LE 5001-Pressure Meter, Letica SA, Barcelona, Spain). Plasma glucose levels were measured by a glucose-oxidase based method, and creatinine levels were determined by a modified Jaffe method. Plasma insulin levels and plasma adiponectin levels were measured using an enzyme-linked immunosorbent assay kit (Linco Research, St Charles, MO, USA). Plasma c-peptide levels were measured using an enzyme-linked immunosorbent assay kit (Shibayagi, Shibukawa, Japan). HOMA-IR was calculated using fasting glucose (mmol/l) × fasting insulin (mU/l)/22.5. Plasma triglyceride and cholesterol analyses were performed using a GPO-TRinder kit (Sigma, St Louis, MO, USA). A GTT was performed to assess the insulin resistance state in each group. GTT was performed following 8-hour fasting, and blood samples were collected through the tail vein. Rats received 2 g dextrose per kg body weight by i.p. injection for GTT, and blood sampling was done to measure blood glucose levels at 0, 30, 60, 90, and 120 min after glucose loading. To determine the urinary albumin excretion, individual rats were caged in a metabolic cage and 24-h urine was collected for the entire study. The urinary albumin concentration was determined by a competitive enzyme-linked immunosorbent assay (Shibayagi, Shibukawa, Japan) and corrected by urinary creatinine concentration. Rats were killed under anesthesia by i.p. injection of sodium pentobarbital (50 mg/kg). Experiments were conducted in accordance with the Korea University Guide for Laboratory Animals.

**Analysis of gene expression by real-time quantitative PCR in tissues and cells**

Total RNA was extracted from epididymal fat tissues, renal cortical tissues, or experimental cells with Trizol reagent and further purified using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Primers were designed from the respective gene sequences using Primer 3 software, and the secondary structures of templates were examined and excluded using the mfold software program. Table 2 shows the nucleotide sequences of the primers. Quantitative gene expression was performed on a LightCycler 1.5 system (Roche Diagnostics Corporation, Indianapolis, IN, USA) using SYBR Green technology. Real-time reverse transcription-PCR was performed for 10 min at 50°C and 5 min at 95°C. Subsequently, 45 cycles were applied, consisting of denaturation for 10 s at 95°C and annealing with extension for 30 s at 60°C. At the end of the PCR cycle, samples were heated to 95°C to check that a single PCR product was obtained. The ratio of each gene and β-actin level (relative gene expression number) was calculated by subtracting the threshold cycle number (Ct) of the target gene from that of β-actin and raising 2 to the power of this difference.

**Histological examination of adipose tissue and kidney**

The adipose tissue obtained from epididymal fat was fixed for 48 h with 10% paraformaldehyde at 4°C, dehydrated, embedded in paraffin, and cut into 4-μm-thick slices, and stained with hematoxylin and eosin and observed under a microscope to compare adipocyte sizes. The kidney tissues embedded in paraffin were cut into 4-μm-thick slices and were stained with Periodic Acid-Schiff. A semiquantitative score (SI) was used to evaluate the degree of glomerulosclerosis on Periodic Acid-Schiff-stained sections according to the method described by Ma et al. The severity of sclerosis for each glomerulus was graded from 0 to 4+ as follows: 0, no lesion; 1+, sclerosis of <25% of the glomerulus; 2+, 3+, and 4+, sclerosis of 25–50, >50–75, and >75% of the glomerulus, respectively. All histological examinations were carried out by a pathologist in a blinded manner, and more than 80 glomeruli were analyzed in kidney sections from each rat.

**Lipid extraction and measurement of lipids from renal tissues**

Lipids from renal cortical tissues were extracted by the method of Bligh and Dyer. Total cholesterol and triglyceride content was measured using commercial kit (Wako Chemicals, Richmond, VA, USA) according to the manufacturer’s instructions.

**Measurement of 8-isoprostane from urine and cultured adipocytes**

8-Isoprostane in either 24 h urine or culture supernatant from adipocytes was measured using EIA kit (Cayman Chemical, Ann Arbor, MI, USA). For measurement of 8-isoprostane from culture supernatant, differentiated adipocytes were serum-starved for 24 h, then treated with 30 mM of high glucose. To study the effect of AT2, adipocytes were treated with or without AT2 at a final concentration.
of 100 nm. In some wells, L158809 or PD-1233319 was pretreated 30 min before stimulation with AT2. All experimental groups were cultured in triplicate and harvested at 24 h, and conditioned media were collected at the end of the treatment period.

Measurement of lipid peroxidation from adipose tissue in experimental animals
The extent of the peroxidative reaction in the adipose tissue was determined by measuring lipid hydroperoxides directly utilizing the redox reactions with ferrous ions from adipose tissue homogenates using lipid hydroperoxide (LPO) assay kit (Cayman Chemical, Ann Arbor, MI, USA).

Culture and differentiation of adipocytes
3T3-L1 cells were obtained from American Type Cell Culture (ATCC, Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 U/ml antibiotics at 37 °C under a humidified 5% CO2 atmosphere. Cultures were fed every 2–3 days during growth and every 2 days after confluence. Differentiation of pre-adipocytes into adipocytes was induced by the addition of a hormone cocktail as described previously. In brief, 2 days after confluence, the medium was removed and replaced with standard adipocyte differentiation induction medium containing 0.5 mmol/l isobutylmethylxanthine, 1 µmol/l dexamethasone, and 10 µg/ml insulin. Three days later, the induction medium was replaced by Dulbecco's modified Eagle's medium with 10% fetal bovine serum plus insulin only, and cells were then fed every 2–3 days. Differentiation of adipocytes was confirmed morphologically by multiple oil red O-stained lipid droplets in the cytoplasm and quantitation of intracytoplasmic lipids (see below). As full maturation of adipocytes occurs 15 days after differentiation, all in vitro experiments were performed using cells 15 days after differentiation. To evaluate the effects of AT2, ATI and AT2 receptor (AT1R and AT2R, respectively) blockers, and high glucose conditions on adipocyte function, cells were treated for 24 h with AT2 at a final concentration of 100 nM, 10 µM of L158809 (AT1R blocker), 10 µM of PD-1233319 (AT2R blocker), and 30 mM of glucose. As a positive control, pioglitazone (Takeda Pharmaceuticals Co., Osaka, Japan) and TNF-α were administered at final concentrations of 10 µM and 10 ng/ml, respectively, to the culture media.

Quantitation of intracytoplasmic triglyceride levels
After being induced for 15 days, adipocytes were treated under different conditions for 48 h and then the cells were monitored by measurement of intracellular lipid accumulation using Oil red O staining as described previously. The cells were fixed with 3% glutaraldehyde for 1.5 h, and then the fixation solution was replaced by 500 µl of 60% isopropl alcohol. After 5 min, isopropl alcohol was evaporated, and the adipocytes were stained in a working solution of 300 µl oil Red O for 2 h. The stain solution was removed, and the cells were rinsed in 500 µl of 60% isopropl alcohol for 5 s. To extract the dye, 700 µl of 60% isopropl alcohol was added per well and then sealed plates were shaken for 2 h. The extracted dye was then quantitated spectrophotometrically at 510 nm. The level of lipid accumulation in each stimuli condition was corrected by that found in the control cells without stimulation. All experimental groups were cultured in triplicate and harvested at 24 h for the extraction of total RNA and protein. For the measurement of secreted MCP-1 in the cultured medium, conditioned media were collected at the end of the treatment period and measured by a quantitative sandwich enzyme-linked immunosorbent assay using a commercial kit (Biosource Inc., Camarillo, CA, USA).

Measurement of glucose uptake in cultured adipocytes
We next performed 2-DOG uptake assay to define the direct effect of AT1 receptor antagonist on insulin sensitivity in adipocytes. Differentiated adipocytes were starved in serum-free medium for 24 h and then washed three times with PBS buffer. Cells were incubated in 1 ml of Krebs-Ringer phosphate HEPES (KRPH) buffer (136 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl2, 2.5 mM Na2HPO4, 10 mM HEPES and 0.1% (w/v) bovine serum albumin, pH 7.4) for 30 min at 37 °C. The cells were then stimulated with 100 nM of insulin with or without 100 nM of AT2 and incubated for 15 min at 37 °C. Cells were next incubated with 1 µCi of 2-deoxy-[1-3H]-D-glucose and incubated for the indicated times from 10 to 30 min. To exclude noncarrier-mediated glucose uptake, 10 µM of cytochalasin B was pretreated for 30 min in control wells. In some wells, L158809 was administered 30 min before treatment with AT2. At the indicated time, the media was collected, cells washed three times with PBS buffer and lysed in 0.1 n NaOH for 1 h at room temperature. Total radioactivity from media (Rm) and total cell-associated radioactivity from lysates (Rc) were counted using a liquid scintillation counter. True glucose uptake was calculated as follows: radioactivity of glucose uptake = [Rc/Rm + R(c)] sample - [Rc/Rm + R(c)] cytochalasin B. The radioactivity was normalized for total protein quantified by the Bradford method. Next, the dose dependency of AT2 in glucose uptake was determined. As peak inhibition of glucose uptake occurred at 20 min, different concentrations of AT2 at final concentrations of 1, 10, and 100 nM were treated for 20 min, and glucose uptake was measured as described previously.

Transient transfection and luciferase reporter activity assay
To test the effect of high glucose stimuli, AT2, and their antagonist on NF-kB transcriptional activity in adipocytes, a luciferase reporter assay was performed. An NF-kB reporter plasmid containing three NFkB consensus sequences was provided by Dr Timothy Blackwell. Cells were plated onto 24-well plates at a density of 1 × 105 cells/well. Following 24 h of growth, the cells were transfected with 1 µg of NF-kB reporter plasmid and 1 µg of plasmid containing Renilla luciferase driven by a TK promoter for 24 h using Superfect (Qiagen, Valencia, CA, USA). After that, cells were treated for 24 h with 100 nM of AT2, 10 µM of L158809, 10 µM of PD-1233319, and 30 mM of glucose. In some wells, pioglitazone and TNF-α were administered at a final concentration of 10 µM and 10 ng/ml, respectively. After 24 h, luciferase activity was determined using the dual luciferase assay system according to the manufacturer’s instructions (Promega Corp., Madison, WI, USA). To control for differences in transfection efficiency, a plasmid containing Renilla luciferase was included in each transfection and used for normalization.

Extraction of nuclear proteins and western blotting
Nuclear proteins from cells were extracted using a commercial nuclear extraction kit according to the manufacturer’s instructions (Active Motif, Carlsbad, CA, USA). The protein concentration was determined using the bicinchoninic acid method (Pierce Chemicals, Rockford, IL, USA). Twenty micrograms of protein were electrophoresed on a 10% SDS-polyacrylamide gel electrophoresis mini-gel under denaturing conditions. The proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA) over 150 min at 250 mA. After incubating
the membranes with blocking solution for 1 h at room temperature, the membranes were hybridized with rabbit polyclonal anti-p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and diluted 1:1000 in blocking buffer overnight at 4 °C. The filter was then incubated with horseradish peroxidase-conjugated secondary antibody, diluted 1:1000, for 60 min at room temperature. The detection of specific signals was performed using the enhanced chemiluminescence method (Amersham, Buckinghamshire, UK). Equal amounts of protein loading were confirmed by β-actin western staining of the gel.

Statistical analysis
Nonparametric analysis was used due to the relatively small number of samples present (animal experiment was six in each group and in vitro experiment was three in each condition). Results were expressed as the mean ± s.e.m. A Kruskall–Wallis test was used for comparison of more than two groups, followed by a Mann–Whitney U-test for comparison using a microcomputer-assisted program with SPSS for Windows 10.0 (Spss Inc., Chicago, IL, USA).

Supplementary Material
Supplementary material is available.

References