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**Beta-adrenoceptor agonists downregulate adiponectin, but upregulate adiponectin receptor 2 and tumor necrosis factor-alpha expression in adipocytes**

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## **Abstract**

Recently, the insulin-sensitizing adipokine adiponectin and the insulin resistance-inducing adipokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were reported to inhibit each other's production in adipocytes. We investigated the effects of two  $\beta_3$ -adrenoceptor agonists, 5-[(2R)-2-[[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL-316,243) and ( $\pm$ )-(R\*,R\*)-[4-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]acetic acid (BRL37344), on the gene expression of adiponectin, two adiponectin receptors, and TNF- $\alpha$  in adipose tissues of C57BL/6J mice. CL-316,243 and BRL37344 downregulated adiponectin, but upregulated adiponectin receptor 2 (not receptor 1) in epididymal or/and subcutaneous white adipose tissues and in brown adipose tissue. TNF- $\alpha$  expression was upregulated only in epididymal adipose tissue. To further explore these effects, we treated differentiated 3T3-L1 adipocytes with the non-selective  $\beta$ -adrenoceptor agonist isoproterenol. As a result, adiponectin receptor 2 (but not receptor 1) gene expression and TNF- $\alpha$  protein expression increased, but gene expression and secretion of adiponectin decreased. The upregulation of adiponectin receptor 2 by isoproterenol is most likely via  $\beta_2, \beta_3$ -adrenoceptors, adenylyl cyclases, and protein kinase A (PKA). However, the accompanying activation of AMP-activated protein kinase (AMPK) may inhibit this upregulation. Our results suggest that upregulation of TNF- $\alpha$  and downregulation of adiponectin by  $\beta$ -adrenoceptor activation may contribute to the pathogenesis of catecholamine-induced insulin resistance, and that upregulation of adiponectin receptor 2 may be a feedback result of reduced adiponectin.

*Keywords:*  $\beta$ -adrenoceptor; Adiponectin; Adiponectin receptor; Tumor necrosis factor- $\alpha$ ; 3T3-L1 adipocyte; Adipose tissue; Catecholamine; Insulin resistance

## 1. Introduction

White adipose tissue is a key regulator of whole-body energy metabolism, playing a central role in the balance between energy storage and energy mobilization. Brown adipose tissue is specific for metabolic heat production. In rodent adipose tissues,  $\beta$ -adrenoceptors, predominantly  $\beta_3$ -adrenoceptors, are expressed. They mediate the major effects of adrenaline and noradrenaline, such as stimulating lipolysis in white adipose tissue and thermogenesis in brown adipose tissue. Regional differences exist between visceral and subcutaneous white adipose tissues. Visceral fat tissue is known to be lipolytically active in response to catecholamines compared to subcutaneous tissue (Lafontan and Berlan, 2003).

In recent years, it has been demonstrated that adipocytes also secrete biologically active molecules called adipokines. Among them, adiponectin and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) are two important adipokines that antagonistically influence insulin sensitivity. TNF- $\alpha$  is thought to be a causative factor of insulin resistance in fat tissue, involving insulin receptor autophosphorylation and insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation (Hotamisligil and Spiegelman, 1994; Hotamisligil et al., 1994, 1996). Adiponectin is a recently discovered insulin-sensitizing adipokine composed of an N-terminal collagenous domain and a C-terminal globular domain. In plasma it can exist as full-length adiponectin or globular domain adiponectin (Kadowaki and Yamauchi, 2005; Kadowaki et al., 2006; Fruebis et al., 2001; Yamauchi et al., 2001). Adiponectin stimulates fatty acid oxidation and glucose transport in muscle and inhibit gluconeogenesis in liver by activating AMP-activated protein kinase (AMPK) (Yamauchi et al., 2002). TNF- $\alpha$  and adiponectin are reported to mutually inhibit each other's production in adipose tissue (Maeda et al., 2001, 2002; Fasshauer et al., 2002).

Adiponectin receptor 1 and 2 mediate most effects of adiponectin. Adiponectin receptor 1, which

is a high-affinity receptor for globular domain adiponectin and a very low-affinity receptor for full-length adiponectin, is most abundantly expressed in skeletal muscle. Adiponectin receptor 2, which serves as a moderate-affinity receptor for both forms of adiponectin, predominates in liver (Yamauchi et al., 2003). Interestingly, both of these receptors are also expressed in adipocytes, where adiponectin itself is expressed and secreted (Fasshauer et al., 2004). A recent report suggests that adiponectin acts as an autocrine factor in adipocytes (Fu et al., 2005).

Studies demonstrate that  $\beta$ -adrenoceptor agonists decrease adiponectin gene expression via activation of a Gs-protein-PKA-dependent pathway (Fasshauer et al., 2001) in 3T3-L1 adipocytes and in white adipose tissue (Delporte et al., 2002). At the present time, however, little is known about the effects of  $\beta$ -adrenoceptor agonists on the regulation of adiponectin receptors and TNF- $\alpha$  in adipose tissues. Only a single study has reported that chronic treatment of diabetic (db/db) mice with  $\beta_3$ -adrenoceptor agonist increased adiponectin levels and decreased insulin levels in plasma, but left adiponectin receptors in adipose tissues unchanged (Oana et al., 2005). These results may only reflect the indirect effects of  $\beta_3$ -adrenoceptor agonists as altered insulin levels (Tsuchida et al., 2004) could, in turn, regulate adiponectin receptors. The direct influences of  $\beta$ -adrenoceptor agonists on adiponectin receptors are still unknown. In the present study, we examined the acute pharmacological effects of  $\beta$ -adrenoceptor agonists on adiponectin, adiponectin receptors and TNF- $\alpha$  expression in white and brown adipose tissues of lean mice *in vivo* and in differentiated 3T3-L1 adipocytes *in vitro*.

## **2. Materials and Methods**

### *2.1. Materials*

Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Gibco (Rockville, MD). H-89 and compound C were obtained from D. Western Therapeutics Institute, Inc. (Nagoya, Japan) and Calbiochem (La Jolla, CA), respectively. The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): 5-[(2R)-2-[[[(2R)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL-316,243), ( $\pm$ )-(R\*,R\*)-[4-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]acetic acid (BRL37344), isoproterenol, dobutamine, fenoterol, forskolin, 5-aminoimidazole-4-carboxamide-riboside (AICAR), insulin, isobutylmethylxanthine and dexamethasone.

## *2.2. Animals and treatments*

Twenty-three male C57BL/6J mice aged 10 weeks were obtained from Charles River Labs (Tokyo, Japan) and were fasted for 8 h before the experiments began.

Mice were injected with  $\beta_3$ -adrenoceptor agonist CL-316,243 (n=8) or BRL37344 (n=7) subcutaneously at a dose of 2 mg/kg of body weight. A second dose was given eight hours later. Control mice (n=8) received vehicle (0.9% saline) only.

Sixteen hours after the first injection, all mice were sacrificed by diethyl ether overdose. Epididymal and inguinal white adipose tissue, as well as interscapular brown adipose tissue were immediately removed, frozen in liquid nitrogen, and stored at -80°C until extraction of RNA.

All procedures involving animals were approved by the Animal Care and Use Committee of University of Tsukuba. Moreover, the protocols complied with European Community guidelines for the use of experimental animals.

### *2.3. Cell culture and differentiation*

3T3-L1 preadipocytes (American Type Culture Collection, Rockville, MD) were grown at 37 °C in 5% CO<sub>2</sub> in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (minimal medium). Two days after reaching confluence, preadipocytes were cultured for 48 h in minimal medium further supplemented with 1.7 μM insulin, 0.5 mM isobutylmethylxanthine, and 1 μM dexamethasone to induce differentiation. After culturing another 48 h in minimal medium supplemented only with 1.7 μM insulin, cells were refed with minimal medium for 4 days. At the time of the experiments, more than 90% of the cells had accumulated fat droplets as determined by Oil Red O staining. Adipocytes were maintained for 6 h in serum-free medium before various effectors were added.

### *2.4. Quantitative real-time PCR*

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), and 1 μg of total RNA was reverse transcribed into cDNA using the RNA PCR kit (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's instructions.

Quantitative real-time PCR was performed using ABI PRISM 7900 Sequence Detector, TaqMan Universal PCR Master MIX and Assays-on-Demand Gene Expression Assay MIX (Product No. adiponectin: Mm00456425ml, adiponectin receptor 1: Mm01291334mH, adiponectin receptor 2: Mm00815950ml, TNF-α: Mm00443258ml and 18S rRNA: 4308329) (Applied Biosystems, Foster City, CA). The thermal cycling protocol was 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Adiponectin, adiponectin receptor 1, adiponectin receptor 2, and TNF-α mRNA levels were normalized to those of 18S rRNA.

## *2.5. Assays for intracellular cAMP, adiponectin release and TNF- $\alpha$ protein level*

Intracellular cAMP, released adiponectin in culture medium, and TNF- $\alpha$  protein levels were measured using ELISA kits from Amersham Biosciences (Amersham, UK), Otsuka Pharmaceutical (Tokyo, Japan) and eBiosciences (San Diego, CA), respectively, according to the manufacturers' instructions.

## *2.6. Western blots*

Cells were lysed with cell lysis buffer (Cell Signaling Technology, Inc., Beverly, MA). Lysates were analyzed by Western blot using either anti-phospho-AMPK antibody (Thr172) or AMPK antibody (Cell Signaling Technology, Inc., Beverly, MA).

## *2.7. Statistical analysis*

Results are expressed as the mean  $\pm$  S.E.M. Significance was assessed by ANOVA with the Dunnett's test. *P* values  $<0.05$  were considered significant and  $<0.01$  highly significant.

# **3. Results**

*3.1.  $\beta_3$ -adrenoceptor agonists downregulate adiponectin, but upregulate adiponectin receptor 2 and TNF- $\alpha$  in adipose tissues of C57BL/6J mice*



As  $\beta_3$ -adrenoceptor is the predominant subtype of  $\beta$ -adrenoceptors expressed in rodent adipocytes, we investigated the effects of two  $\beta_3$ -adrenoceptor agonists, CL-316,243 and BRL37344, on adiponectin, adiponectin receptor, and TNF- $\alpha$  gene expression in adipose tissues of C57BL/6J mice *in vivo*.

Steady-state adiponectin mRNA levels in epididymal white adipose tissue were 2.3-fold higher than that of subcutaneous white adipose tissue and 3-fold higher than that of brown adipose tissue. When we measured levels from the CL-316,243-treatment group, adiponectin gene expression was significantly reduced in epididymal white adipose tissue and brown adipose tissue by 52% and 56%, respectively, as compared to the control group ( $P<0.01$ ). In the BRL37344-treatment group, adiponectin mRNAs were decreased by 31% in subcutaneous white adipose tissue ( $P<0.05$ ) and by 46% in brown adipose tissue ( $P<0.01$ ) (Fig. 1A).

Adiponectin receptor 1 gene expression in subcutaneous white adipose tissue was less than in epididymal white adipose tissue and brown adipose tissue. Treatment with  $\beta_3$ -adrenoceptor agonists did not affect expression in any group (Fig. 1B).

Adiponectin receptor 2 mRNAs, however, were upregulated in subcutaneous white adipose tissue and brown adipose tissue in the CL-316,243-treatment group (2.3-fold,  $P<0.01$  and 2.2-fold,  $P<0.05$ , respectively). In the BRL37344-treatment group, adiponectin receptor 2 mRNAs were increased in epididymal white adipose tissue (1.8-fold,  $P<0.01$ ), subcutaneous white adipose tissue (2.0-fold,  $P<0.01$ ) and brown adipose tissue (2.9-fold,  $P<0.01$ ) (Fig. 1C).

Steady-state TNF- $\alpha$  mRNA levels in subcutaneous white adipose tissue were notably higher than those in epididymal white adipose tissue and brown adipose tissue. TNF- $\alpha$  mRNAs were upregulated in epididymal white adipose tissue in the CL-316,243-treatment group (8.9-fold,  $P<0.05$ ) and in the BRL37344-treatment group (14.5-fold,  $P<0.01$ ) (Fig. 1D).

### *3.2. Non-selective $\beta$ -adrenoceptor agonist isoproterenol downregulates adiponectin, but upregulates adiponectin receptor 2 and TNF- $\alpha$ in differentiated 3T3-L1 adipocytes*

The *in vivo* data led us to speculate that  $\beta$ -adrenoceptor agonists could downregulate adiponectin while upregulating adiponectin receptor 2 and TNF- $\alpha$  gene expression in adipocytes. To test this hypothesis *in vitro*, we added the non-selective  $\beta$ -adrenoceptor agonist isoproterenol to differentiated 3T3-L1 adipocytes.

Treatment of 3T3-L1 adipocytes with 10  $\mu$ M isoproterenol for 16 h reduced the level of adiponectin mRNA by 50% ( $P<0.01$ ). Treatment did not change adiponectin receptor 1 levels, but it upregulated adiponectin receptor 2 gene expression 2.2-fold ( $P<0.01$ ) (Fig. 2A). Suppressed adiponectin and elevated adiponectin receptor 2 mRNA levels were significant after 8 h and persisted for up to 24 h after 10  $\mu$ M isoproterenol treatment ( $P<0.01$ ) (Fig. 2B).

Treatment with isoproterenol for 24 h decreased adiponectin release by about 30% (Fig. 2C), but increased TNF- $\alpha$  protein expression by about 50% (Fig. 2D). Adiponectin receptor 2 protein expression, TNF- $\alpha$  mRNA expression and the amount of TNF- $\alpha$  released into the medium were too low to measure accurately.

### *3.3 $\beta_2, \beta_3$ -adrenoceptor agonists upregulate adiponectin receptor 2 in differentiated 3T3-L1 adipocytes*

To determine which subtype(s) of  $\beta$ -adrenoceptor mediated the effect of isoproterenol on adiponectin receptor 2 gene expression, different subtype-specific  $\beta$ -adrenoceptor agonists were

tested. The  $\beta_1$ -adrenoceptor agonist dobutamine was without effect, while the  $\beta_2$ -adrenoceptor agonist fenoterol and  $\beta_3$ -adrenoceptor agonists CL-316,243 and BRL37344 (10  $\mu$ M) each significantly enhanced adiponectin receptor 2 mRNA expression by about 2-fold ( $P<0.01$ ) after 16 h treatment (Fig. 3A). These stimulatory effects of  $\beta_2, \beta_3$ -adrenoceptor agonists were statistically significant at concentrations as low as 100 nM. However, none of the selective  $\beta$ -adrenoceptor agonists affected adiponectin receptor 1 gene expression (data not shown).

#### *3.4. Isoproterenol stimulates adiponectin receptor 2 mainly via adenylyl cyclase and PKA*

Typically, the main signaling pathway of  $\beta$ -adrenoceptors leads to activation of Gs-proteins that, in turn, activate adenylyl cyclases for cAMP generation. This second messenger then activates protein kinase A (PKA). To look for activation of adenylyl cyclases, we measured cAMP levels following  $\beta$ -adrenoceptor activation. A 6-fold induction of intracellular cAMP was observed when differentiated 3T3-L1 adipocytes were treated with 10  $\mu$ M isoproterenol for 10 min ( $P<0.01$ ) (Fig. 3B). Treatment with forskolin, a direct stimulator of adenylyl cyclase, for 8 h increased adiponectin receptor 2 mRNAs 2-fold (2  $\mu$ M,  $P<0.01$ ) and 3-fold (10  $\mu$ M,  $P<0.01$ ) (Fig. 3C), but did not change adiponectin receptor 1 mRNAs (data not shown).

To look for downstream activation of PKA, we used the selective antagonist H-89. After 1 h of pre-incubation with 10  $\mu$ M H-89, isoproterenol was added for 16 h. H-89 reversed the stimulatory effect of isoproterenol on adiponectin receptor 2 gene expression (Fig. 3D).

#### *3.5. Activation of AMPK inhibits isoproterenol-induced upregulation of adiponectin receptor 2*

AMP-activated protein kinase (AMPK) is a protein which is activated in response to an increase in the ratio of AMP to ATP within the cell and therefore acts as an efficient sensor for the cellular energy state. Binding of AMP activates AMPK allosterically and induces phosphorylation the threonine residue (Thr-172), the site whose phosphorylation is essential for activity. Reports show that isoproterenol can promote the activation of AMPK in adipocytes (Moule et al., 1998; Yin et al. 2003). Therefore, we also examined the effects of AMPK activation on adiponectin receptor mRNA expression.

Culture of 3T3-L1 adipocytes in the presence of 10  $\mu$ M isoproterenol for 15 min or 1 mM and 2 mM 5-aminoimidazole-4-carboxamide-riboside (AICAR), a cell-permeable AMPK activator, for 30 min increased phosphorylation of AMPK at Thr172 but did not affect total AMPK levels (Fig. 3E).

Adiponectin receptor 2 gene expression was reduced 40% and 60% by 1 mM and 2 mM 5-aminoimidazole-4-carboxamide-riboside (AICAR), respectively ( $P<0.01$ ) (Fig. 3F), but adiponectin receptor 1 expression was not altered (data not shown).

3T3-L1 adipocytes were preincubated with 10  $\mu$ M compound C, a selective inhibitor of AMPK, for 2 h before treatment with isoproterenol for 24 h. The addition of compound C enhanced the stimulatory effect of isoproterenol on adiponectin receptor 2 gene expression (Fig. 3G).

#### **4. Discussion**

In the present study, we demonstrate for the first time that the  $\beta_3$ -adrenoceptor agonists CL-316,243 and BRL37344 increased TNF- $\alpha$  gene expression in rodent epididymal white adipose tissue *in vivo*, and that isoproterenol increased TNF- $\alpha$  protein expression in differentiated 3T3-L1 adipocytes *in vitro*. The reason TNF- $\alpha$  expression is upregulated only in epididymal white adipose

tissue may be that lipid metabolism responses to catecholamines differ in epididymal and subcutaneous white adipose tissue and brown adipose tissue. Epididymal adipose tissue, a type of visceral fat tissue, is lipolytically active compared to subcutaneous fat (Lafontan and Berlan, 2003). As high concentrations of (1 mM) free fatty acid, the product of triglyceride hydrolysis, are reported to increase TNF- $\alpha$  secretion in adipocytes (Nguyen et al., 2005), the abundantly produced free fatty acids in epididymal adipose tissue may contribute to the upregulation of TNF- $\alpha$ , while the amount of free fatty acids in subcutaneous adipose tissue is inadequate. Compared with white adipose tissue, in which free fatty acids are produced and released to provide energy to other organs and tissues in response to catecholamines, in brown adipose tissue free fatty acids are oxidized in mitochondria as an energy source for thermogenesis. The concentration of free fatty acids in brown adipose tissue may therefore be inadequate to regulate TNF- $\alpha$  expression.

Growing evidence suggests that catecholamines and increased activity of the sympathetic nervous system induce insulin resistance (Reaven et al., 1996; Hoiegggen et al., 2000; Facchini et al., 1996; Maison et al, 2000; Blüher et al., 2000). However, the mechanisms regulating these effects are still not completely understood. In our study, TNF- $\alpha$ , which induces insulin resistance (Hotamisligil, 1999), is upregulated by  $\beta$ -adrenoceptor agonists. Furthermore, the expression and release of adiponectin, an insulin-sensitizing adipokine, were confirmed to be downregulated as previously reported (Delporte et al., 2002; Fasshauer et al., 2001). As it is thought that adiponectin and TNF- $\alpha$  mutually inhibit each other's production in adipose tissue (Maeda et al., 2001, 2002), mutual influences of the two adipokines might exist if  $\beta$ -adrenergic stimulation changes the regulation of one of them first. The upregulation of TNF- $\alpha$  and downregulation of adiponectin by  $\beta$ -adrenoceptor activation in adipocytes may contribute to the pathogenesis of catecholamine-induced insulin resistance.

This is also the first report that  $\beta$ -adrenoceptor agonists increase adiponectin receptor 2 gene expression, but not adiponectin receptor 1, in white and brown adipose tissue of C57BL/6J mice *in vivo* and in differentiated 3T3-L1 adipocytes *in vitro*. The upregulation of adiponectin receptor 2 might partly ameliorate insulin resistance by improving adiponectin affinity, but it could not completely rescue  $\beta$ -adrenoceptor activation-induced insulin resistance.

As an autocrine factor, adiponectin is reported to promote adipocyte differentiation and lipid accumulation, and is proposed to act to maintain adipocyte size and mass at baseline levels (Fu et al., 2005). Our results showing that isoproterenol-induced adiponectin receptor 2 upregulation is accompanied by a reduced adiponectin expression (Fig. 2B) are consistent with the recent report indicating that adiponectin inhibits adiponectin receptor 2 gene expression (Bauche et al., 2006). Based on these findings, we propose the upregulation of adiponectin receptor 2 may be a consequence of reduced adiponectin. Under the conditions where  $\beta$ -adrenergic stimulation downregulates adiponectin, we propose that adiponectin receptor 2, which accounts for almost half of the total adiponectin receptor expression in fat tissue (Beylot et al., 2006), is locally upregulated to compensate for the loss of adiponectin. This response may augment the overall autocrine effect of adiponectin, which in turn, helps to reaccumulate lipid lost by lipolysis upon  $\beta$ -adrenergic stimulation. Thus, the use of the feedback response may facilitate lipid homeostasis of adipocytes.

The  $\beta_2$ -adrenoceptor agonist fenoterol also shows the same stimulatory effect as  $\beta_3$ -adrenoceptor agonists on adiponectin receptor 2 in adipocytes *in vitro* (Fig. 3A). As  $\beta_2, \beta_3$ -adrenoceptor agonists are not completely subtype-specific at high concentrations, we confirmed the effects of fenoterol, CL-316,243 and BRL37344 at concentrations as low as 100 nM to make sure that the effects are through  $\beta_2$  or  $\beta_3$ -adrenoceptors, respectively. This finding suggests that the effect of  $\beta_2$ -adrenoceptor agonists on adiponectin receptor 2 may also exist in other organs and tissues where

both  $\beta_2$ -adrenoceptor and adiponectin receptor 2 are present, such as liver and pancreatic islets.

In adipocytes,  $\beta$ -adrenoceptor activation result in the simultaneous stimulation of both PKA and AMPK.  $\beta$ -adrenergic stimulation-induced AMPK activation in adipocytes is associated with an increased AMP/ATP ratio (Koh et al., 2007). Both PKA and AMPK are likely to work in synergy to regulate key enzymes involved in metabolism (Cohen and Haride, 1991; Nielsen and Richter, 2003; Dyck et al., 1999; Boone et al., 1999; Winder et al., 1997). However, they can also antagonize one another, as is the case in the regulation of hormone-sensitive lipase, which regulates lipolysis in adipose and muscle tissue (Wat et al., 2005). In our study, we demonstrate that isoproterenol regulates adiponectin receptor 2 mainly via activation of adenylyl cyclases and PKA (Fig. 3B-D). However, we also found that AMPK activation may inhibit the upregulation of adiponectin receptor 2 (Fig. 3E-G). The findings indicate that PKA and AMPK antagonize one another in the regulation of adiponectin receptor 2 in adipocytes.

AMPK is thought to improve insulin sensitivity in muscle and liver (Kahn et al., 2005; Long and Zierath, 2006). But it is also reported that activation of AMPK inhibits basal and insulin-stimulated glucose uptake and lipogenesis in isolated adipocytes (Gaidhu et al., 2006). Our results show that AMPK activation reduces adiponectin receptor 2 gene expression. These data suggest that the AMPK signal cascade may oppose insulin effects in fat cells.

Our study demonstrates that adiponectin receptor 2 is upregulated by  $\beta$ -adrenoceptor agonists while adiponectin receptor 1 is unchanged. A possible reason underlying this discrepancy may be the receptor's affinity for the various adiponectin forms. Adiponectin receptor 2 is the receptor that mediates action of the main full-length form of adiponectin in human plasma (Fruebis et al., 2001; Yamauchi et al., 2003). Adiponectin receptor 1 binds globular domain adiponectin, the proteolytic cleavage product of full-length adiponectin, reported to be produced by leukocyte elastase secreted from activated monocytes and/or neutrophils (Fruebis et al., 2001; Yamauchi et al., 2003; Waki et

al., 2005). Since full-length adiponectin newly produced by adipocytes is bound by its high affinity receptor 2 (but not receptor 1), adiponectin receptor 2 may play a major role in mediating the metabolic effects of adiponectin as an autocrine factor in adipose tissues.

In our study, adiponectin receptor 2 is also regulated by the adenylyl cyclase stimulator forskolin (Fig. 3C) and the AMPK activator 5-aminoimidazole-4-carboxamide-riboside (AICAR) (Fig. 3F), while adiponectin receptor 1 is unaffected. It is also reported that growth hormone and PPAR $\alpha,\gamma$  agonists upregulate only adiponectin receptor 2 in adipocytes *in vitro* (Fasshauer et al., 2004; Tsuchida et al., 2005). These findings indicate that the regulatory mechanism of adiponectin receptor 2 is wholly different from that of adiponectin receptor 1.

A previous report showed that  $\beta$ -adrenoceptor agonists decrease adiponectin gene expression in white adipose tissue (Delporte et al., 2002). We demonstrate for the first time that adiponectin is also suppressed in brown adipose tissue (Fig. 1A). Although brown adipose tissue, which regulates body temperature, plays a different role from white adipose tissue, the effects of  $\beta_3$ -adrenoceptor agonists CL-316,243 and BRL37344 on the regulation of adiponectin receptor 2 and adiponectin are the same in both types of adipose tissue.

Taken together, we demonstrate *in vivo* and *in vitro* that  $\beta$ -adrenoceptor agonists downregulate adiponectin, but upregulate adiponectin receptor 2 (not receptor 1) and TNF- $\alpha$  expression in adipocytes. Our results suggest that upregulation of TNF- $\alpha$  and downregulation of adiponectin by  $\beta$ -adrenoceptor activation may contribute to the pathogenesis of catecholamine-induced insulin resistance, and that upregulation of adiponectin receptor 2 may be a feedback result of reduced adiponectin.

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## Figure legends

Fig. 1. The effects of two  $\beta_3$ -adrenoceptor agonists, CL-316,243 (CL) and BRL37344 (BRL), on (A) adiponectin, (B) adiponectin receptor 1, (C) adiponectin receptor 2 and (D) TNF- $\alpha$  gene expression in epididymal white adipose tissue (EPI WAT), subcutaneous adipose tissue (SC WAT), and brown adipose tissue (BAT) in C57BL/6J mice. Mice were injected subcutaneously with two doses (2mg/kg) of CL-316,243, BRL37344 or vehicle. Adipose tissues were collected and the mRNA expression of adiponectin, adiponectin receptor 1, adiponectin receptor 2 and TNF- $\alpha$  were quantified by real-time PCR and normalized to 18s rRNA mRNA levels. Results are expressed as the means  $\pm$  S.E.M. Open bars, control (CON) mice (n=8); light gray bars, CL316,243-treated mice (n=8); dark gray bars, BRL37344-treated mice (n=7). \* $P$ <0.05, \*\* $P$ <0.01

Fig. 2. The effect of non-selective  $\beta$ -adrenoceptor agonist isoproterenol (ISO) on adiponectin, adiponectin receptor 1, and adiponectin receptor 2 gene expression, adiponectin release and TNF- $\alpha$  protein expression in fully differentiated 3T3-L1 adipocytes. (A) Adiponectin, adiponectin receptor 1 and adiponectin receptor 2 gene expression following 16 h treatment of isoproterenol (10  $\mu$ M). (B) Time course effects of 10  $\mu$ M isoproterenol on adiponectin and adiponectin receptor 2 gene expression. (C) Adiponectin release following 24 h treatment with isoproterenol. (D) TNF- $\alpha$  protein expression following 24 h treatment with isoproterenol. The mRNA expression of adiponectin, adiponectin receptor 1 and adiponectin receptor 2 were quantified by real-time PCR and normalized to 18s rRNA mRNA levels. Adiponectin release and TNF- $\alpha$  protein expression were quantified by ELISA and normalized to total protein concentration. Results are expressed as the means  $\pm$  S.E.M.; (n=3) \* $P$ <0.05, \*\* $P$ <0.01

Fig. 3. Mechanisms for upregulating adiponectin receptor 2 gene expression by isoproterenol. (A) Adiponectin receptor 2 gene expression following 16 h treatments of  $\beta_1$ -adrenoceptor agonist dobutamine (DOBU),  $\beta_2$ -adrenoceptor agonist fenoterol (FENO), and  $\beta_3$ -adrenoceptor agonists CL-316,243 (CL) and BRL37344 (BRL) (10  $\mu$ M each). (B) Intracellular cAMP following 10 min treatment with isoproterenol (ISO). (C) Adiponectin receptor 2 gene expression following 8 h treatment with adenylyl cyclase stimulator forskolin (FOR). (D) Effect of PKA inhibitor H-89 on isoproterenol-induced adiponectin receptor 2 gene expression. After pre-incubation with H-89 (10  $\mu$ M) for 1 h, isoproterenol (10  $\mu$ M) was added for 16 h. (E) P-AMPK (Thr172) (top panel) and total AMPK protein (bottom panel) following 15 min treatment with isoproterenol (ISO) or 30 min with AMPK stimulator 5-aminoimidazole-4-carboxamide-riboside (AICAR). (F) Adiponectin receptor 2 gene expression following 16 h treatment with AICAR. (G) Effect of AMPK inhibitor

compound C (Comp) on isoproterenol-induced adiponectin receptor 2 gene expression. After pre-incubation with compound C (10  $\mu$ M) for 2 h, isoproterenol (10  $\mu$ M) was added for 24 h. Intracellular cAMP levels were measured by ELISA. The mRNA expression of adiponectin, adiponectin receptor 1 and adiponectin receptor 2 were quantified by real-time PCR and normalized to 18s rRNA mRNA levels. P-AMPK and total AMPK proteins were analyzed by Western blot using either anti-phospho-AMPK antibody (Thr172) or AMPK antibody. Results are expressed as the means  $\pm$  S.E.M.; (n=3) \*\* $P$ <0.01



Fig.1.

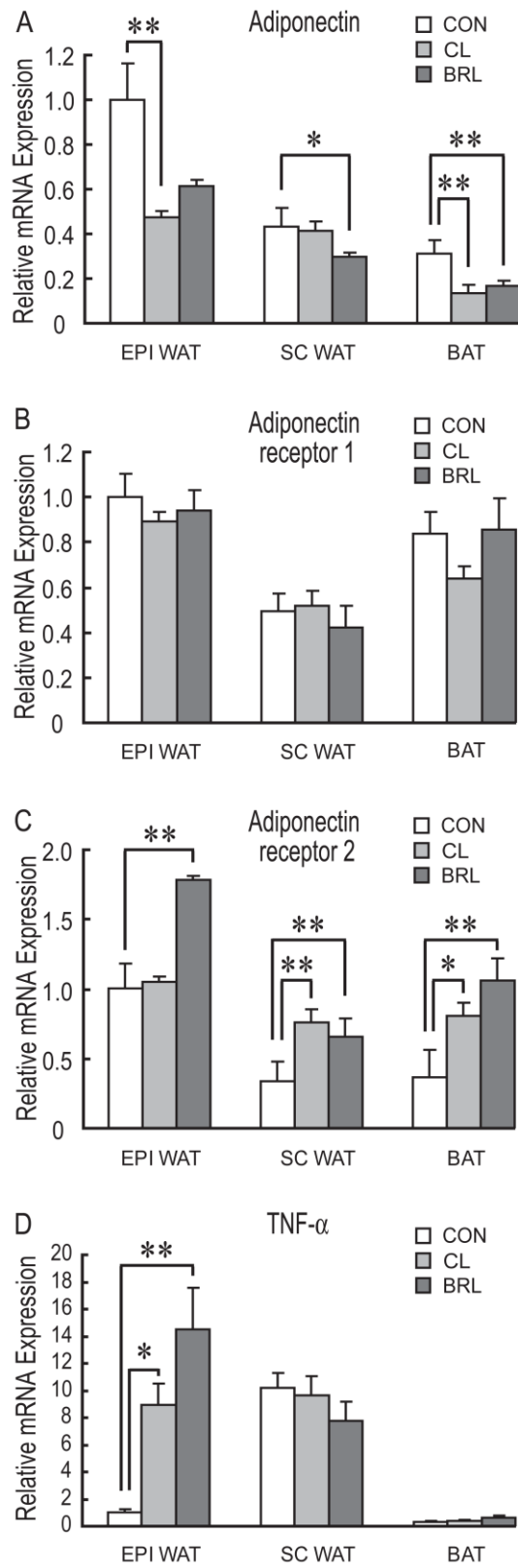


Fig.2.

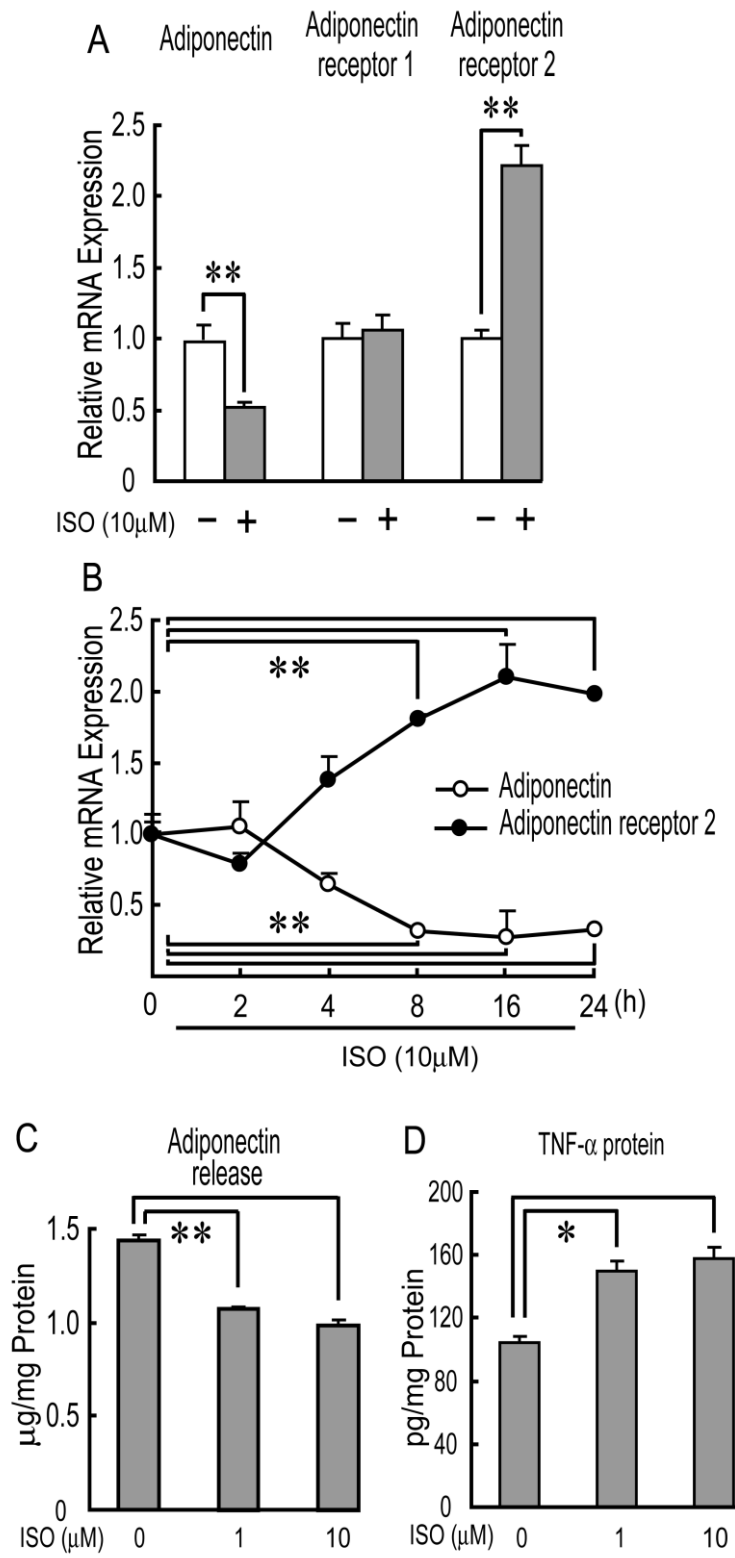


Fig.3.

