A Postweaning Reduction in Circulating Ghrelin Temporarily Alters Growth Hormone (GH) Responsiveness to GH-Releasing Hormone in Male Mice But Does Not Affect Somatic Growth

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Ghrelin was initially identified as an endogenous ligand for the GH secretagogue receptor. When administrated exogenously, ghrelin stimulates GH release and food intake. Previous reports in ghrelin-null mice, which do not exhibit impaired growth nor appetite, question the physiologic role of ghrelin in the regulation of the GH/IGF-I axis. In this study, we generated a transgenic mouse that expresses human diphtheria toxin (DT) receptor (DTR) cDNA in ghrelin-secretion cells [ghrelin-promoter DTR-transgenic (GPDTR-Tg) mice]. Administration of DT to this mouse ablates ghrelin-secretion cells in a controlled manner. After injection of DT into GPDTR-Tg mice, ghrelin-secreting cells were ablated, and plasma levels of ghrelin were markedly decreased [nontransgenic littermates, 70.6 ± 10.2 fmol/ml *vs.* GPDTR-Tg, 5.3 ± 2.3 fmol/ml]. To elucidate the physiological roles of circulating ghrelin on GH secretion and somatic growth, 3-wk-old GPDTR-Tg mice were treated with DT twice a week for 5 wk. The GH responses to GHRH in male GPDTR-Tg mice were significantly lower than those in wild-type mice at 5 wk of age. However, those were normalized at 8 wk of age. In contrast, in female mice, there was no difference in GH response to GHRH between GPDTR-Tg mice and controls at 5 or 8 wk of age. The gender-dependent differences in response to GHRH were observed in ghrelin-ablated mice. However, GPDTR-Tg mice did not display any decreases in IGF-I levels or any growth retardation. Our results strongly suggest that circulating ghrelin does not play a crucial role in somatic growth. **(*Endocrinology* 151: 1743–1750, 2010)**

GH secretion is predominantly regulated by two hypothalamic peptides, one factor is GHRH and a second is somatostatin (SST). In 1999, Kojima *et al.* (1) discovered ghrelin as an endogenous ligand for the GH secretagogue receptor (GHS-R or ghrelin receptor) from rats' stomach. Ghrelin, an acylated peptide of 28 amino acids, is synthesized primarily in endocrine cells of the stomach, named X/A-like or ghrelin cells (2). Peripheral administration of ghrelin strongly stimulates GH secretion (1, 3). Because coadministration of GHRH and ghrelin produces synergistic effects on pituitary GH release (4), circulating ghrelin may play a role in augmentation of GHRH-stimulated GH pulses. Therefore, circulating ghrelin was thought to be the third peptide which

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Abbreviations: BMD, Bone mineral density; CT, computed tomography; DT, diphtheria toxin; DTR, DT receptor; GHS-R, GH secretagogue receptor; GPDTR-Tg, ghrelin-promoter DTR-transgenic; HB-EGF, heparin-binding epidermal growth factor-like growth factor; SST, somatostatin; WT, nontransgenic littermates.

regulates GH secretion. Indeed, patients with a functional mutation in GHS-R, ghrelin receptor, display familial short stature (5). Okimura *et al.* (6), however, demonstrated that circulating ghrelin levels do not correlated with those of GH; also, administration of a GHS antagosist to freely moving rats did not reduce plasma GH levels. Ghrelin knockout mice also exhibit normal growth patterns (7). On the other hand, ghrelin receptor knockout mice exhibit modest, but significant, body weight reductions and decreased serum IGF-I levels (8). Together, these findings question the physiologic significance of ghrelin in the regulation of GH secretion. As always with such model mice, there may be confounding factors, such as developmental adaptation and other compensatory mechanisms. To avoid these factors, it may be necessary to ablate ghrelin after birth or before puberty. Moreover, during the prepubertal and pubertal period, GH-dependent proportional body growth is observed in many mammalian species. The fetal growth is GH-independent, and growth during the early postnatal is only partial dependent upon GH. Therefore, to evaluate whether an absence of circulating ghrelin can influence a somatic growth through GH/IGF-I axis modification, we think that it is appropriate to choose a postweaning model.

In this study, we adopted a diphtheria toxin (DT) receptor (DTR)-mediated conditional and targeted cell ablation strategy to ablate ghrelin secretion cells, X/A-like cell, in a specific and controlled manner (9). We generated a transgenic mouse expressing human DTR cDNA, which encodes human heparin-binding epidermal growth factor-like growth factor (HB-EGF), under the control of the transcriptional regulatory regions of ghrelin. In this mouse, ghrelin-secreting cells express the human DTR and can be ablated after the administration of a small amount of DT. By using this transgenic mouse, we ablated ghrelin-secretion cells after weaning, which allowed us to evaluate the physiologic significance of ghrelin in GH secretion and somatic growth.

Materials and Methods

All animal experiments were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research. Procedures were performed in accordance with the principles and guidelines established by that committee.

Plasmid construction and generation of transgenic mice [ghrelin-promoter DTR-transgenic (GPDTR-Tg) mice]

The pGPDTR plasmid was constructed by replacement of the mouse albumin enhancer/promoter region of pMS7 (9) with a 4.1-kb *MulI-Hin*dIII fragment containing the 5′-flanking region of the human ghrelin gene (−4110/−33) derived from the p-4110/-33GHRE plasmid (human ghrelin promoter in pGL3) (Fig. 1A) (10). The 6.4-kb *NotI-XhoI* fragment of pGPDTR was microinjected into the pronucleus of fertilized eggs obtained from C57/B6 mice (SLC, Shizuoka, Japan). The viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (Japan CLEA, Osaka, Japan) by using standard techniques (11). Founder transgenic mice, identified by PCR analysis, were bred with C57BL/6 mice. Mice were housed in air-conditioned animal quarters, with light between 0800 and 2000 h. Except where noted, animals were fed standard rat chow (CE-2, 352 kcal/100 g; Japan CLEA) and water *ad libitum*.

Semiquantitative PCR

Total RNA was extracted using a Sepasol-RNA kit (Nacalai Tesque, Kyoto, Japan). RT used a high capacity cDNA RT kit (Applied Biosystems, Foster City, CA).

Semiquantitative PCR determined the distribution of the DTR in GPDTR-Tg mice, using the following primers: sense 5′-CCTCCTCTCGGTGCGGG-3′ and antisense 5′-AGTCACCAGTGCCGAGAGAACTG-3′. Thirty-five cycles of thermal was performed with 94 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec. Human heart mRNA (purchased from Clontech, Palo Alto, CA) was used as a positive control.

DT injection

DT was purchased from Sigma-Aldrich Japan (Tokyo, Japan). According to the previous report using DTR-mediated cell ablation systems (9), DT was injected im.

Histological procedures

Formalin-fixed, paraffin-embedded tissue sections were immunostained using avidin-biotin peroxidase complex methods (Vectastain "ABC" Elite kit; Vector Laboratories, Burlingame, CA) as described (11). Sections were incubated overnight at 4 C with antighrelin-(1–11) antiserum that specifically recognizes acylated ghrelin (final dilution, 1:5000). Tissue sections were also stained with hematoxylin and eosine.

Measurement of plasma ghrelin levels

Measurement of plasma ghrelin levels was performed as reported previously (12). Blood samples drawn from the retro-orbital vein at 1000 h were immediately transferred to chilled siliconized glass tubes containing Na2EDTA (1 mg/ml) and

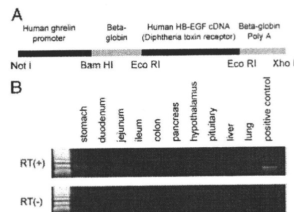

FIG. 1. Generation of GPDTR-Tg mice. A, The GPDTR-Tg construct contained a fusion gene comprised of the 5′flanking lesion of human ghrelin (4085 bp) and the DTR cDNA (human HB-EGF). B, Expression of DTR mRNA in various tissues of GPDTR-Tg mice at 8 wk of age. The human heart mRNA was used as a positive control.

TABLE 1. PCR primers and TaqMan probes

Ghrelin	Sense	5′-GCATGCTCTGGATGGACATG-3′
	Antisense	5′-TGGTGGCTTCTTGGATTCCT-3′
	Probe	5′-AGCCCAGAGCACCAGAAAGCCCA-3′
GH	Sense	5′-AAGAGTTCGAGCGTGCCTACA-3′
	Antisense	5′-GAAGCAATTCCATGTCGGTTC-3′
	Probe	5′-CCATTCAGAATGCCCAGGCTGCTTTC-3′
GHRH	Sense	5′-AGGATGCAGCGACACGTAGA-3′
	Antisense	5′-TCTCCCCTTGCTTGTTCATGA-3′
	Probe	5′-CCACCAACTACAGGAAACTCCTGAGCCA-3′
SST	Sense	5′-AGCTGAGCAGGACGAGATGAG-3′
	Antisense	5′-ACAGGATGTGAATGTCTTCCAGTT-3′
	Probe	5′-CGAACCCAGCAATGGCACCCC-3′
IGF-1	Sense	5′-ACCCGGACCTACCAAAATGAC-3′
	Antisense	5′-GGTGTGAAGACGACATGATGTGT-3′
	Probe	5′-CACCTGCAATAAAG-3′
GHS-R	Sense	5′-CACCAACCTCTATCCAGCAT-3′
	Antisense	5′-CTGACAAACTGGAAGCGTTTGCA-3′
	Probe	5′-TCCGATCTGCTCATCTTCCTGTGCATG-3′

aprotinin (1000 KIU/ml; Ohkura Pharmaceutical, Kyoto, Japan). After centrifugation at 4 C to separate out the plasma, hydrochloric acid was added to samples at a final concentration of 0.1 N. Plasma was immediately frozen and stored at −80 C until assayed. Plasma ghrelin concentrations were determined using a ghrelin ELISA kit (Mitsubishi Kagaku Iatron, Tokyo, Japan).

Real-time PCR analysis

Extraction of total RNA from various tissues and RT was performed as described above. Real-time quantitative PCR used an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using the primers and TaqMan probes described in Table 1. The mRNA expression levels of each gene were normalized to that of 18S rRNA. All samples were examined in triplicate in 96-well plates using an ABI Prism 7500 sequence detector according to the manufacturer's protocol.

GH provocative test

GH provocative test was carried out as previously described (12). These experiments were conducted in unanesthelized mice. Human GHRH was purchased from Sumitomo Pharmaceuticals Co., Ltd. (Osaka, Japan). Serum samples were collected at 15 and 30 min after sc injection of 180 mcg/kg of GHRH.

Ghrelin-rescue experiments

Osmotic infusion pumps (Alzet Micro-Osmotic pump, Model 1002; Durect Corp., Cupertino, CA) were implanted sc in 3-wk-old male GPDTR-Tg mice. Ghrelin (60 mcg/kg·d; Peptide Institute, Osaka, Japan) or saline was continuously infused through the osmotic infusion pumps. Then mice were started to treat with DT (50 ng/kg twice a week) a day after pump implantation. The average plasma ghrelin levels during continuous infusion of ghrelin were 31.6 ± 5.3 fmol/ml in the DT-treated GPDTR-Tg mice, whereas those without ghrelin infusion were 1.7 ± 0.2 fmol/ml. GH provocative test were carried out in these mice at the age of 5 wk.

Measurement of serum GH and IGF-I levels

Blood samples were collected from the tail veins of mice. Serum was isolated by centrifugation and stored at −20 C until assayed. Serum GH levels and IGF-I levels were measured using the appropriate EIA kits from SPI-BIO (Bonde, France) and Diagnostic Systems Laboratories, Inc. (Webster, TX), respectively, according to the manufacturers' instructions.

Measurement of body lengths

Mouse body length was measured by manual immobilization and extension of mice to determine nose-to-anus length. All measurements were performed by the same individual in a blind fashion.

Measurement of fat mass and bone mineral density (BMD)

The fat mass (% fat) and BMD of mice were measured by computed tomography (CT) (Laboratory CT; Lacita, Aloka, Japan) under pentobarbital anesthesia.

Statistical analysis

Results are expressed as the means ± SEM. Multiple comparisons between groups were made by Turkey-Krammer test, with α set at $P < 0.05$. The results on body weight and serum GH levels after GHRH injection were analyzed by a two-way ANOVA followed by Tukey's *post hoc* test, with α set at $P < 0.05$. Statistical analyses were carried out with STATVIE 4.0 software (Abacus Concepts, Inc., Berkley, CA).

Results

Generation of transgenic mice in which ghrelin can be ablated in a controlled manner

Transgenic mice

To elucidate physiologic role of ghrelin in GH secretion and somatic growth, we developed transgenic mice in which ghrelin can be ablated in controlled manner. We adopted a DTR-mediated conditional and targeted cell ablation strategy. We created transgenic mice that expressed the gene for the human DTR, human HB-EGF,

under the control of the ghrelin promoter. By injecting transgenes into 184 eggs, we obtained three lines of transgenic mouse (Tg 1-2, Tg 5-1, and Tg 5-8). We continued with the Tg 5-1 transgenic line, because Tg 1-2 animals did not exhibit decreases in plasma ghrelin levels after injection of high-dose DT and Tg 5-8 required high doses of DT (50 mcg/kg) to ablate ghrelin-producing cells (data not shown). In Tg 5-1 transgenic animals, semiquantitative PCR analysis revealed high expression of DTR mRNA in the stomach and weak expression in the duodenum and jejunum. No expression, however, could be detected in the ileum, colon, pancreas, hypothalamus, pituitary, liver, or lung (Fig. 1B). In Tg 5-1 mice, the ghrelin-producing cells of the stomach were ablated by injection with low-dose DT (10 or 50 ng/kg) (Fig. 2, A, B, and D). We therefore designated the Tg 5-1 transgenic line and nontransgenic littermates as GPDTR-Tg mice and wild-type (WT) mice, respectively.

Ablation of ghrelin-producing cell

To determine the dose and timeframe of DT injection, preliminarily studies were performed: GPDTR-Tg mice were injected with saline or DT twice a week at a dose of 10, 30, 50, 100, and 500 ng/kg (on d 0 and 2). Plasma ghrelin levels on d 4 were decreased to approximately 60, 30, 5, 5, and 5% of control mice (Tg mice treated with saline) after 10, 30, 50, 100, and 500 ng/kg of DT injection, respectively. Thus, we judged that 50 ng/kg of DT is the smallest effective dose to reduce plasma ghrelin. The final results using 10 and 50 ng/kg of DT were described below. Next, GPDTR-Tg mice were injected with 50 ng/kg of DT with four schedules: once a week (on d 0), twice a week (on d 0 and 2), three times a week (on d 0, 2, and 4), or daily (from d 0 to 6), and plasma ghrelin levels were measured on d 7. The once-a-week injection of DT was insufficient, but the twice-a-week injection of DT had enough effect on reduction in plasma ghrelin concentration.

To ablate ghrelin-producing cells, 8-wk-old male WT and GPDTR-Tg mice were injected im with 10 or 50 ng/kg DT daily on d 0 and 2 and analyzed on d 4. WT mice treated with saline or DT and GPDTR-Tg mice treated with saline were used as control mice.

To evaluate the effects of DT injection on ghrelin-producing cell, we analyzed stomach by immunohistochemical analysis with antighrelin antisera (Fig. 2A) and real-time PCR (Fig. 2, B and C). DT injection reduced in a dose-dependent manner both the number of ghrelin-positive cells and the expression of ghrelin mRNA in the stomach of GPDTR-Tg mice (Fig. 2, A and B). DT injection did not produce in any abnormalities in WT mice, because

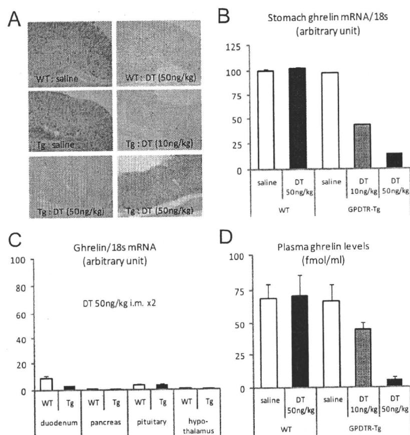

FIG. 2. Ablation of ghrelin-secretion cells. Eight-week-old male GPDTR-Tg mice (Tg) and nontransgenic littermates (WT) were injected with saline or 10 or 50 ng/kg of DT (im) on d 0 and 2, then analyzed on d 4. A, Histological analysis of stomach sections. Immunohistochemical analysis of ghrelin peptide expression and hematoxylin and eosin staining. Original magnification, ×100. B, Ghrelin mRNA levels in the stomach. C, Ghrelin mRNA levels in the duodenum, pancreas, pituitary, and hypothalamus of GPDTR-Tg and WT mice injected with 50 ng/kg of DT. D, Plasma ghrelin levels in GPDTR-Tg and WT mice. For B–D, data represent the means ± SEM (n = 8).

these mice do not possess the DTR, making them insensitive to DT. In transgenic animals, DT injection also reduced ghrelin mRNA expression in the duodenum, but not the pancreas, pituitary, or hypothalamus (Fig. 2C). Plasma ghrelin levels in GPDTR-Tg mice treated with 10 and 50 ng/kg of DT were decreased to approximately 60 and 5–7% of control mice, respectively (Fig. 2D). These results suggested that this transgenic mouse model is a useful tool for evaluating the physiologic role of circulating ghrelin.

Histological analysis with hematoxylin and eosin staining revealed that no inflammatory cell infiltration was seen in the stomach (Fig. 2A), small intestine, colon, pancreas, pituitary, and hypothalamus of the GPDTR-Tg mice with 50 ng/kg of DT injection. Other historical abnormalities were also not observed in these tissues (data not shown).

The effects of a reduction in circulating ghrelin after weaning on the GH/IGF-I axis and somatic growth

To study the effects of postweaning reductions in circulating ghrelin on the GH/IGF-I axis and somatic growth, 3-wk-old WT and GPDTR-Tg mice were treated with DT

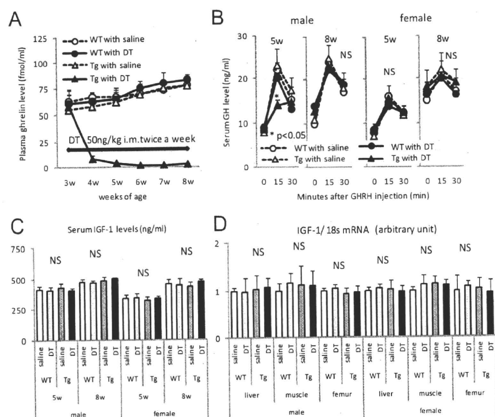

FIG. 3. The effects of a postweaning reduction in circulating ghrelin on the GH/IGF-I axis. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). A, Plasma ghrelin levels before and after DT injection. B, GH response to GHRH administration (180 μg/kg sc) in GPDTR-Tg and WT mice at 5 and 8 wk of age. C, Serum IGF-I levels of GPDTR-Tg and WT mice at 5 and 8 wk of age. D, IGF-I mRNA levels in liver, skeletal muscle, and femur in GPDTR-Tg and WT mice at 5 wk of age. Data represent the means ± SEM (n = 12).

(50 ng/kg) or saline twice a week for 5 wk (from 3 to 8 wk old). After DT injection, plasma ghrelin levels of GPDTR-Tg mice decreased rapidly. In GPDTR-Tg mice, ghrelin levels were undetectable by 5 wk of age, remaining so thereafter (Fig. 3A). The data obtained from GPDTR-Tg mice were compared with those from three groups of control mice (WT with saline, WT with DT, and GPDTR-Tg with saline).

To elucidate whether a postweaning reduction in circulating ghrelin can influence GH secretion, we measured basal serum GH levels and performed GH provocative test with GHRH. There were no differences in basal serum GH levels between GPDTR-Tg mice treated with DT and control mice in either males or females at 5 or 8 wk of age. GH provocative test with GHRH showed some intriguing results (Fig. 3B). The GH responses to GHRH in male GPDTR-Tg mice treated DT were significantly lower than those in three controls at 5 wk of age. However, those responses were normalized at 8 wk of age. On the other hand, there were no differences in GH response to GHRH among four groups (WT with saline or DT, and Tg with saline or DT) in females at 5 or 8 wk of age.

To elucidate whether temporarily attenuation of GH responses to GHRH can affect IGF-I regulation, we investigated serum IGF-I levels and IGF-I mRNA expressions in the liver, skeletal muscle, and distal femur. There were no differences in serum IGF-I levels among any animal groups in either males or females at 5 or 8 wk of age (Fig. 3C). There were also no differences in IGF-I mRNA expressions in the liver, skeletal muscle, or distal femur among any animal groups at 5 wk of age (Fig. 3D). We then investigated the effects of decreases in circulating ghrelin on the expression of mRNA encoding GHRH and SST within the hypothalamus and encoding GH and GHS-R in the pituitary. There were no differences in mRNA expression levels of these mediators among any animal groups in male and female at 5 wk of age (Fig. 4).

As expected from the results of the IGF-I studies, no evidence of growth retardation could be found in either male or female GPDTR-Tg mice treated with DT during the observation period. There were no difference in body weight or length in comparison with three groups of control mice at any point (Fig. 5, A and B, for male; and Fig. 5, D and E, for female animals). CT analysis of body composition demonstrated that there were no differences in percent fat or BMD among any animal groups at 5 and 8 wk of age (Fig. 5C for male, and Fig. 5F for female animals).

There were no differences in weekly food intake from 3 to 8 wk of age [WT *vs.* GPDTR-Tg (treated with DT); male, 18.4 ± 0.5 *vs.* 18.9 ± 0.7; female, 18.4 ± 1.0 *vs.* 18.5 ± 0.6 (g/wk)]. These results suggested that although GH responses to GHRH were temporarily reduced under conditions of decrease in circulating ghrelin, somatic growth was not impaired.

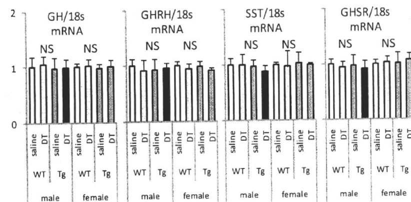

FIG. 4. The effects of a postweaning reduction in circulating ghrelin on the expression of mRNA encoding GHRH, SST,GH and GHS-R. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). Pituitary mRNA levels of GH and GHS-R and hypothalamic mRNA levels of GHRH and SST in GPDTR-Tg and WT mice at 5 wk of age. Data represent the means ± SEM (n = 12).

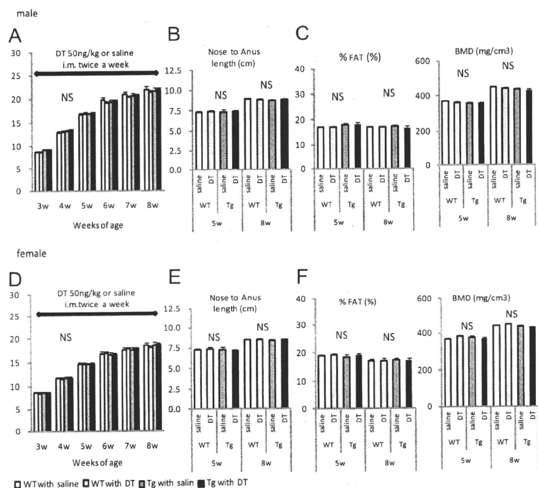

FIG. 5. The effects of a postweaning reduction in circulating ghrelin levels on somatic growth. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). A and D, Changes in body weight in male (A) and female mice (D). B and E, Nose to anus length in male (B) and in female mice (E) at 5 and 8 wk of age. C and F, Body composition (% Fat) and BMD as analyzed by CT in male (C) and in female mice (F) at 5 and 8 wk of age. Data represent the means ± SEM (n = 12).

GH response to GHRH in the ghrelin-rescued GPDTR-Tg mice

To elucidate whether GH responsiveness to GHRH can be ameliorated by ghrelin replacement in the ghrelin-ablated mice, GH provocative test were carried out in the DT-treated GPDTR-Tg mice whose circulating ghrelin were rescued by continuously administration of ghrelin with osmotic pump. The average plasma ghrelin levels during continuous infusion of ghrelin were 31.6 ± 5.3 fmol/ml in the DT-treated GPDTR-Tg mice, whereas those without ghrelin infusion were 1.7 ± 0.2 fmol/ml. GH provocative test were carried out at the age of 5 wk.

GH responsiveness to GHRH was ameliorated by ghrelin replacement. Serum GH levels at 0, 15, and 30 min after GHRH administration in the ghrelin-rescued mice were 7.8 ± 1.6, 26.2 ± 4.2, and 12.3 ± 0.8 ng/ml, respectively, whereas those in mice without ghrelin replacement were 6.8 ± 1.5, 10.9 ± 2.6, and 11.3 ± 1.6 ng/ml, respectively. These results suggested that attenuated response to GHRH seen in ghrelin-ablated mice without ghrelin replacement was due to acute ghrelin deficiency.

Discussion

In this study, we generated transgenic mice expressing the DTR driven by the transcriptional regulatory machinery of ghrelin. Injection of DT into this mouse can ablate ghrelin-secreting cells. Approximately 70–80% of circulating ghrelin originates from the stomach (13). Ghrelin-producing cells are also found throughout the small intestine, with the duodenum producing approximately one-tenth that of the stomach (14). Semiquantitative PCR revealed that DTR was only expressed in stomach and not in pituitary, hypothalamus, and pancreas and the intensity of the band of DTR in stomach was very low. Three possibilities might be considered to explain this result. The first is the low efficiency of gene transfection. Three lines of GPDTR-Tg mice that we generated in this study were inserted with low copy numbers of transgene (DTR cDNA). Thus, the expression levels of DTR mRNA could be very low even in stomach. The second is the efficiency of gene expression. In this study, we designed a fusion gene comprising the 4085-bp fragment contained a partial sequence of the 5′-flanking region of the human ghrelin gene and human DTR. The efficiency of gene expression driven by this fragment might be lower than those driven by the original ghrelin promoter region. The last, except gastrointestinal tract, transcription of ghrelin gene might be driven by a different size of fragment of the 5′-franking region. Immunohistochemical and PCR analyses demonstrated that ghrelin-secreting cells in the stomach and duodenum were ablated after DT injection into GPDTR-Tg mice, resulting in marked reduction of plasma ghrelin levels. In contrast, ghrelin-producing cells of the pituitary and hypothalamus were unaffected. Thus, this transgenic mouse is a useful model to explore the role of circulating ghrelin, because plasma ghrelin levels can be abrogated in a controlled manner without altering pituitary and hypothalamic ghrelin mRNA expression levels.

The physiologic roles of ghrelin in the regulation of GH secretion remain unclear, because previous reports using rodents deficient or reduced in ghrelin signals have given conflicting results (7, 8, 15, 16). Sun *et al.* (7) reported that ghrelin-deficient mice did not exhibit any growth retardation or decreases in serum IGF-I levels. Wortley *et al.*

(15) also were unable to observe any significant differences between ghrelin-deficient mice and WT mice in body weight or basal serum GH levels, when fed a standard diet. Moreover, Zigman *et al.* (16) demonstrated there was no significant difference in serum IGF-I levels between ghrelin receptor knockout and WT mice. Sun *et al.* (8), however, showed that ghrelin receptor knockout mice exhibited only a small reduction in body weight and serum IGF-I levels. In addition, Pantel *et al.* (5) showed that two unrelated families with short statue have a missense mutation of GHS-R. This mutation impairs the constitutive activity of the GHS-R. They also reported a young patient with growth delay who has a recessive partial isolated GH deficiency due to GHS-R mutations (17). These results indicate importance of ghrelin/GHS-R signals in GH secretion and somatic growth.

The purpose of this study is to evaluate whether an absence of circulating ghrelin can influence GH secretion and somatic growth via GH/IGF-I axis in mammals. First, we investigated basal serum GH levels and the GH response to GHRH. Although basal serum GH levels in the ghrelin-abrogated mice did not differ from those seen in WT mice, the GH responses to GHRH in male GPDTR-Tg mice were significantly lower than those in WT mice at 5 wk of age. As coadministration of GHRH and ghrelin produces synergistic effects on pituitary GH release (4), circulating ghrelin may play a role in augmentation of GHRH-stimulated GH pulses. Indeed, GH responsiveness to GHRH was ameliorated by ghrelin replacement in the ghrelin-ablated mice. However, the attenuated response to GHRH in the ghrelin-ablated mice had persisted only for a short term. The GH responses to GHRH in male GPDTR-Tg mice were recovered and were not different from those in WT mice at 8 wk of age. It is possible that an adaptation to reduced circulating ghrelin occurred within a short term. Indeed, Popovic *et al.* (18) reported that 10 patients who underwent total-gastrectomy at least 2 yr ago, a state of acquired chronic hypoghrelinemia, exhibited normal GH response to GHRH compared with normal subjects. Meanwhile, in female mice, there were no differences in either basal serum GH levels or GH response to GHRH between WT and GPDTR-Tg mice at 5 or 8 wk of age. The secretory pattern of GH in rodents is sexually differentiated. In male rats, GH is secreted in episodic pattern with low levels between pulses, whereas in females, the pulses are lower and plasma GH levels between pluses are higher than males (19). The secretory pattern of GH differs between male and female by 30 d of age (20). Gonadal steroids are thought to produce the sexual differences in GH secretion. We assumed that the sexual differences in GH response to GHRH in ghrelin-ablated mice may depend on gonadal steroids.

As GH secretion is pulsatile in nature, a single measurement of GH concentration in blood would not adequately reflect endogenous GH secretion. To estimate the amplitude and frequency of GH pulses, short-interval blood sampling under a conscious state is required. Such studies are difficult to perform in mice. Instead, we investigated serum IGF-I levels, skeletal muscle IGF-I mRNA expression, and anthropometric parameters that reflect pulsatile GH release under similar nutritional conditions (21). Serum IGF-I levels and IGF-I mRNA expression in skeletal muscle did not decrease in the ghrelin-abrogated mice in comparison with WT mice. These results suggest that circulating ghrelin does not play a dominant role in the GH/IGF-I axis. Due to significant differences between species in the regulation of GH secretion (21), we have to give careful considerations to apply the results of animal experiments concerning GH secretion directly to humans; insulin-induced hypoglycemia is a potent stimulus of GH secretion in humans, whereas rats respond to the stress of hypoglycemia by decreasing GH secretion (22, 23). L-arginine is a potent GH secretagogue in humans, but does not (or less overtly) stimulate GH secretion in rats (21, 24).

Somatic growth is affected not only by GH and IGF-I but also by thyroid hormones, sex steroids, and glucocorticoids. It also depends on genetic background and nutrition. Adequate nutrition is one of the most important factors affecting somatic growth. In present study, there were no differences in food intake between the ghrelin-abrogated mice and WT mice. Body weight, length, and body composition also were not influenced by plasma ghrelin levels. These results suggest that circulating ghrelin does not play a dominant role in somatic growth.

We cannot exclude the possibility that hypothalamic ghrelin may regulate GH secretion, as hypothalamic ghrelin-secreting cells were preserved in this animal model. Shuto *et al.* (25) demonstrated that transgenic rats expressing antisense GHS-R mRNA within the arcuate nucleus of the hypothalamus displayed growth retardation, suggesting that ghrelin/GHS-R systems in the hypothalamus function in the regulation of GH. Further studies will be needed to elucidate the role of hypothalamic ghrelin in GH secretion.

In summary, we have succeeded in generating transgenic mice in which circulating ghrelin can be abrogated in a controlled manner after birth. Our results suggest that circulating ghrelin does not play a crucial role in somatic growth.

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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

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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 $mg \cdot kg^{-1}$, 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	CGAGCAGTGCCAGACACAGCCATCG	*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-

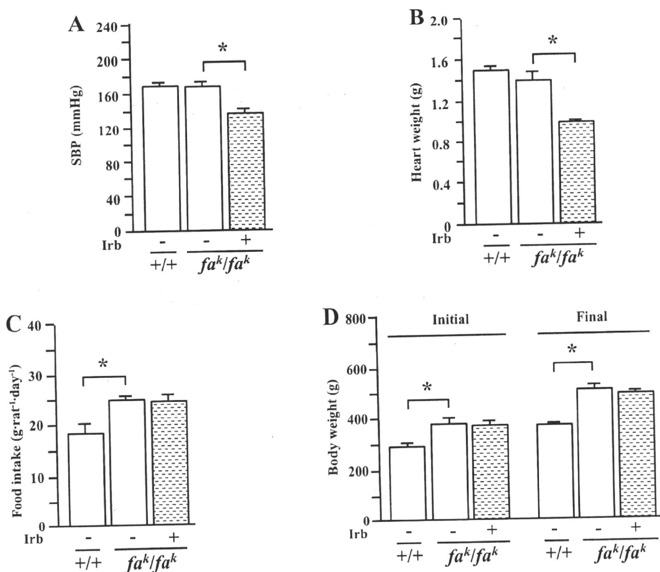

Figure 1
Systolic blood pressure (SBP) (A), heart weight (B), food intake (C) and initial and final body weight (D) in male lean (+/+) and obese (fa^k/fa^k) Koletsky rats. Animals received irbesartan (Irb) (40 mg·kg^{-1}·day^{-1}) or vehicle by oral gavage for 7 weeks. SBP was measured with a tail-cuff 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

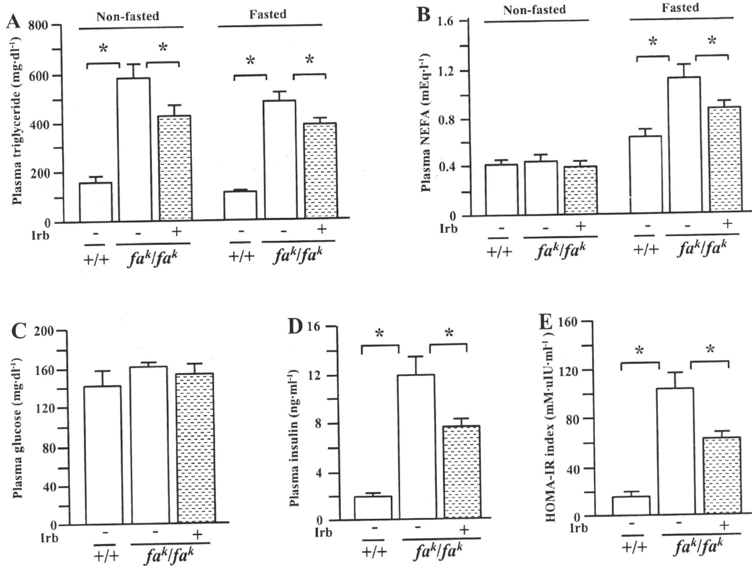

Figure 2
Non-fasted and fasted (rats deprived of food) plasma concentrations of triglyceride (A) and non-esterified fatty acids (NEFA) (B), fasted plasma glucose (C) and insulin (D) concentrations, and the index of the homeostasis model assessment of insulin resistance (HOMA-IR) (E) in male lean (+/+) 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.

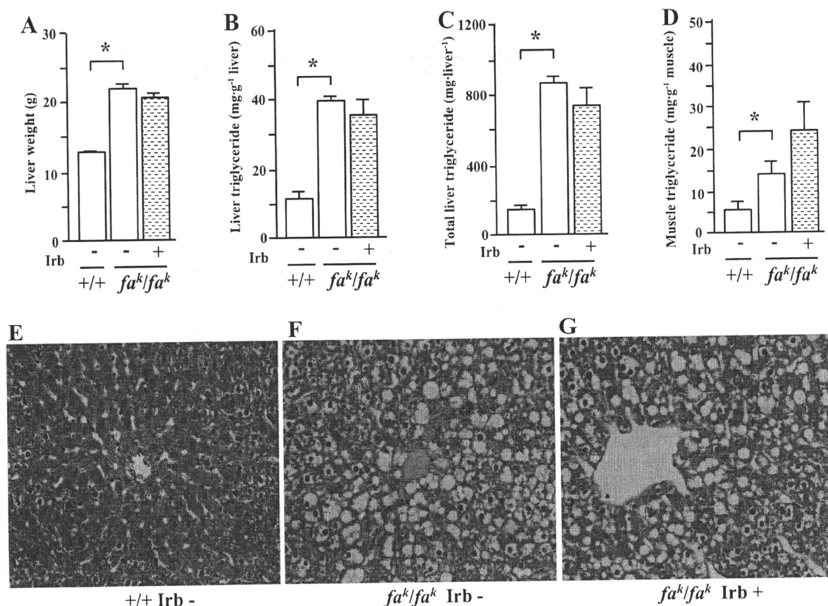

Figure 3

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

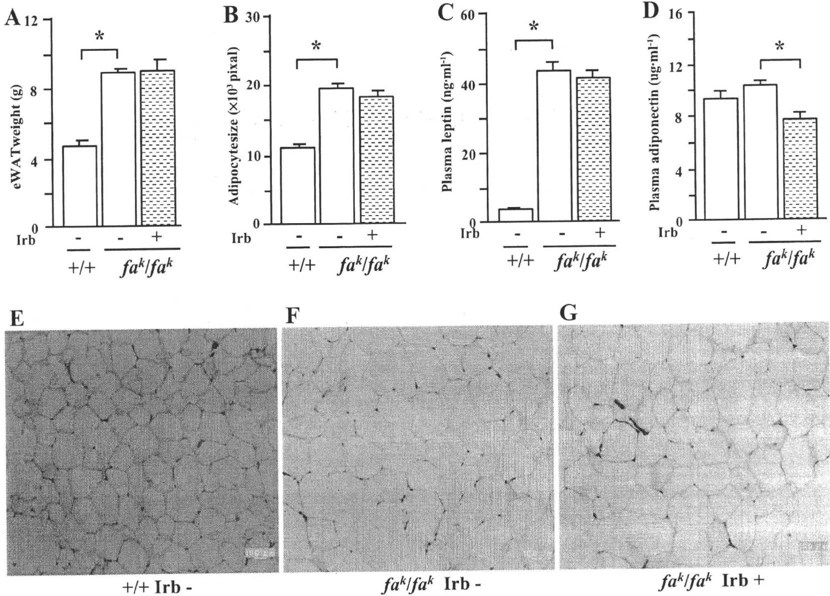

Figure 4
Epididymal white adipose tissue (eWAT) weight (A), adipocyte size (B), fasted (rats deprived of food) plasma concentrations of leptin (C) and adiponectin (D), and representative images showing histology of eWAT (haematoxylin and eosin–staining, X200) (E-G) in male lean (+/+) and obese (fa^k/fa^k) 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

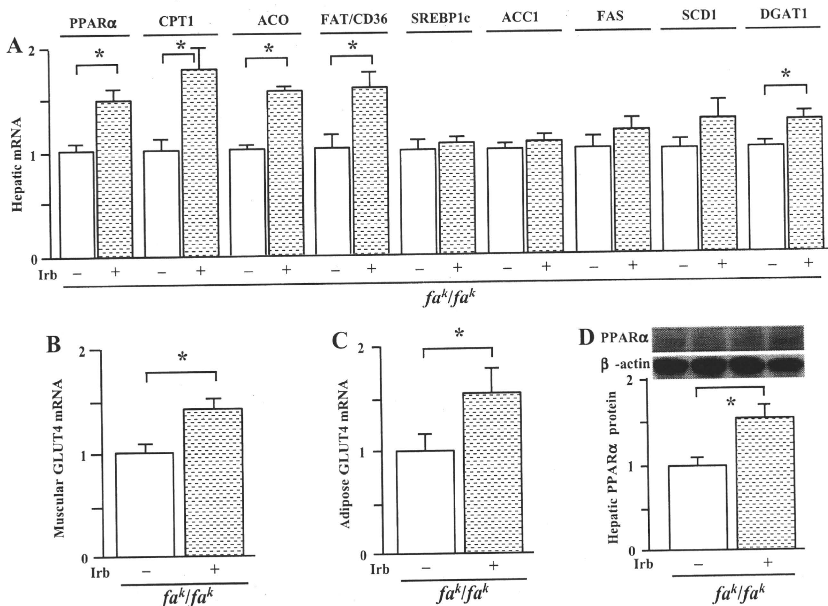

Figure 5

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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