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An Adipose Tissue-Independent Insulin-Sensitizing Action of Telmisartan: a Study in Lipodystrophic Mice

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ABSTRACT

Adipose tissue plays an important role in energy balance and metabolism and is the major target for insulin-sensitizing peroxisome proliferator-activated receptor (PPAR) γ agonists. The angiotensin II type 1 receptor blocker telmisartan, a partial agonist of PPAR-γ, has been demonstrated to improve insulin sensitivity. However, there is uncertainty about the sites of its action. Here, we demonstrate that treatment with telmisartan (3 mg/kg p.o.) for 7 weeks decreased plasma glucose levels in oral glucose and insulin tolerance tests and the index of the homeostasis model assessment of insulin resistance in A-ZIP/F-1 transgenic mice, an animal model of lipodystrophy. These effects were accompanied by decreases in circulating triglyceride and fatty acid levels. However, this treatment did not affect body weight and plasma adiponectin, leptin, and corticosterone levels. In A-ZIP/F-1 mouse liver the transcripts encoding PPAR-γ and its downstream lipogenic genes were highly up-regulated, consistent with increased hepatic triglyceride content and lipid droplet accumulation. Telmisartan reversed these effects and also down-regulated mRNAs encoding gluconeogenic genes. Thus, the present findings are consistent with a novel mode of insulin-sensitizing action of telmisartan, involving an adipose tissue-independent pathway. Telmisartan-elicited down-regulation of hepatic expression of PPAR-γ-regulated lipogenic genes is associated with amelioration of fatty liver.

Insulin resistance is a key component of the metabolic syndrome and both precedes and predicts the development of type 2 diabetes. Moreover, even in the absence of diabetes, insulin resistance increases the risk of nonalcoholic steatohepatitis and cardiovascular disease (Prasad and Quyyumi, 2004).

Telmisartan is a well established angiotensin II type 1 receptor (AT1) blocker. It has been demonstrated that telmisartan improves insulin sensitivity in rodents that have received high-fat–containing diets (Benson et al., 2004; Araki et al., 2006; Sugimoto et al., 2006) and in diabetic and nondiabetic patients (Pershadsingh and Kurtz, 2004; Honjo et al., 2005; Miura et al., 2005). However, uncertainty exists about the sites of its action.

As the primary site for energy storage, adipose tissue plays an important role in energy balance and metabolism and also produces bioactive substances that regulate insulin sensitivity (Engeli et al., 2003). For example, adipocyte-derived leptin regulates food intake and energy expenditure and, in conjunction with the adipokine adiponectin, modulates insulin sensitivity. The peroxisome proliferator-activated receptor (PPAR) γ is a member of the ligand-activated nuclear receptor superfamily and is expressed at high levels in adipose tissue (Evans et al., 2004). PPAR-γ regulates genes that modulate lipid utilization and storage, and lipoprotein metabolism and adipocyte differentiation and insulin action (Evans et al., 2004). Thus, PPAR-γ is the master regulator of adipogenesis and is activated by the thiazolidinediones that are used clinically to stimulate the action of insulin in adipose tissue (Evans et al., 2004). In addition to its actions at AT1, telmisartan has been shown recently to facilitate the differentiation of 3T3-L1 preadipocytes and to activate PPAR-γ-responsive genes in adipocytes in vitro (Benson et al., 2004; Fujimoto et al., 2004). In light of these findings, we speculated that the antidiabetic effects of telmisartan might depend on PPAR-γ in adipose tissues.

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ABBREVIATIONS: AT1, angiotensin II type 1 receptor; ACC, acetyl-CoA carboxylase; AUC, area under curve; FAS, fatty acid synthase; G6P, glucose-6-phosphatase; HOMA-IR, the homeostasis model assessment of insulin resistance; ITT, insulin tolerance test; NEFA, nonesterified fatty acids; OGTT, oral glucose tolerance test; PEPCK, phosphoenolpyruvate carboxykinase; PPAR, peroxisome proliferator-activated receptor; SBP, systolic blood pressure; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element-binding protein; WT, wild type; PCR, polymerase chain reaction; ANOVA, analysis of variance.

The A-ZIP/F-1 transgenic mouse is an animal model of lipodystrophy in which a dominant-negative protein that impairs gene activation by leucine zipper transcription factors results in the absence of adipose tissue (Moitra et al., 1998). The mice eat, drink, and urinate copiously, grow heavier than their littermates, and exhibit decreased fecundity (Moitra et al., 1998). A-ZIP/F-1 mice are profoundly insulin-resistant, hyperlipidemic, hepatosteatotic, and hypertensive (Chao et al., 2000; Ebihara et al., 2001; Gavrilova et al., 2003; Takemori et al., 2007). In the present study, we tested the role of adipose tissue in the insulin-sensitizing effect of telmisartan in A-ZIP/F-1 mice.

Materials and Methods

Animals and Treatments. The principles of laboratory animal care were followed in the present study. All procedures were conducted in accordance with institutional guidelines and were approved by the Animal Ethics Committee, Kyoto University, Japan. Mice were housed in a temperature-controlled facility (21 ± 1°C; 55 ± 5% relative humidity) with a 12-h light/dark cycle.

The generation and characterization of the "fatless" A-ZIP/F-1 mice have been reported previously (Ebihara et al., 2001). A-ZIP/F-1 mice were on the FVB/N background, produced by breeding of A-ZIP/F-1 males and wild-type (WT) females. Female A-ZIP/F-1 mice and their WT littermates were used in experiments because females are sterile, possibly as a consequence of leptin deficiency, whereas the males were used for breeding purposes. The animals received a standard diet (CLEA, Tokyo, Japan) and water ad libitum. At 16 to 18 weeks of age, systolic blood pressure (SBP) and body weight were measured. For determination of nonfasting plasma levels of glucose, triglyceride, and nonesterified fatty acids (NEFA) by enzymatic methods, five small droplets of blood were collected carefully by orbital puncture under light ether anesthesia. The A-ZIP/F-1 mice were selected and grouped (n = 7 each group) for experiments based on these parameters; these parameters did not differ between animals in the vehicle- and telmisartan-treated groups. Two experiments were performed. 1) Higher-dosage experiment: telmisartan (3 mg/kg, a generous gift from Boehringer Ingelheim Co., Ltd, Tokyo, Japan, suspended in 5% gum arabic) was administered by oral gavage, once daily (11:00–12:00 AM) for 7 weeks, whereas controls received 5% gum arabic alone. Mice were weighed every 3 to 4 days, and food intake was estimated weekly. SBP was measured at week 2. Eight small droplets of nonfasted and/or fasted (12 h) blood were collected for determination of plasma levels of adiponectin, leptin, corticosterone, glucose, and lipids at week 4. Oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (ITT) were performed at weeks 5 and 6, respectively. Liver was dissected and weighed after animals were killed, and a segment of liver was immediately frozen in liquid nitrogen and stored at −80°C for subsequent determination of triglyceride and gene expression. 2) Lower dosage experiment: the protocol was the same as that of the higher-dosage experiment except for replacement of 3 mg/kg with 1 mg/kg telmisartan.

SBP Measurement. SBP was measured in conscious mice by a tail-cuff method 2 to 5 h after administration of drug or vehicle (MK-2000ST; Muromachi Kikai Co Ltd, Tokyo, Japan). At least six readings were taken for each measurement.

OGTT and ITT. Mice were fasted for 12 h with free access to water. In the OGTT, mice received a glucose solution (2 g/kg in 10 ml) by the oral route and in the ITT, mice received aqueous insulin (0.75 IU/kg in 10 ml i.p.). Two to three small droplets of blood were collected at each time point for determination of plasma glucose levels with use of a commercial kit (Wako, Osaka, Japan) before and 20, 60, and 120 min after administration of glucose or insulin (Humulin *R*-Insulin, Eli Lilly and Co., Indianapolis, IN), respectively. The area under the curve (AUC) of glucose was calculated from the plasma concentration-time relationships. Plasma insulin concentrations at 0 min were also measured by enzyme-linked immunosorbent assay (Morinaga, Tokyo, Japan). The index of the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as an indicator of insulin sensitivity according to the following formula: [insulin (μIU) × glucose (mM)]/22.5.

Histological Examination. A portion of liver was fixed with 10% formalin and embedded in paraffin. Twenty-micron sections were cut and stained with hematoxylin and eosin for examination of liver histology (IX-81, Olympus Corporation, Tokyo, Japan).

Determination of Blood Biochemistry and Liver Triglyceride Content. Plasma triglyceride and NEFA levels were determined by use of commercial kits (Wako). Plasma adiponectin (Otsuka Pharmaceutical, Tokushima, Japan), leptin (Morinaga), and corticosterone (Cayman Chemical, Ann Arbor, MI) levels were also assayed by enzyme-linked immunosorbent assay.

Tissue triglyceride content was determined as described previously (Oakes et al., 2001). In brief, 100 mg of liver was homogenized and extracted with 2 ml of isopropyl alcohol. After centrifugation, triglyceride content in the supernatant was determined with an enzymatic colorimetric method (Wako).

Gene Expression Analysis. RNA was extracted from the livers of individual mice using TRIzol (Invitrogen, Osaka, Japan). Single-stranded cDNA was synthesized from 1 μg of total RNA by use of the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed with an AB 7300 Real-Time PCR System using TaqMan (Applied Biosystems, Foster City, CA). The primers and probes (σ-Genosys, Hokkaido, Japan) used are shown in Table 1. Mouse mitochondrial subunit 18S rRNA was selected as the endogenous control gene.

PPAR-γ protein was quantified by Western blotting (Lorenzo et al., 2002). Tissue proteins were resolved on 4 to 12% polyacrylamide gels in the presence of sodium dodecyl sulfate, transferred electrophoretically to polyvinylidene difluoride membranes, blocked (in buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 5% bovine serum albumin, 0.1% Tween 20), and incubated at 4°C for 18 h with PPAR-γ-specific antibody (1:800; Cell Signaling, Danvers, MA). Detection was performed with peroxidase-conjugated secondary antibody by enhanced chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Immunoblotting with a monoclonal anti-β-actin antibody (Cell Signaling) was conducted to ensure equal protein loading.

Data Analysis. All results are expressed as means ± S.E.M. Data from more than two experimental groups were analyzed by one-way analysis of variance (ANOVA). The Student-Newman-Keuls test was performed to identify the differences between groups. Data from two experimental groups (Fig. 6) were analyzed by use of the Student's t-test. $P < 0.05$ was considered significant.

Results

Higher-Dosage Experiment. In accordance with the previous reports (Moitra et al., 1998; Takemori et al., 2007), A-ZIP/F-1 mice that received a standard diet exhibited higher SBP (Fig. 1A), increased food intake (Fig. 1B), heavier body weights (Fig. 1C), compared with WT mice. It has been reported that there is a 90% reduction in brown adipose tissue and the remaining 10% is inactive in A-ZIP/F-1 mice (Moitra et al., 1998). In contrast to WT mice (Fig. 1D) in the present study, A-ZIP/F-1 mice showed minimal adipose tissue (Fig. 1E). We first tested the effects of telmisartan at higher dosage (3 mg/kg), although this was lower than the dose used in previous studies (5 mg/kg) (Benson et al., 2004; Araki et al., 2006; Sugimoto et al., 2006). Telmisartan treatment lowered SBP to the level observed in WT mice (Fig. 1A), but exerted minimal effects on parameters relating to food

TABLE 1
Primer and probe sequences for real-time PCR assays

Gene	Probe	Primers[a]
18S	CGCGCAAATTACCCACTCCCGA	*f* CGGCTACCACATCCAAGGA *r* CCAATTACAGGGCCTCGAAA
PPAR-γ	CTTCCATCACGGAGAGGTCCACAGAGC	*f* AGAGCATGGTGCCTTCGC *r* ATGTCAAAGGAATGCGAGTGG
PEPCK	CAACTGTTGGCTGGCTCTCACTGACCC	*f* GTGTCATCCGCAAGCTGAAGA *r* CTTTCGATCCTGGCCACATCT
G6P	CTCTGTATGGGAACCCTCGCCACG	*f* GAGGCCTTGTAGGAAGCATTG *r* CCATCCCAGCCATCATGAGTA
SCD1	CCACCACCACCATCACTGCACCTC	*f* ATGCTCCAAGAGATCTCCAGTTCT *r* CTTCACCTTCTCTCGTTCATTTCC
SREBP1c	CAGCTCATCAACAACCAAGACAGTGACTTC	*f* GGAGCCATGGATTGCACATT *r* CCTGTCTCACCCCCAGCATA
FAS	AACCACCCTCTGGGCATGGCTATCTTCT	*f* GGCTCAGCATGGTCGCTT *r* CTCCCGCCAGCTGTCATT
ACC1	CTCAACCTGGATGGTTCTTTGTCCCAGC	*f* GCCATTGGTATTGGGGCTTAC *r* CCCGACCAAGGACTTTGTTG

[a] Sequences: 5′ to 3′. Forward primers are designated by *f* and reverse primers by *r*.

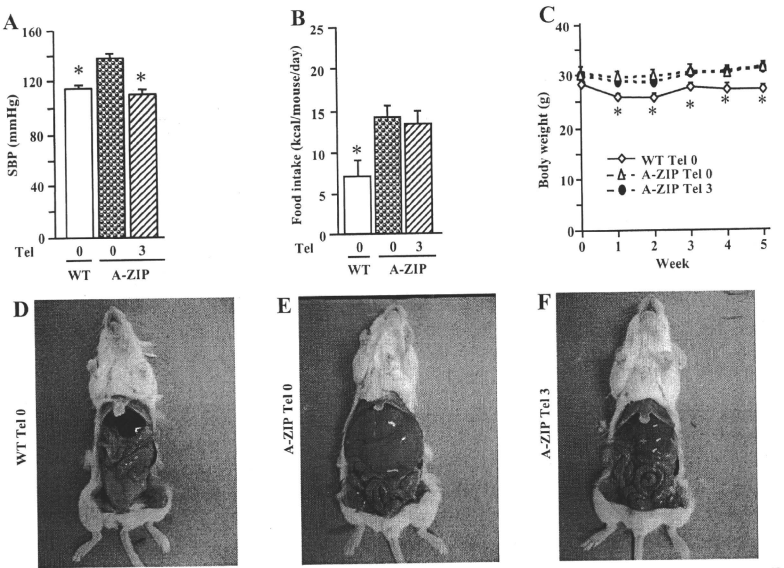


Fig. 1. SBP (A), food intake (B), body weight (C), and abdominal appearance (adipose tissues and liver) (D and F) in female WT and A-ZIP/F-1 mice. Animals were treated orally with vehicle (control, Tel 0) or telmisatan (3 mg/kg, Tel 3) once daily for 7 weeks. SBP was measured with a tail-cuff method at week 2 after treatment; 24-h food intake was determined at week 5. All values are means ± S.E.M. (n = 7). *, $P < 0.05$ compared with A-ZIP/F-1 control (Tel 0) (ANOVA).

intake (Fig. 1B), body weight (Fig. 1C), or adipose tissue (Fig. 1F, by gross necropsy) in A-ZIP/F-1 mice.

At week 4, plasma glucose levels in A-ZIP/F-1 mice were significantly increased over those in WT mice under non-fasted conditions, but not after a 12-h fast (Fig. 2A). Fasted plasma insulin levels (Fig. 2B) and the HOMA-IR index (Fig. 2C) were much higher in A-ZIP/F-1 than in WT mice. Telmisartan treatment markedly decreased plasma insulin concentrations (Fig. 2B) and the HOMA-IR index (Fig. 2C) in A-ZIP/F-1 mice, but was without effect on plasma glucose concentrations (Fig. 2A).

In further studies the responses of plasma glucose to challenge with exogenous glucose and insulin were assessed. Plasma glucose concentrations and glucose AUC did not differ significantly between A-ZIP/F-1 and WT mice either before or after OGTTs were conducted (2 g/kg) at week 5 (Fig. 2, D and E). Telmisartan treatment prevented the increases in plasma glucose concentrations at 20 min and in glucose AUC

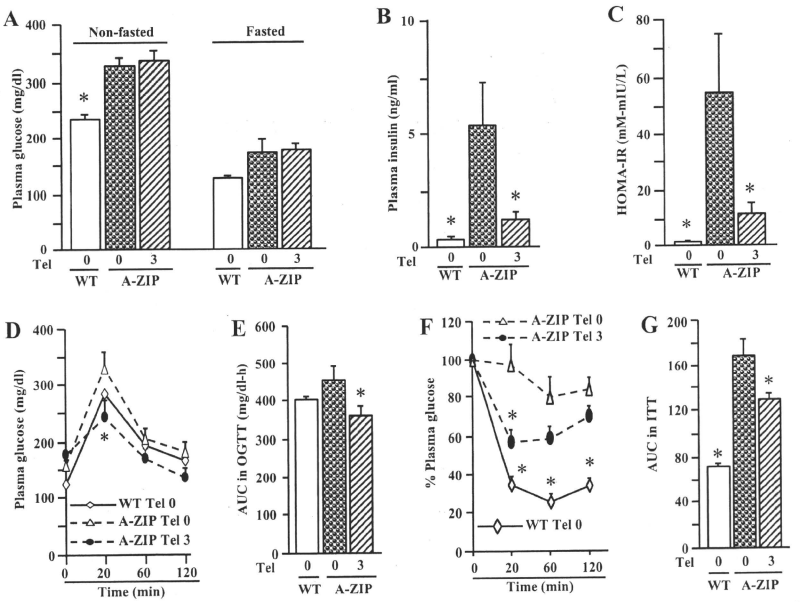


Fig. 2. Nonfasted and fasted (12 h) plasma glucose (A) and fasted insulin (B) levels, the index of the HOMA-IR (C) at week 4, and plasma glucose response to exogenous glucose (D and E) and insulin (F and G) challenge in female WT and A-ZIP/F-1 mice. OGTT (glucose: 2 g/kg p.o.) and insulin tolerance test (ITT, insulin: 0.75 IU/kg i.p.) were performed after fasting (12 h) at week 5 and 6, respectively. All values are means ± S.E.M. (n = 7). *, $P < 0.05$ compared with A-ZIP/F-1 control (Tel 0) (ANOVA). Tel 3, telmisartan 3 mg/kg.

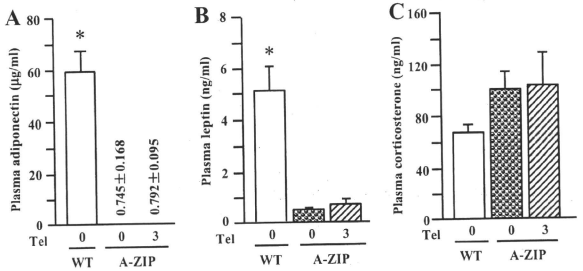
in OGTT. In contrast, the decrease in plasma glucose concentrations elicited by insulin was minimal in A-ZIP/F-1 mice compared with WT control (Fig. 2F), but was more pronounced when telmisartan was coadministered. Consistent with these findings, the glucose AUC after insulin was higher in A-ZIP/F-1 than in WT mice (Fig. 2G), and was decreased by telmisartan treatment.

Plasma adiponectin and leptin levels were extremely low in A-ZIP/F-1 mice and were unaffected by telmisartan treatment (Fig. 3, A and B). In contrast, plasma corticosterone in A-ZIP/F-1 mice was slightly, but not significantly, increased over WT control (Fig. 3C); telmisartan treatment did not affect plasma corticosterone levels in A-ZIP/F-1 mice. The generalized lipodystrophy also led to redistribution of lipids. Nonfasted plasma levels of triglyceride and NEFA were elevated in A-ZIP/F-1 mice compared with WT mice (Fig. 3, D and E). Treatment with telmisartan for 4 weeks decreased plasma triglyceride and NEFA levels by 42% and 28%, respectively.

The hepatomegaly exhibited by A-ZIP/F-1 mice (Fig. 1E) relative to control animals (Fig. 1D) was partially reversed by telmisartan (Fig. 1F). Indeed, the increase in hepatic triglycerides in A-ZIP/F-1 mice to 7- to 12-fold of WT control was substantially ameliorated by telmisartan (Fig. 4, B and C). In accord with these findings, the extensive fatty infiltration in A-ZIP/F-1 livers (Fig. 4E) compared with control (Fig. 4D), was also reversed in part by telmisartan (Fig. 4F).

Expression of the mRNAs encoding the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P) was similar in liver of A-ZIP/F-1 and control mice; both mRNAs were decreased by treatment with telmisartan by 62% and 61%, respectively (Fig. 5, A and B). In livers of A-ZIP/F-1 mice PPAR-γ mRNA expression was increased relative to WT control, which was substantially resolved by telmisartan treatment (Fig. 5C). In accord with these findings, PPAR-γ2 immunoreactive protein was up-regulated in A-ZIP/F-1 mouse liver and normalized by telmisartan treatment, but PPAR-γ1 protein expression was very low and unaltered (Fig. 5D). Hepatic stearoyl-CoA desaturase (SCD) 1, fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) 1 mRNA levels in the A-ZIP/F-1 mice were markedly increased over those in WT mice (Fig. 5, E–G), but the slight increase in sterol regulatory element-binding protein (SREBP) 1c mRNA did not attain statistical significance (Fig. 5H). Consistent with the effect of telmisartan on PPAR-γ gene expression, treatment of A-ZIP/F-1 mice with the AT1 blocker markedly suppressed hepatic mRNAs corresponding to the PPAR-γ-regulated downstream genes SCD1, FAS, ACC1, and SREBP1c.

Lower-Dosage Experiment. We also tested the effects of telmisartan at lower dosage. Treatment with 1 mg/kg telmisartan significantly decreased SBP in A-ZIP/F-1 mice (Fig. 6A). However, this treatment did not affect plasma glucose



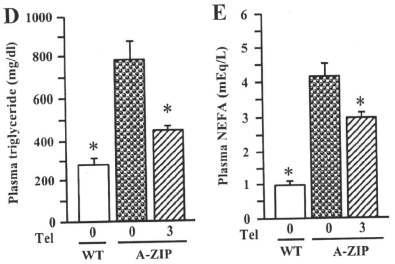
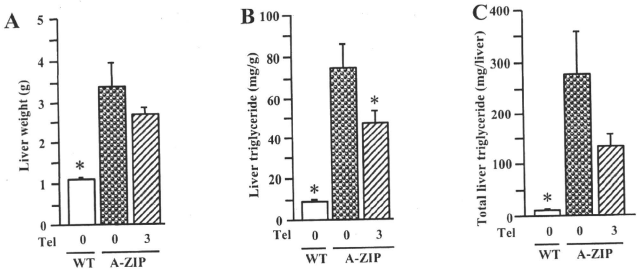


Fig. 3. Nonfasted plasma adiponectin (A), leptin (B), corticosterone (C), triglyceride (D), and NEFA (E) levels in female WT and A-ZIP/F-1 mice (week 7). All values are means ± S.E.M. (n = 7). Versus A-ZIP/F-1 control (Tel 0), *, $P <$ 0.05 (ANOVA). Tel 3, telmisartan 3 mg/kg.



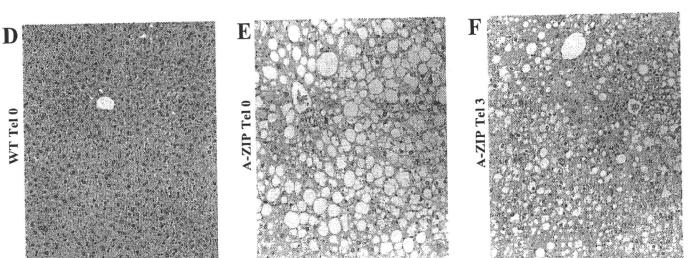


Fig. 4. Liver weight (A), liver triglyceride (B) ,and total liver triglyceride (C) contents, and liver histology in female WT and A-ZIP/F-1 mice (week 7). All values are means ± S.E.M. (n = 7). *, $P < 0.05$ compared with A-ZIP/F-1 control (Tel 0) (ANOVA). Representative liver pathological changes (hematoxylin and eosin staining, ×200) (D and F). Tel 3, telmisartan 3 mg/kg.

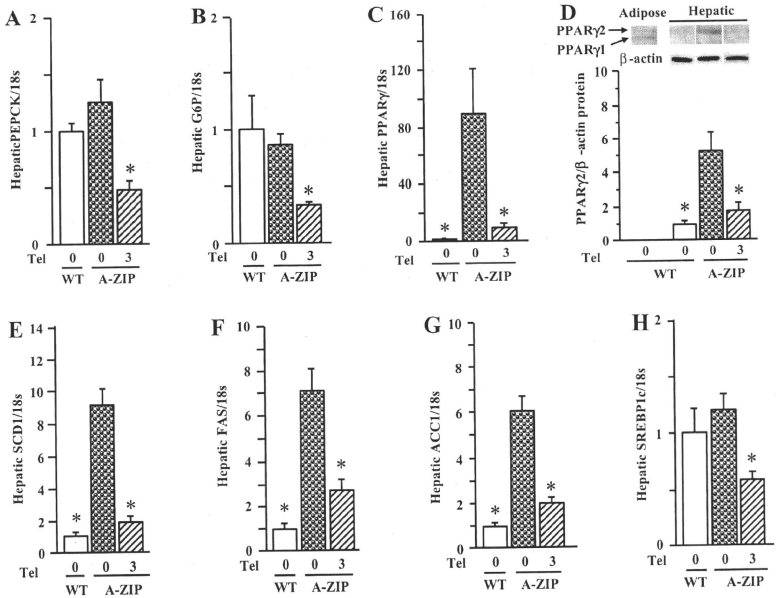


Fig. 5. Hepatic expression of PEPCK (A), G6P (B), PPAR-γ (C), SCD1 (E), FAS (F), and ACC1 (G), and SREBP1c (H) mRNAs, and PPARγ protein (D) in female WT and A-ZIP/F-1 mice (week 7). Quantitative real-time PCR results were normalized to 18 s, whereas the results from Western blot analysis were normalized to β-actin. Levels in WT mice were arbitrarily assigned a value of 1. All values are means ± S.E.M. (n = 7). *, $P < 0.05$ compared with A-ZIP/F-1 control (Tel 0) (ANOVA). Tel 3, telmisartan 3 mg/kg.

levels under fasting conditions (Fig. 6B) and during OGTT (Fig. 6C) and ITT (Fig. 6D); similarly, nonfasting triglyceride and NEFA levels (Fig. 6E), liver weight, and triglyceride content (Fig. 6F) remained unchanged. Real-time PCR analysis demonstrated no difference between the vehicle- and telmisartan-treated groups in the expression of hepatic PPAR-γ (Fig. 6G), SCD1 (Fig. 6H), SREBP1c (Fig. 6I), FAS (Fig. 6J), and ACC1 (Fig. 6K) mRNAs.

Discussion

PPAR-γ is expressed predominantly in adipose tissue, which facilitates lipid uptake and storage in that tissue and alters the release of leptin and adiponectin, which modulates insulin sensitivity. Adipose PPAR-γ is the molecular target for the insulin-sensitizing thiazolidinediones (Evans et al., 2004). The principal findings to emerge from the present study were that telmisartan treatment (3 mg/kg) enhanced insulin sensitivity and improved the abnormalities of lipid metabolism observed in A-ZIP/F-1 mice. Thus, the present findings indicate that telmisartan has additional beneficial effects that are distinct from adipose tissues. It seems that the improvement in insulin sensitivity in adipose tissue-deficient mice affected by telmisartan is independent of PPAR-γ agonism.

Although the mechanisms underlying insulin resistance in patients with lipodystrophies are unclear, accumulation of triglycerides in liver and skeletal muscle is probably important (Garg, 2004). Intracellular accumulation of fatty acids and triglycerides in nonadipose tissues has been implicated in insulin resistance (Shimabukuro et al., 1997). It has been demonstrated that a decrease in hepatic triglyceride pools leads to improved insulin sensitivity (Neschen et al., 2005; Savage et al., 2006). On the other hand, an increase in blood lipid levels, especially NEFA, modulates the action of insulin. A high plasma NEFA concentration is a risk factor for deterioration of glucose tolerance that is independent of the other parameters relating to insulin resistance or insulin secretion (Charles et al., 1997). Chronically elevated plasma NEFA concentrations stimulate gluconeogenesis, exacerbate hepatic/muscle insulin resistance, and impair insulin secretion in genetically predisposed individuals (Boden 1997; Bergman and Ader, 2000). Decreased availability of precursor substrates, including free fatty acids and glucose, also diminishes hepatic synthesis and export of esterified lipids (Ran et al., 2004). In the present study, amelioration of insulin resistance in the lipodystrophic A-ZIP/F-1 mice by telmisartan treatment was accompanied by a decrease in hepatic triglyceride accumulation and circulating lipid concentrations.

PPAR-γ is expressed normally at low levels in liver, but is strongly up-regulated in the liver of A-ZIP/F-1 mice and seems to contribute to the development of hepatic steatosis

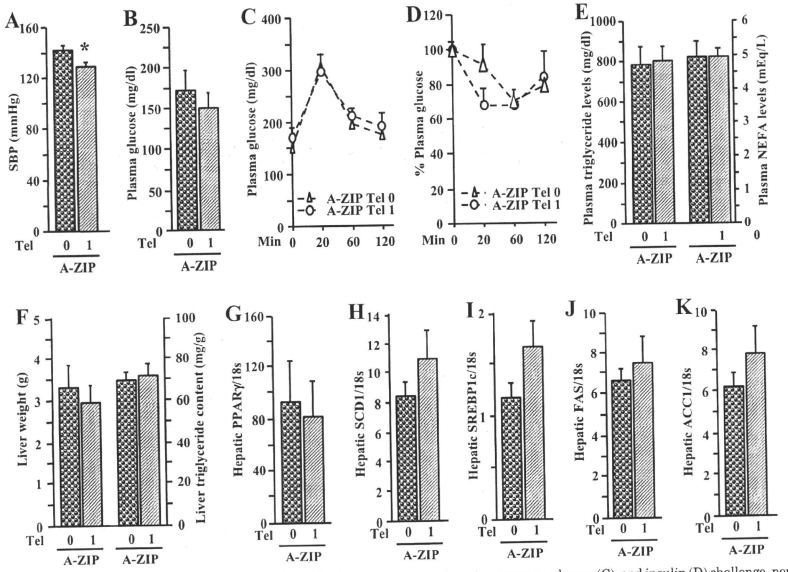


Fig. 6. SBP (A), fasted (12 h) plasma glucose concentrations (B), plasma glucose response to exogenous glucose (C), and insulin (D) challenge, nonfasted plasma triglyceride and NEFA levels (E), liver weight and triglyceride content (F), and hepatic expression of PPAR-γ (G), SCD1 (H), SREBP1c (I), FAS (J), and ACC1 (K) in female WT and A-ZIP/F-1 mice. Animal treatments and sample processing were conducted as described, with the exception that a dose of 1 mg/kg telmisartan was used instead of 3 mg/kg. All values are means ± S.E.M. ($n = 7$). *, $P < 0.05$ compared with A-ZIP/F-1 control (Tel 0) (Student's t-test). Tel 1, telmisartan 1 mg/kg.

(Chao et al., 2000; Gavrilova et al., 2003). Although treatment of A-ZIP/F-1 mice with rosiglitazone, a thiazolidinedione that acts as a PPAR-γ agonist, lowered circulating lipid levels, hepatic steatosis was exacerbated (Chao et al., 2000). Moreover, ablation of hepatic PPAR-γ decreased hepatic steatosis in A-ZIP/F-1 mice and promoted hyperlipidemia (Gavrilova et al., 2003). Thus, steatosis seems to result from increased triglyceride formation and uptake by the liver in a PPAR-γ-regulated fashion (Gavrilova et al., 2003). In the present study, telmisartan reversed the increase in hepatic PPAR-γ expression in A-ZIP/F-1 mouse liver and also normalized the expression of several PPAR-γ-responsive genes that participate in fatty acid and triglyceride synthesis. Whereas thiazolidinediones decreased serum triglycerides and free fatty acids, glucose and insulin regulation was not restored (Chao et al., 2000). Thus, the present findings clearly distinguish the effects of telmisartan from PPAR-γ agonist thiazolidinediones. The precise underlying molecular mechanism by which telmisartan modulates hepatic PPAR-γ activity should now be explored further.

Energy homeostasis is regulated at the level of food intake, overall activity, sympathetic tone, energy expenditure, and insulin sensitivity (Flier, 1997). Serum leptin concentrations in A-ZIP/F-1 mice are ~5% of those in WT mice (Moitra et al., 1998). The adipokine adiponectin acts in concert with leptin to enhance insulin sensitivity and regulate glucose metabolism (Berg and Scherer, 2005). Leptin is secreted by white and brown adipose tissue in proportion to tissue mass (Moitra et al., 1998). Leptin acts through the hypothalamic-pituitary-adrenal axis to regulate insulin sensitivity indirectly (Cusin et al., 1998; Liu et al., 1998). Hepatic leptin overexpression decreased substantially the steatosis in A-ZIP/F-1 mice and also normalized muscle lipid and serum concentrations of glucose and insulin, as well as triglycerides and free fatty acids (Ebihara et al., 2001). Thus, restitution of leptin compensated for the lack of adipose tissue and prevented the hyperglycemia, hyperinsulinemia, hypertriglyceridemia, and increased free fatty acid levels that are characteristic of the A-ZIP/F-1 mouse. In the present study, telmisartan did not affect plasma adiponectin and leptin levels, which indicates that the primary defect due to adipocyte deletion was not overcome.

Although disturbance of the regulatory actions of adipose tissue-derived mediators is important, adrenocortical dysregulation may contribute to insulin resistance (Roberge et al., 2007). Thus, glucocorticoids regulate food intake and metabolism that influence energy utilization (Haluzik et al., 2002) and also stimulate gluconeogenesis via PEPCK and G6P (Pilkis and Granner, 1992). Leptin deficiency may contribute to hypercorticosteronemia in A-ZIP/F-1 mice (Haluzik et al., 2002). Indeed, combined leptin infusion and adrenalectomy decreased plasma corticosterone levels and improved diabetes in A-ZIP/F-1 mice (Haluzik et al., 2002). In the present study, the small increase in plasma corticosterone

levels in A-ZIP/F-1 mice was not statistically significant and was minimally affected by telmisartan. Thus it is unlikely that telmisartan improves insulin resistance by modulating adrenal corticosterone production in A-ZIP/F-1 mice.

Telmisartan at lower dosage (1 mg/kg) significantly decreased SBP in A-ZIP mice, which is attributed to AT1 inhibition. However, it did not affect insulin resistance, hyperlipidemia, or fatty liver, and did not decrease the expression of hepatic PPAR-γ-regulated lipogenic genes. These results were distinct from those made after treatment with telmisartan at 3 mg/kg (see above) or rosiglitazone (Chao et al., 2000). Thus, these findings may suggest that AT1 inhibition alone is unlikely to account for the improved insulin sensitivity and fatty liver in the lipodystrophic mice. Genetic blockade of AT1 or PPAR-γ signaling may be necessary to dissociate the actions of telmisartan at AT1 and PPAR-γ.

Lipodystrophies, such as the Seip-Berardinelli syndrome (Moitra et al., 1998) are characterized by the selective loss of adipose tissue. These conditions may be genetic in origin or may emerge during the treatment of HIV patients with protease inhibitors. Affected patients are predisposed to insulin resistance and its attendant complications, including diabetes, hypertriglyceridemia, hepatic steatosis and, hypertension (Garg, 2004; Agarwal and Garg, 2006). Morbidity and mortality in patients with lipodystrophies are frequently manifested as diabetes mellitus, hepatic cirrhosis as a result of steatosis, atherosclerosis, and recurrent acute pancreatitis caused by prolonged hypertriglyceridemia (Garg, 2004; Agarwal and Garg, 2006). Current treatments are restricted to managing the metabolic abnormalities of insulin resistance, diabetes, dyslipidemia, and hypertension; to date, no specific therapies have emerged. The present study has identified an adipose-tissue-independent insulin-sensitizing effect of telmisartan. Amelioration of hepatic steatosis was associated with down-regulation of hepatic expression of PPAR-γ-mediated genes responsible for lipid synthesis. Therefore, the present finding that telmisartan improves insulin resistance and its associated complications in A-ZIP/F-1 mice may constitute important information for the development of new strategies to manage patients with lipodystrophy.

Taken together, the present findings are consistent with a novel mode of insulin-sensitizing action of telmisartan, involving an adipose tissue-independent pathway. The precise underlying molecular mechanism by which telmisartan modulates hepatic PPAR-γ activity should now be explored further.

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RESEARCH PAPER

Irbesartan treatment up-regulates hepatic expression of PPARα and its target genes in obese Koletsky (fa^k/fa^k) rats: a link to amelioration of hypertriglyceridaemia

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BACKGROUND AND PURPOSE

Hypertriglyceridaemia is associated with an increased risk of cardiovascular disease. Irbesartan, a well-established angiotensin II type 1 receptor (AT_1) blocker, improves hypertriglyceridaemia in rodents and humans but the underlying mechanism of action is unclear.

EXPERIMENTAL APPROACH

Male obese Koletsky (fa^k/fa^k) rats, which exhibit spontaneous hypertension and metabolic abnormalities, received irbesartan (40 $mg \cdot kg^{-1} \cdot day^{-1}$) or vehicle by oral gavage over 7 weeks. Adipocyte-derived hormones in plasma were measured by ELISA. Gene expression in liver and other tissues was assessed by real-time PCR and Western immunoblotting.

KEY RESULTS

In Koletsky (fa^k/fa^k) rats irbesartan lowered plasma concentrations of triglycerides and non-esterified fatty acids, and decreased plasma insulin concentrations and the homeostasis model assessment of insulin resistance index. However, this treatment did not affect food intake, body weight, epididymal white adipose tissue weight, adipocyte size and plasma leptin concentrations, although plasma adiponectin was decreased. Irbesartan up-regulated hepatic expression of mRNAs corresponding to peroxisome proliferator-activated receptor (PPAR)α and its target genes (carnitine palmitoyltransferase-1a, acyl-CoA oxidase and fatty acid translocase/CD36) that mediate hepatic fatty acid uptake and oxidation; the increase in hepatic PPARα expression was confirmed at the protein level. In contrast, irbesartan did not affect expression of adipose PPARγ and its downstream genes or hepatic genes that mediate fatty acid synthesis.

CONCLUSIONS AND IMPLICATIONS

These findings demonstrate that irbesartan treatment up-regulates PPARα and several target genes in liver of obese spontaneously hypertensive Koletsky (fa^k/fa^k) rats and offers a novel insight into the lipid-lowering mechanism of irbesartan.

Abbreviations

ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; AT1, angiotensin II type 1 receptor; ARB, angiotensin II type 1 receptor blocker; CPT, carnitine palmitoyltransferase; DGAT, diacylglycerol acyltransferase; eWAT, epididymal white adipose tissue; FAS, fatty acid synthase; FAT, fatty acid translocase; GLUT, glucose transporter; HOMA-IR, homeostasis model assessment of insulin resistance; NEFA, non-esterified fatty acids; PPAR, peroxisome proliferator-activated receptor; SBP, systolic blood pressure; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element-binding protein

Introduction

The metabolic syndrome is a cluster of conditions arising from many factors including genetic mutation, overnutrition and a sedentary lifestyle. Common components of the metabolic syndrome include abdominal obesity, hypertension, dislipidaemia and insulin resistance. Insulin resistance and type 2 diabetes are associated with abnormalities in lipid and lipoprotein homeostasis, including elevated triglycerides, which increase the risk of cardiovascular disease (Krauss, 2004). Hypertriglyceridaemia is considered to be an important risk factor for atherosclerosis and other cardiovascular complications in patients with type 2 diabetes (Ginsberg, 1996), and may also be associated with premature coronary artery disease (Brunzell, 2007). In addition, elevated plasma concentrations of non-esterified fatty acids (NEFA) have been associated with deterioration of glucose tolerance independent of other markers of insulin resistance that characterize subjects who are at risk from type 2 diabetes (Charles *et al.*, 1997). Prolonged elevations of NEFA in plasma can exacerbate the impairment in glucose homeostasis in individuals with obesity and type 2 diabetes (Saloranta and Groop, 1996) and may stimulate gluconeogenesis, the development of insulin resistance in muscle and liver, and may also impair insulin secretion in genetically predisposed individuals (Bergman and Ader, 2000). It has been suggested that NEFAs are a major link between obesity and insulin resistance/type 2 diabetes (McGarry, 2002; Bays *et al.*, 2004). Therefore, pharmacological treatments that decrease circulating triglycerides and NEFAs may improve insulin resistance and reduce the risk of cardiovascular disease.

Irbesartan, one of the earliest angiotensin II type 1 receptor blocker (ARBs) to enter clinical use, is a well-established and widely used antihypertensive agent. Irbesartan has been shown to decrease plasma triglyceride concentrations in the obese Zucker rat (Janiak *et al.*, 2006; Muñoz *et al.*, 2006) and the corpulent JCR:LA-cp rat (Russell *et al.*, 2009). Large-scale clinical trials have also demonstrated that irbesartan improves metabolic parameters, including plasma triglyceride concentrations, in patients with hypertension and the metabolic syndrome (Kintscher *et al.*, 2007; Parhofer *et al.*, 2007). However, the underlying mechanism of these lipid-lowering effects remains unknown.

The genetically obese Koletsky (fa^k/fa^k) rat strain carries a nonsense mutation in the leptin receptor gene (Takaya *et al.*, 1996). The fa^k mutation results in hyperphagia, obesity, insulin resistance and hyperlipidaemia (Koletsky, 1973; Koletsky and Ernsberger, 1992; Friedman *et al.*, 1997) superimposed on the background of the spontaneously hypertensive lean Koletsky (+/+) littermates. As ARBs are prescribed to the patients with hypertension, the present study investigated the mechanism of the lipid-lowering effect of irbesartan using the obese spontaneously hypertensive Koletsky (fa^k/fa^k) rats. The principal findings to emerge were that, irbesartan decreased plasma triglyceride and NEFA concentrations, in addition to decreases in plasma insulin concentrations and the index of homeostasis model assessment of insulin resistance (HOMA-IR). Peroxisome proliferator-activated receptor (PPAR)α and several PPARα-responsive genes were up-regulated in liver, thus increasing the capacity for uptake and oxidation of fatty acids. In contrast, irbesartan did not significantly affect the expression of PPARγ and downstream genes in white adipose tissues, and the genes responsible for fatty acid synthesis in liver. Thus, irbesartan improves hypertriglyceridaemia and high free fatty acid concentrations via a hepatic PPARα pathway in insulin resistant rats with obesity and hypertension.

Methods

Animals, diet and experimental protocol

All animal procedures were in accordance with the 'Principles of laboratory animal care' (http://grants1.nih.gov / grants / olaw / references / phspol.htm) and were approved by the Animal Ethics Committee, Kyoto University, Japan.

Male obese Koletsky (fa^k/fa^k) rats and their lean (+/+) littermates aged 10–11 weeks were generous gifts from Japan SLC, Inc., Shizuoka, Japan. Rats were housed in a temperature-controlled facility (21 ± 1°C, 55 ± 5% relative humidity) with a 12-h light/dark cycle (2 rats per cage). Animals were allowed free access to water and the standard diet (CLEA Tokyo, Japan) for 1 week before starting the experiments. Rats were divided into three groups (n = 6 per group): lean control (+/+ Irb -), obese control (fa^k/fa^k Irb -) and obese with irbesartan treatment (fa^k/fa^k Irb +). There was no difference in body weight between two obese groups before treatments. Animals in the fa^k/fa^k Irb + group were administered irbesartan (nomenclature follows Alexander *et al.*, 2008) (40 mg·kg^{-1}, a generous gift from Shionogi & Co., Ltd, Japan, suspended in 5% Gum Arabic) by oral gavage once daily (11 h 00 min–12 h 00 min) for 7 weeks. The rats in the +/+ Irb – and fa^k/fa^k Irb – groups received vehicle (5% gum arabic) alone. The rats were weighed once every 3–4 days to determine gavage volume and daily food intake was estimated from weekly measurements. Systolic blood pressure

(SBP) was measured at Week 1. Blood samples were collected by retro-orbital venous puncture under ether anaesthesia at Week 5 in animals that had been deprived of food for 6 h, for determination of plasma concentrations of triglyceride and NEFA using enzymatic methods (kits from Wako, Osaka, Japan), leptin (Morinaga, Tokyo, Japan) and adiponectin (Otsuka Pharmaceutical, Tokushima, Japan) using commercial ELISAs. Plasma glucose and insulin concentrations were determined using enzymatic (kit from Wako, Osaka, Japan) and ELISA (kit from Morinaga, Tokyo, Japan) methods, respectively, at Week 6 after the rats had been deprived of food for 12 h. The HOMA-IR index was calculated as an indicator of insulin sensitivity according to the following formula: [insulin ($\mu IU \cdot mL^{-1}$) × glucose (mM)]/22.5. Animals were weighed at Week 7 and then killed by prompt dislocation of the neck vertebra. Epididymal white adipose tissue (eWAT) and liver were collected and weighed. The gastrocnemius muscle [contains red (mostly type IIa muscle fibres) and white (primarily type IIb fibres) skeletal muscle] was also collected. Segments of each of eWAT, liver and skeletal muscle were snap frozen in liquid nitrogen and stored at −80°C for subsequent determination of triglyceride content and/or gene analysis.

SBP

Systolic blood pressure was measured (2–5 h after administration of irbesartan or vehicle) in conscious rats by a tail-cuff method (MK-2000ST; Muromachi Kikai Co Ltd, Tokyo, Japan). At least six readings were taken for each measurement.

Determination of triglyceride content in liver and skeletal muscle

Triglyceride contents in liver and skeletal muscle were determined as described previously (Oakes *et al.*, 2001). Briefly, 100 mg of tissue was homogenized and extracted with 2 mL of isopropanol. After centrifugation (1000× *g*), the triglyceride content in the supernatants was determined enzymatically (Wako, Osaka, Japan).

Histological examination

A portion of eWAT or liver was fixed with 10% formalin and embedded in paraffin. Four-micron sections were cut and stained with haematoxylin and eosin for examination of adipose tissue and liver histology (IX-81, Olympus Corporation, Tokyo, Japan). The adipocyte cross-sectional area was measured using an image analysing system (KS 400 Imaging System; Carl Zeiss Vision, Eching, Germany).

Gene expression analysis

Total RNA was isolated from the eWAT, livers and skeletal muscle of individual mice using TRIzol (Invitrogen, Osaka, Japan). Single-stranded cDNA was synthesized from 1 μg of total RNA using SuperScript III First-Strand Synthesis System for RT-PCR, according to the manufacturer's instructions (Invitrogen, Osaka, Japan). Quantitative real-time PCR was performed with an AB 7300 Real-Time PCR System using TaqMan (Applied Biosystems, USA). The sequences of primers and probes (Sigma-Genosys, Japan) used in the present study are shown in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as an endogenous control (housekeeping gene).

Protein expression was quantified by Western blotting (Lorenzo *et al.*, 2002). Tissue proteins were resolved on 4–12% polyacrylamide gels in the presence of sodium dodecylsulphate, transferred electrophoretically to polyvinylidene difluoride membranes, blocked (in buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 5% bovine serum albumin, 0.1% Tween-20) and incubated at 4°C for 18 h with PPARα-specific antibody (1:300; Santa Cruz, CA, USA). Detection was performed with peroxidase-conjugated secondary antibody, by enhanced chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Immunoblotting with a monoclonal anti-β-actin antibody (Cell Signaling, Beverly, MA, USA) was conducted to ensure equal protein loading.

Data analysis

All results are expressed as means ± SEM. Data obtained from experiments with three groups of animals (Figures 1–4) were analysed by one-way analysis of variance (ANOVA). If a difference was detected (*F*-ratio), the Student-Newman-Keuls test was performed to locate the differences between groups. Data obtained from experiments with two groups of animals (Figure 5) were analysed by Student's *t*-test; $P < 0.05$ was considered to be statistically significant.

Results

Metabolic abnormalities and effects of irbesartan in obese Koletsky (fa^k/fa^k) rats

Animals were genotyped by the supplier. Obese Koletsky (fa^k/fa^k) rats appeared to be somewhat larger than their lean littermates; rats of both genotype were hypertensive (SBP: ≈170 mmHg; Figure 1A) compared with normal controls (SBP: ≈120 mmHg). Food intake (Figure 1C) and body

Table 1

Primer and probe sequences for real time RT-PCR assays

Gene	Probe	Primers[a]
GAPDH	TTGTGCAGTGCCAGCCTCGTCTCA	*f* TGTTCTAGAGACAGCCGCATCTT
		r CCGACCTTCACCATCTTGTCTAT
PPARγ	CCTGCGGAAGCCCTTTGGTGACT	*f* TGACCAGGGAGTTCCTCAAAA
		r AGCAAACTCAAACTTAGGCTCCAT
FAS	ACCATCTCTGGACCTCAGGCTGCAGT	*f* TGCCTGCCTGCCACAAC
		r CTTGCTTTAGCTGCTCCACAAATT
ACC1	CAGCACAGCTCCAGATTGCCATGG	*f* GGTGGCTGATGTCAATCTTCCT
		r TCATACGAATATCCTTGATCCTAAATAGAG
CD36	CTTGGATGTGGAACCCATAACTGGATTC	*f* CCTAACGAAGATGAGCATAGGACAT
		r GTTGACCTGCAGTCGTTTTGC
SCD1	CCGGGCCCATTCATACACATCGTTCT	*f* CCTCATCATTGCCAACACCAT
		r GGCGTGTGTCTCAGAGAACTTG
SREBP1c	CAAAGCTGAATAAATCTGCTGTCTTGCGCA	*f* CCTGGTGGTGGGCACTGA
		r GTGCTGTAAGAAGCGGATGTAGTC
PPARα	CTGCAAGGGCTTCTTTCGGCGA	*f* CTATGGAGTCCACGCATGTGAA
		r TTGTCGTACGCCAGCTTTAGC
CPT1a	CCCCGCGAATCCGTCCAGC	*f* GGTTCAAGAATGGCATCATCACT
		r TCACACCCACCACCACGATA
ACO	CAGACGGAGATGGGCCACGGAAC	*f* AAGAACTCCAGATAATTGGCACCTA
		r TGGTTTCCAAGCCTCGAAGAT
CPT1b	CGAGCAGTGCCAGACAGCCATCG	*f* CGGATGCAGTGGGACATTC
		r CCAGGGCCTTGGCTACTTG
aP2	TGGGAGTTGGCTTCGCCACCAG	*f* TCCAGTGAGAACTTCGATGATTACA
		r GGCCATACCGGCCACTTT
Adiponectin	TTCTCTCCAGGAGTGCCATCTCTGCC	*f* GGACCAAGAACACCTGCGTCT
		r TCCTGGTCACAATGGGATACC
DGAT1	CAGAACTCCATGAAGCCCTTCAAGGACAT	*f* CAGCAGTGGATGGTCCCTACTAT
		r AAGAGACGCTCAATGATTCGTG
GLUT4	CATCAACGCCCCACAGAAAGTGATTG	*f* GCTCCCTTCAGTTTGGCTATAACA
		r GCCAAGTTGCATTGTAGCTCTGT

[a]Forward primers are designated by *f* and reverse primers by *r*.
Sequences: 5′ to 3′.
GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; CPT, carnitine palmitoyltransferase; DGAT, diacylglycerol acyltransferase; FAS, fatty acid synthase; GLUT, glucose transporter; PPAR, peroxisome proliferator-activated receptor; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element-binding protein.

weights (Figure 1D) were greater in obese rats than in lean controls, but there was no difference in heart weight between the genotypes (Figure 1B). Irbesartan treatment (40 mg·kg^{-1}) decreased SBP by ~40 mmHg (Figure 1A) and heart weight (Figure 1B) in obese rats, consistent with its cardiovascular actions. However, this treatment did not significantly affect food intake (Figure 1C) and body weight (Figure 1D).

Compared with lean controls, plasma triglyceride concentrations were higher in obese Koletsky (fa^k/fa^k) rats under both fasting (deprived of food) and non-fasting conditions (Figure 2A). Plasma NEFA concentrations were also higher in obese rats under fasting, but not non-fasting, conditions (Figure 2B). Treatment of obese rats with irbesartan for 5 weeks decreased both fasted and non-fasted plasma triglyceride and fasted NEFA concentrations, but it was without effect on non-fasted NEFA concentrations in plasma of obese rats (Figure 2A and B).

In fasted animals, plasma glucose concentrations did not differ between rats of either genotype (Figure 2C). However, plasma insulin concentra-

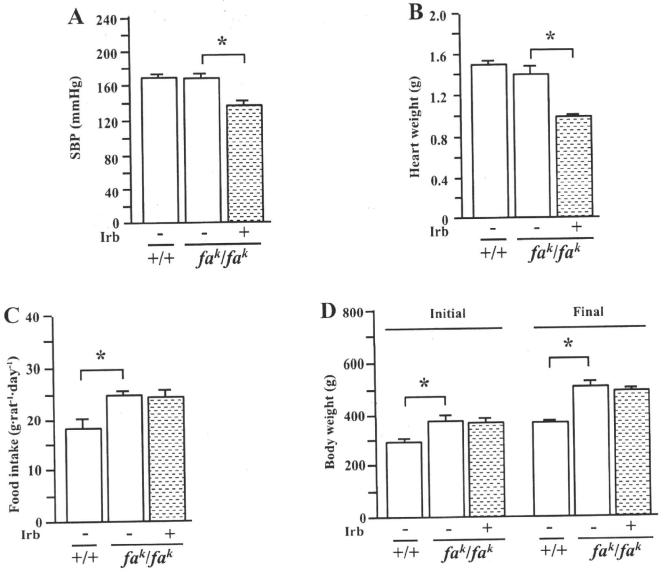

Figure 1

Systolic blood pressure (SBP) (A), heart weight (B), food intake (C) and initial and final body weight (D) in male lean (+/+) and obese (fa^k/fa^k) Koletsky rats. Animals received irbesartan (Irb) (40 mg·kg^{-1}·day^{-1}) or vehicle by oral gavage for 7 weeks. SBP was measured with a tail-cuff method at Week 1 after treatment. Food intake over 24 h was determined at Week 3. All values are means ± SEM ($n = 6$ each group). Irb -: vehicle; Irb +: irbesartan; *$P < 0.05$.

tions (Figure 2D) and the HOMA-IR index (Figure 2E) were considerably higher in the obese rats than in their lean counterparts. Although irbesartan treatment was without effect on plasma glucose concentration, plasma insulin concentrations and the HOMA-IR index in obese rats were decreased by this treatment (Figure 2D and E).

Hepatomegaly (Figure 3A) was evident in the obese Koletsky (fa^k/fa^k) rat; consistent with this finding, hepatic triglyceride content was increased markedly in these animals (Figure 3B and C), compared with corresponding lean controls. Increased vacuolization was evident on histological examination of liver sections from obese rats (Figure 3F) compared with lean rats (Figure 3E), indicative of excess lipid droplet accumulation. In skeletal muscle, triglyceride content was also increased in obese rats (Figure 3D); irbesartan treatment did not significantly alter these parameters (Figure 3A–D and G).

Compared with lean control rats, eWAT weight (Figure 4A), adipocyte size (Figure 4B and F) and plasma leptin concentrations (Figure 4C) were greater in obese Koletsky rats, but plasma adiponectin concentrations (Figure 4D) were not different between lean and obese animals. In obese rats irbesartan decreased plasma adiponectin concentrations (Figure 4D) but did not affect eWAT weight (Figure 4A), adipocyte size (Figure 4B and G) and plasma leptin concentrations (Figure 4C).

Gene expression profile in obese Koletsky (fa^k/fa^k) rats

By real-time PCR obese Koletsky (fa^k/fa^k) rats showed a significant increase in hepatic and adipose, but not muscular, expression of GAPDH, compared with lean rats; irbesartan treatment did not significantly affect GAPDH expression (data not shown). Thus, comparisons in gene expression were restricted

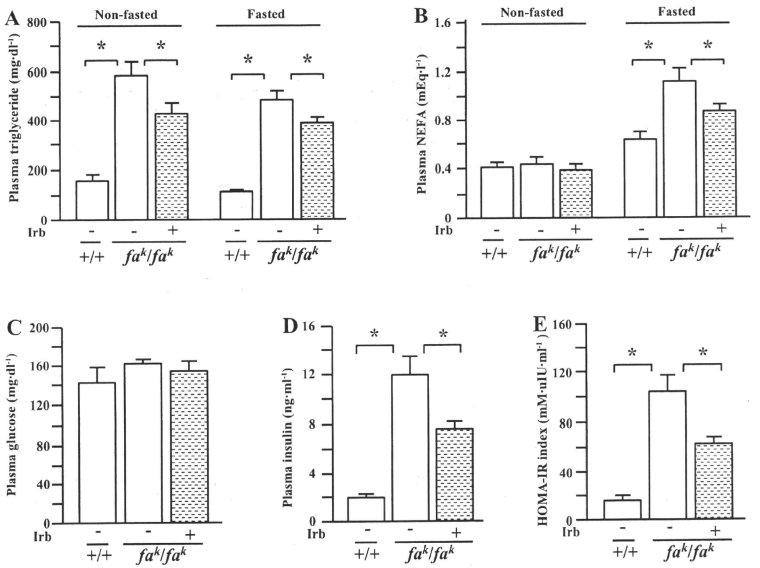

Figure 2

Non-fasted and fasted (rats deprived of food) plasma concentrations of triglyceride (A) and non-esterified fatty acids (NEFA) (B), fasted plasma glucose (C) and insulin (D) concentrations, and the index of the homeostasis model assessment of insulin resistance (HOMA-IR) (E) in male lean (+/+) and obese (fa^k/fa^k) Koletsky rats; animals received irbesartan (Irb) or vehicle as described in the legend to Figure 1. All values are means ± SEM ($n = 6$ each group). Irb -: vehicle; Irb +: irbesartan; $^*P < 0.05$.

to obese animals, with or without irbesartan treatment.

Interestingly, irbesartan treatment up-regulated PPARα, carnitine palmitoyltransferase (CPT)1a, acyl-CoA oxidase (ACO) and fatty acid translocase (FAT)/CD36 (Figure 5A) mRNAs in liver of obese Koletsky (fa^k/fa^k) rats; the increase in PPARα expression was confirmed at the protein level by Western immunoblotting (Figure 5D). However, irbesartan treatment did not alter hepatic sterol regulatory element-binding protein (SREBP)1c, acetyl-CoA carboxylase (ACC)1, fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD)1 mRNA expression, but increased the level of diacylglycerol acyltransferase (DGAT)1 mRNA (Figure 5A).

In contrast with these findings in liver, irbesartan treatment did not alter the expression of PPARα, CPT1a, ACO and FAT/CD36 mRNAs in skeletal muscle of obese Koletsky (fa^k/fa^k) rats (data not shown). Consistent with decreased HOMA-IR index, irbesartan up-regulated muscular glucose transporter (GLUT)4 expression (Figure 5B).

In white adipose tissue from obese Koletsky (fa^k/fa^k) rats, irbesartan treatment did not significantly change the expression of mRNAs encoding PPAR&gamma, aP2, adiponectin, FAS, ACC1, CD36, SCD1, SREBP1c and DGAT1 (data not shown). However, GLUT4 mRNA level was increased by irbesartan treatment (Figure 5C).

Discussion

Consistent with recent clinical findings (Kintscher *et al.*, 2007; Parhofer *et al.*, 2007), the present study demonstrates that irbesartan treatment improves hypertriglyceridaemia and reduces free fatty acid concentrations in obese Koletsky (fa^k/fa^k) rats that exhibit hypertension and metabolic abnormalities.

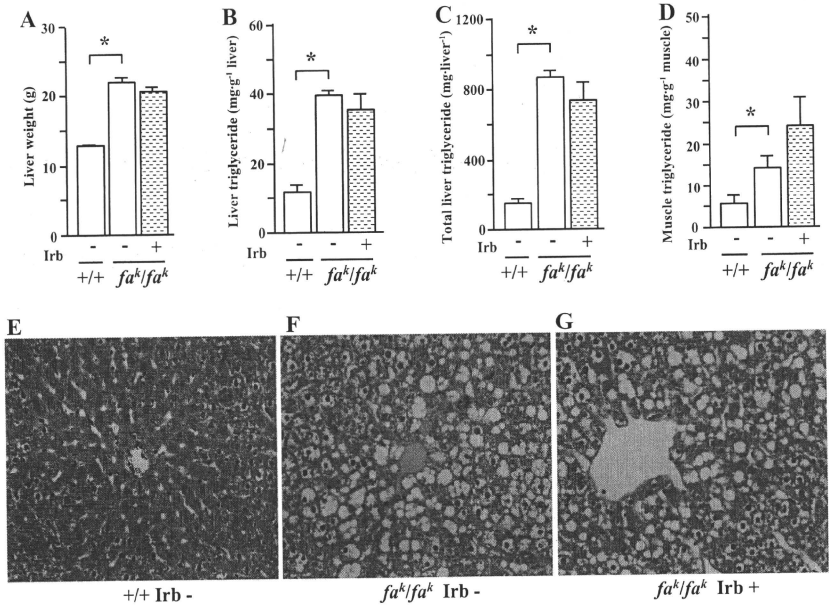

Figure 3

Liver weight (A), triglyceride contents in liver (B, C) and skeletal muscle (D), and representative images showing histology of liver (Hematoxylin and eosin–staining, X200) (E-G) in male lean (+/+) and obese (fa^k/fa^k) Koletsky rats; animals received irbesartan (Irb) or vehicle as described in the legend to Figure 1. All values are means ± SEM ($n = 6$ each group). Irb -: vehicle; Irb +: irbesartan; *$P < 0.05$.

Improvements in plasma lipid concentrations are not produced by all ARBs. Telmisartan, valsartan, candesartan, olmesartan and losartan were without effect on serum triglyceride concentrations in hypertensive patients with metabolic syndrome and/or type 2 diabetes (Bahadir *et al.*, 2007; Ichikawa, 2007; Tomiyama *et al.*, 2007; Nakayama *et al.*, 2008). Genetic blockade of AT_1 also failed to decrease serum triglyceride concentrations in mice fed high fat (Kouyama *et al.*, 2005) or methionine-choline deficient diets (Nabeshima *et al.*, 2009). Similarly, telmisartan and valsartan treatment did not alter serum triglyceride concentrations in rats fed a diet that was high in fat and carbohydrate (Sugimoto *et al.*, 2006). Losartan (Xu *et al.*, 2005) and olmesartan (Yokozawa *et al.*, 2009) did not significantly affect serum triglyceride and/or NEFA concentrations in obese Zucker rats. Thus, it appears that mechanisms other than AT_1 inhibition may mediate the lipid-lowering actions of irbesartan.

peroxisome proliferator-activated receptor α is a member of the nuclear receptor superfamily. PPARα is predominantly expressed in liver and, to a lesser extent, in skeletal muscle and heart, where it has a crucial role in controlling fatty acid oxidation (Reddy and Hashimoto, 2001). Fibrates are established pharmacological activators of PPARα that decreases circulating triglycerides and improves insulin sensitivity. Interestingly, in the present study irbesartan treatment was found to up-regulate PPARα and several target genes that mediate fatty acid oxidation in liver of obese Koletsky (fa^k/fa^k) rats. Furthermore, hepatic expression of FAT/CD36, another PPARα target gene that is important in facilitating cellular fatty acid uptake and ameliorating insulin resistance in rodents and humans (Pravenec *et al.*, 2001; Su and Abumrad, 2009), was also up-regulated by irbesartan. However, irbesartan did not significantly enhance the expression of these genes in skeletal muscle. Irbesartan also did

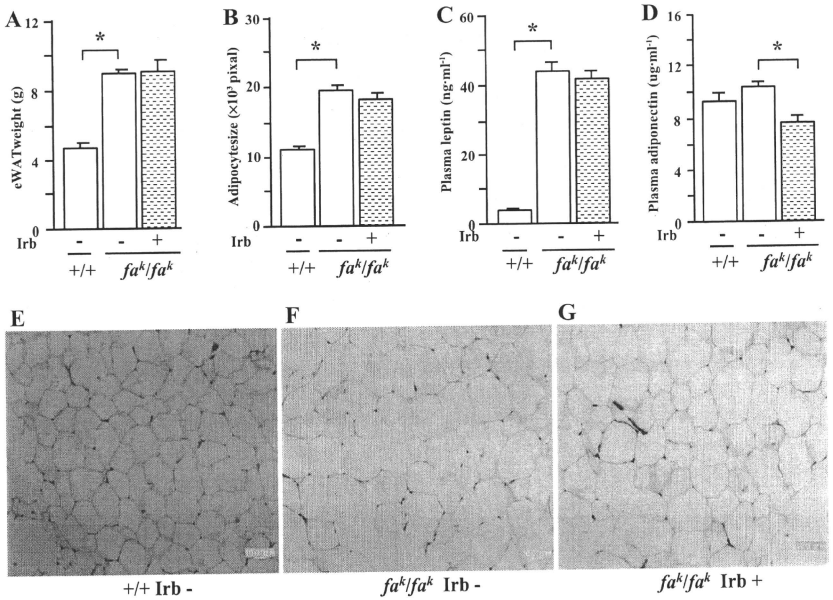

Figure 4

Epididymal white adipose tissue (eWAT) weight (A), adipocyte size (B), fasted (rats deprived of food) plasma concentrations of leptin (C) and adiponectin (D), and representative images showing histology of eWAT (haematoxylin and eosin–staining, X200) (E-G) in male lean (+/+) and obese (fa^k/fa^k) Koletsky rats; animals received irbesartan (Irb) or vehicle as described in the legend to Figure 1. All values are means ± SEM (n = 6 each group). Irb -: vehicle; Irb +: irbesartan; *$P < 0.05$.

not affect the hepatic expression of the genes that mediate fatty acid synthesis, including SREBP1c, FAS, ACC1 and SCD1, in obese Koletsky (fa^k/fa^k) rats. Thus, the present findings suggest that the actions of irbesartan in increasing fatty acid uptake and oxidation by the liver are mediated by a hepatic PPARα pathway and lead to decreased plasma triglyceride and free fatty acid concentrations. However, irbesartan also increased hepatic expression of DGAT1, a key enzyme in triglyceride synthesis (Villanueva *et al.*, 2009). It is possible that the unchanged hepatic lipid content after irbesartan treatment may reflect minimal overall effects on the balance between fatty acid uptake, β-oxidation, esterification and lipid secretion/storage.

In contrast to the situation with PPARα, PPARγ is expressed predominantly in adipose tissue and at only low levels in liver and skeletal muscle. PPARγ-activating ligands improve adipose tissue function by altering fat topography and adipocyte phenotype and by up-regulating genes involved in fatty acid metabolism and triglyceride storage (Sharma and Staels, 2007). Furthermore, PPARγ activation is associated with potentially beneficial effects on the secretion of a range of factors from adipose tissue, including adiponectin, thereby improving insulin sensitivity (Sharma and Staels, 2007). Indeed, adiponectin is an important mediator of the improvement in insulin sensitivity elicited by PPARγ agonists (Sharma and Staels, 2007). The increase in plasma adiponectin concentrations observed after thiazolidinedione therapy is closely associated with a decline in hepatic fat content (Sharma and Staels, 2007). Thus, treatment with rosiglitazone enhances insulin sensitivity, and is accompanied by decreased plasma triglyceride and NEFA concentrations, increased plasma adiponectin and leptin concentrations, and increased eWAT weight in mice fed high

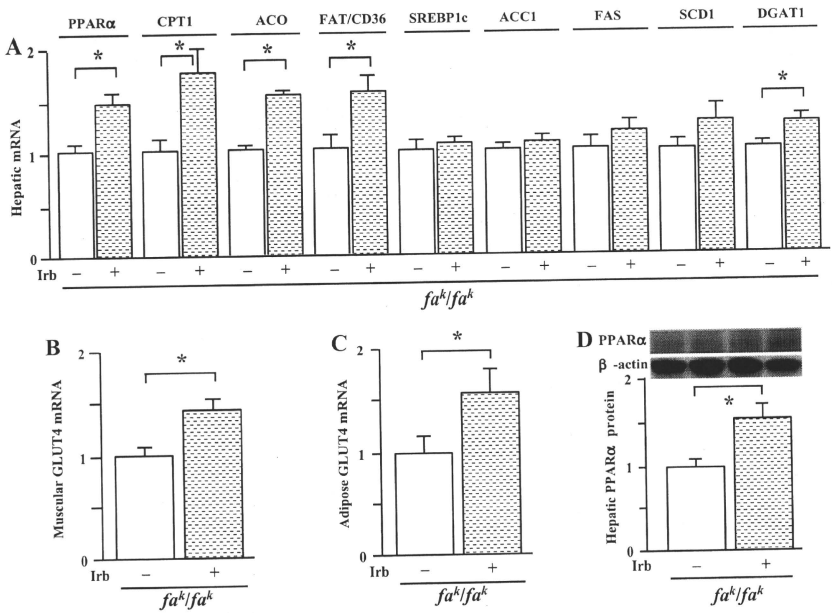

Figure 5

Gene expression profile. (A) mRNAs encoding peroxisome proliferator-activated receptor (PPAR)α, carnitine palmitoyltransferase (CPT)1a, acyl-CoA oxidase (ACO), fatty acid translocase (FAT)/CD36, sterol regulatory element-binding protein (SREBP)1c, acetyl-CoA carboxylase (ACC)1, fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD)1 and diacylglycerol acyltransferase (DGAT)1 (B) GLUT4 mRNA in skeletal muscle (C) GLUT4 mRNA in eWAT and (D) protein expression of PPARα in liver of male obese Koletsky (fa^k/fa^k) rats that were either untreated (-) or treated (+) with irbesartan (Irb) as described in Figure 1. Total RNA was isolated from liver, skeletal muscle or eWAT of individual rats using TRIzol. Quantitative PCR results were normalized to GAPDH, while the results from Western blot analysis were normalized to β-actin. Levels in obese control rats were arbitrarily assigned a value of 1. All values are means ± SEM ($n = 6$ each group). *$P < 0.05$. eWAT, epididymal white adipose tissue; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT, glucose transporter.

fat-containing diets (Stienstra *et al.*, 2008; Kuda *et al.*, 2009). Rosiglitazone treatment also decreased plasma glucose and triglyceride concentrations, but increased plasma adiponectin and leptin concentrations, as well as body weight in obese Zucker rats (Cai *et al.*, 2000; Reifel-Miller *et al.*, 2005). In addition, rosiglitazone stimulated adipose activity of DGAT, a key enzyme catalysing triglyceride synthesis, which is accompanied by a specific increase in the mRNA corresponding to adipose DGAT1, but not DGAT2, in rats (Festuccia *et al.*, 2009). Irbesartan has been shown to enhance PPARγ-dependent 3T3-L1 adipocyte differentiation, as reflected by increases in expression of certain adipogenic marker genes (Benson *et al.*, 2004; Schupp *et al.*, 2004). However, findings from immunohistochemical studies indicated that irbesartan treatment decreased PPARγ expression in the white and brown adipose tissues of obese Zucker rats in a dose-dependent fashion (Di Filippo *et al.*, 2005). In the present study, treatment of obese Koletsky (fa^k/fa^k) rats with irbesartan decreased plasma adiponectin concentrations, but was without effect on eWAT weight, adipocyte size and plasma leptin concentrations. In adipose tissue of Koletsky (fa^k/fa^k) rats, irbesartan was without effect on expression of PPARγ and its downstream target genes aP2, adiponectin, FAS, ACC1, CD36, SCD1, SREBP1c and DGAT1. Thus, our present findings do not support the contention that irbesartan improves hyperlipidaemia

and insulin sensitivity by modulating PPARγ signalling in adipose tissue of obese Koletsky rats. Further investigations are required to evaluate whether irbesartan also modulates expression of PPARβ/&delta, the other PPAR isoform involving lipid metabolism.

Muñoz *et al.* reported that irbesartan (50 mg·kg^{-1} for 6 months) decreased lipid accumulation in the liver of obese Zucker rats (Muñoz *et al.*, 2006; Toblli *et al.*, 2008). This group also demonstrated that irbesartan reduced adipocyte size and increased adiponectin expression in eWAT from these animals (Muñoz *et al.*, 2009). However, the present findings demonstrated that irbesartan (40 mg·kg^{-1} for 7 weeks) did not significantly affect hepatic steatosis, eWAT weight, plasma leptin concentration, adipocyte size or adipose adiponectin gene expression in obese Koletsky (fa^k/fa^k) rats. Further, both Russell *et al.* (2009), using the insulin-resistant JCR:LA-cp rat and the present study found that irbesartan treatment decreased plasma adiponectin concentrations. There are several possible factors that may be responsible for these discrepancies. First, the animal strains are different: although both obese Zucker rats and obese Koletsky rats carry leptin receptor mutations, there are still some important differences. In the fatty Zucker rat the *Lepr*^{fa} gene carries a point mutation in codon 269 that produces an amino acid sequence change adjacent to the ligand-binding domain of the receptor (Chua *et al.*, 1996); these animals are still responsive to leptin (Cusin *et al.*, 1996; Wang *et al.*, 1998; Wildman *et al.*, 2000). In contrast, the Koletsky rat (*Lepr*^{fak}) carries a premature stop codon and the mutant receptor lacks a transmembrane domain. This truncates all known isoforms of the receptor and, unlike the *Lepr*^{fa} mutation, the *Lepr*^{fak} mutation is null (Takaya *et al.*, 1996; Wu-Peng *et al.*, 1997; Wildman *et al.*, 2000). Second, blood pressure differs between the two rat strains: young obese Zucker rats (at least until 18 weeks of age) are normotensive (Muñoz *et al.*, 2006; Toblli *et al.*, 2008). In contrast, obese Koletsky rats are spontaneously hypertensive (SBP ≈ 170 mmHg at 10 weeks of age); which remained in excess of 130 mmHg after irbesartan treatment (Figure 1A). Finally, the different doses of irbesartan (50 mg·kg^{-1} vs. 40 mg·kg^{-1}) and durations of treatments (6 months vs. 7 weeks) were used in the Zucker and Koletsky rats so that comparisons are not straightforward.

In the present study, irbesartan treatment also decreased plasma insulin concentration and the HOMA-IR index in obese Koletsky rats. The results of quantitative PCR analysis demonstrated that GLUT4 gene expression in adipose tissue and skeletal muscle was increased by irbesartan treatment. These results suggest that insulin sensitivity improves after irbesartan treatment. NEFAs are a major link between obesity and insulin resistance/type 2 diabetes (McGarry, 2002; Bays *et al.*, 2004). Although a decrease in plasma NEFAs by irbesartan treatment may be associated with enhanced insulin sensitivity, the underlying molecular mechanisms require further investigation. It has been demonstrated that angiotensin II decreases local blood flow both in adipose and skeletal muscle tissue of normal-weight and obese subjects (Goossens *et al.*, 2004). Increased muscular blood flow is associated with increased glucose utilization (Baron *et al.*, 1992; Wiernsperger, 1994). Further specific investigations are required to determine the involvement of this increased blood flow in the enhanced insulin sensitivity induced by irbesartan

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Conflicts of interest

None.

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