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Pioglitazone upregulates adiponectin receptor 2 in 3T3-L1 adipocytes

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ABSTRACT

Aims: Pioglitazone, a full peroxisome proliferator-activated receptor (PPAR)-γ agonist, improves insulin sensitivity by increasing circulating adiponectin levels. However, the molecular mechanisms by which pioglitazone induces insulin sensitization are not fully understood. In this study, we investigated whether pioglitazone improves insulin resistance via upregulation of either 2 distinct receptors for adiponectin (AdipoR1 or AdipoR2) expression in 3T3-L1 adipocytes.

Main methods: Glucose uptake was evaluated by 2-[^{3}H] deoxy-glucose uptake assay in 3T3-L1 adipocytes with pioglitazone treatment. AdipoR1 and AdipoR2 mRNA expressions were analyzed by qRT–PCR.

Key findings: We first confirmed that pioglitazone significantly increased insulin-induced 2-deoxyglucose (2-DOG) uptake in 3T3-L1 adipocytes. Next, we investigated the mRNA expression and regulation of AdipoR1 and AdipoR2 after treatment with pioglitazone. Interestingly, pioglitazone significantly induced AdipoR2 expression but it did not affect AdipoR1 expression. In addition, adenovirus-mediated PPARγ expression significantly enhanced the effects of pioglitazone on insulin-stimulated 2-DOG uptake and AdipoR2 expression in 3T3-L1 adipocytes. These data suggest that pioglitazone enhances adiponectin's autocrine and paracrine actions in 3T3-L1 adipocytes via upregulation of PPARγ-mediated AdipoR2 expression. Furthermore, we found that pioglitazone significantly increased AMP-activated protein kinase (AMPK) phosphorylation in insulin-stimulated 3T3-L1 adipocytes, but it did not lead to the phosphorylation of IRS-1, Akt, or protein kinase Cλ/ζ.

Significance: Our results suggest that pioglitazone increases insulin sensitivity, at least partly, by PPARγ-AdipoR2-mediated AMPK phosphorylation in 3T3-L1 adipocytes. In conclusion, the upregulation of AdipoR2 expression may be one of the mechanisms by which pioglitazone improves insulin resistance in 3T3-L1 adipocytes.

Introduction

Insulin resistance is a primary defect underlying the development of type 2 diabetes mellitus and a central component defining the metabolic syndrome, a constellation of abnormalities that include obesity, hypertension, glucose intolerance, and dyslipidemia (Flier, 2004). Thiazolidinediones (TZDs) are pharmacologic agents that improve glucose homeostasis in type 2 diabetes by increasing insulin sensitivity, largely through improved insulin action in skeletal muscle (Olefsky, 2000). In addition, pioglitazone, which is one of the TZDs, significantly reduces the incidence of major adverse cardiovascular events, strokes, and all-cause mortality in high-risk patients with type 2 diabetes (Dormandy et al., 2005).

The antidiabetic actions of TZDs are believed to be mediated by their interaction with the nuclear receptor PPARγ (Lehmann et al., 1995). In both rodents and humans, adipose tissue is the main expression site for PPARγ, where in PPARγ is a key regulator of adipogenesis (Auboeuf et al., 1997). PPARγ is expressed at much lower levels in other tissues, including other major insulin target tissues, such as the skeletal muscle and liver (Spiegelman, 1998). The distribution of PPARγ expression suggests that the adipose tissue may be the primary target for the insulin-sensitizing effect of PPARγ agonists such that changes in adipose tissue activity lead to improved insulin sensitivity in other tissues (Spiegelman, 1998). Indeed, TZDs markedly change gene expression in adipose tissue, yielding smaller adipocytes whose altered secretion of adipocytokines may improve insulin resistance in other tissues (Okuno et al., 1998). Furthermore, TZDs are well known to cause an elevation of adiponectin levels in vivo and in vitro (Hirose et al., 2002).

Adiponectin (Hu et al., 1996; Maeda et al., 1996; Nakano et al., 1996; Scherer et al., 1995) is a hormone secreted by adipocytes, which function as the key antidiabetic and antiatherogenic adipocytokine (Scherer, 2006). Plasma adiponectin levels are decreased in obesity, insulin resistance, and type 2 diabetes mellitus (Scherer, 2006). Many studies have shown that the high levels of adiponectin are associated with insulin sensitization, whereas low levels are found in insulin resistance (Berg et al., 2001; Fruebis et al., 2001; Hotta et al., 2001; Satoh et al., 2005; Yamauchi et al., 2001). This insulin-sensitizing effect of adiponectin appears to be mediated by the inhibition of gluconeogenesis (Berg et al., 2001) and the stimulation of fatty acid oxidation (Fruebis et al., 2001; Yamauchi et al., 2001) via the activation of AMPK (Yamauchi et al., 2002).

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Recently, 2 distinct receptors for adiponectin (AdipoR1 and AdipoR2) have been identified (Yamauchi et al., 2003). In mice, AdipoR1 is abundantly expressed in the skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver (Yamauchi et al., 2003). To elicit its cellular actions, adiponectin binds to AdipoR1 and AdipoR2 cell surface receptors, triggering signaling cascades that culminate in improved insulin action and/or sensitivity. Adiponectin receptors mediate the activation of AMPK, PPARα, and fatty acid oxidation, which increases glucose uptake and improves lipid metabolism (Yamauchi et al., 2003; Yamauchi et al., 2002). AMPK is a fuel-sensing enzyme that has been implicated in the regulation of glucose and lipid homeostasis and insulin sensitivity (Fisher et al., 2002). Activation of AMPK increases the insulin sensitivity of the muscle glucose transport involved in a step beyond the Akt/protein kinase B (PKB) pathway (Salt et al., 2000). However, the molecular mechanisms by which pioglitazone induces insulin sensitization are not fully understood.

In this study, we investigated whether pioglitazone improves insulin resistance via upregulation of either AdipoR1 or AdipoR2 expression in 3T3-L1 adipocytes.

Materials and methods

Materials

Pioglitazone was donated by Takeda Co (Tokyo, Japan). 3T3-L1 preadipocytes were purchased from American Type Cell Collection (Manassas, VA, USA). pAxCAwt plasmid vector was obtained from TAKARA Biomedical (Shiga, Japan). DMEM, streptomycin, trypsin, fetal bovine serum (FBS), TRIzol reagent, pCR2.1-TOPO vector, LDS sample buffer, and Sample Reducing Agent were from Invitrogen Life Technologies (Carlsbad, CA, USA). The RNeasy kit was obtained from QIAGEN Inc. (Valencia, CA, USA). Anti-phospho-specific Akt (Ser473 and Thr308), anti-Akt, anti-phospho-specific AMPK (Thr172), anti-AMPK, anti-phospho-specific-PKCλ/ζ (Thr410/403) and anti-β-actin antibodies were from Cell Signaling Technology (Boston, MA, USA). Anti-insulin receptor substrate (IRS)-1 (Tyr508) antibody was obtained from Millipore Corp (Billerica, MA, USA). PPARγ siRNA (sc29455), Anti-IRS-1, Anti-PPARγ1, and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidine difluoride (PVDF) transfer membranes were purchased from Millipore Corp. (Bedford, MA, USA). iScript cDNA Synthesis Kit and iQ SYBR Green Supermix were from Bio-Rad Laboratories (Richmond, CA, USA). 2-[^{3}H] Deoxyglucose was purchased from PerkinElmer Inc. (Waltham, MA, USA).

Subcloning of the mouse PPARγ1 cDNA by RT–PCR

The mouse full-length PPARγ1 cDNA (1428 bp) was amplified from mouse liver using 5′-CCATGGTTGACACAGAGATGCCATTC-3′ sense, and 5′-GGGTGGGACTTTCCTGCTAATAC-3′ antisense primers, and subcloned into the pCR2.1-TOPO and sequenced, confirming that the clones corresponded to the mouse PPARγ1 (GenBank accession number U01841).

Construction of recombinant adenoviruses

PPARγ1 adenovirus (Ad-PPARγ1) and lacZ adenovirus (Ad-lacZ) were generated and purified using a previously described protocol (Satoh et al., 2004). Briefly, the mouse PPARγ1 cDNA was inserted in the pAxCAwt plasmid to generate pAxCAwt-mouse PPARγ1. The resulting plasmid, which contains the PPARγ1 cDNA under the control of a CAG promoter (CMV enhancer, chicken β-actin promoter, and part of an untranslated region of rabbit β-globin), was transfected into 293 cells. Recombinant Ad from a single plaque was expanded and purified twice by cesium chloride gradient ultracentrifugation. Viral titer was determined by plaque assay. Control Ad-lacZ that carries the β-galactosidase cDNA was isolated using the same procedure. Both recombinant viruses were dialyzed in PBS, pH 7.4, and stored in 10% glycerol/PBS at −80 °C until use.

Cell culture and cell treatment

3T3-L1 cells were cultured and differentiated as described previously (Nguyen et al., 2005). Differentiated 3T3-L1 adipocytes were incubated with the indicated concentration of pioglitazone, or 0.1% dimethyl sulfoxide vehicle for 3 or 6 h before each assay. For adenovirus infection, 3T3-L1 adipocytes were transduced for 2 h in DMEM high glucose with 2% heat-inactivated serum with the following multiplicity of infection (m.o.i.) and with either the recombinant adenovirus of PPARγ1 (40 m.o.i.) or control recombinant adenovirus of lacZ. The total amount of adenovirus was adjusted to the same m.o.i. as the control adenovirus in each experiment. On the other hand, for PPARγ knockdown experiments, 3T3-L1 adipocytes were transfected with optimized concentrations of either human PPARγ small interfering RNA (siRNA) (Cat. # sc-29455), control nonsense fluorescein conjugate siRNA (Cat. # sc-36869) using siRNA transfection reagent alone according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc). Transduced cells were incubated for 48 h at 37 °C in 10% CO_2 and Dulbecco's modified Eagle's high glucose medium with 10% heat-inactivated serum, followed by incubation in the starvation media required for the assays.

2-Deoxyglucose uptake assay

Glucose uptake was initiated as described previously (Nguyen et al., 2005), with some modifications. After 48 h of adenovirus infection, 3T3-L1 adipocytes were serum-starved for 6 h, and the cells were stimulated with 100 ng/ml insulin in KRP-Hepes buffer (10 mM Hepes, pH7.4, 131.2 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 2.5 mM NaH_2PO_4) for 30 min at 37 °C. Glucose uptake was determined in triplicate at each point at the addition of 2-[^{3}H] deoxyglucose (0.1 μCi, final concentration 0.1 mM) in KRP-Hepes buffer for 5 min at 37 °C. The cells were washed in ice-cold PBS 3 times and solubilized in 1N NAOH. Each sample was subjected to liquid scintillation counting (Nguyen et al., 2005).

Quantitative real-time RT–PCR analysis

Total RNA samples were extracted from cells with TRIzol reagent, and total RNA was further purified using the RNeasy kit with RNase-free DNase I treatment according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed with iScript cDNA Synthesis Kit according to the manufacturer's instructions (Bio-Rad Laboratories, Inc). Quantitative real-time PCR was performed with a Bio-Rad system using iQ SYBR Green Supermix and specific primer pairs (Table 1) selected with Primer Express software (Applied Biosystems). The relative mass of specific RNAs was calculated by the comparative cycle of threshold detection method according to the manufacturer's instructions.

Table 1
Primers used for real-time RT–PCR.

	Forward	Reverse
mACRP30	5′-GCAACTACCCATAGCCCATAC-3′	5′-GCAACTACCCATAGCCCATAC-3′
mAdipoR1	5′-AGATGGAGGAGTTCGTGTATAAG-3′	5′-ATGTAGCAGGTAGTCGTTGTC-3′
mAdipoR2	5′-CCACAACCTTGCTTCATCTACC-3′	5′-ACGAACACTCCTGCTCTGAC-3′
CPH	5′-CACCACATGCTTGCCATCC-3′	5′-CTCCTTTGAGCTGTTTGCAG-3′