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The Antidiabetic Agent Thiazolidinedione Stimulates the Interaction between PPARγ and CBP

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The peroxisome proliferator-activated receptor (PPAR) γ is a member of the nuclear hormone receptor family, which is predominantly expressed on adipocytes and considered to be involved in the process of adipogenesis. The new thiazolidinedione (TZD) compound, T-174, developed as an antidiabetic drug, stimulated the transcription of PPARγ and the adipocyte differentiation of 3T3-L1 cells. Interestingly, T-174 induced the interaction between PPARγ and CBP (cAMP response element binding protein (CREB) binding protein), a co-factor of various transcription regulators. CBP mRNA was expressed in both preadipocytes and adipocytes. These results suggest that CBP plays a role in the PPARγ-mediated signaling pathway activated by TZD, including adipogenesis.

The peroxisome proliferator-activated receptor (PPAR) γ, is a member of the nuclear hormone receptor family, which is comprised of many ligand-dependent transcription factors including the retinoic acid receptor (RAR), thyroid hormone receptor (T_3R), and steroid receptor (e.g. estrogen receptor, ER) (1-3). PPARγ was shown to be expressed in an adipose-specific manner and functions as a key regulator of adipocyte differentiation (4-9).

Several thiazolidinediones (TZDs), insulin-sensitizing drugs for non-insulin-dependent diabetes mellitus (NIDDM), have been reported to be ligands for PPARγ (10). TZDs were also reported to stimulate the differentiation of preadipocytes to adipocytes (11-15). The mechanisms underlying the actions of TZDs, including those on adipogenesis which are mediated through the PPARγ, are unclear, but may be predicted using observations from other members of the nuclear receptor superfamily.

The cAMP response element binding protein (CREB) binding protein (CBP) was originally cloned by virtue of its specific binding to the protein kinase-A activated form of CREB (16). CBP interacts with a variety of transcription factors and signaling molecules, and is believed to participate as a co-factor in a variety of cellular processes which require changes in gene expression (Ref.17,18 for reviews). Recently, CBP has been reported to interact physically with several members of the nuclear receptor superfamily (RAR, T_3R, ER, and retinoid X receptor) in a specific ligand-dependent manner (19,20). Therefore, to determine whether CBP might also contribute to the PPARγ-mediated pathway, we examined whether CBP interacts with PPARγ in a TZD-dependent manner.

MATERIALS AND METHODS

Chemical. T-174 (5-[[2-(2-naphthalenylmethyl)-5-benzoxazolyl]-methyl]-2,4-thiazolidinedione) was synthesized via the standard chemical techniques as described previously (21).

Plasmids. The full length human PPARγ cDNA was amplified by the polymerase chain reaction (PCR) from human fat cell cDNA (Clontech). The PCR product was verified as PPARγ cDNA by sequencing. To generate a fusion protein between the GAL4 DNA binding domain (amino acids 1-147) and the human PPARγ cDNA, a cDNA encoding the ligand binding domain of the human PPARγ2 (a.a. 204-505) was subcloned into the pM mammalian expression plasmid (Clontech). The reporter plasmid 6xUAS-luc contains six copies of a 17-mer upstream activating sequence (UAS) for the GAL4 DNA binding domain and a βRAR minimal promoter in front of a luciferase cDNA (gift from Dr. B. Gloss, University of California at San Diego). For the yeast two-hybrid assays, the ligand binding domain of the human PPARγ2 (a.a. 181-505) was subcloned into the pGBT9 yeast expression plasmid (Clontech). The CBP cDNA corresponding to the interaction domain of nuclear receptor (a.a. 1-464) was amplified by PCR of reverse-transcribed RNA from 3T3-L1 cells. The PCR product was verified as CBP cDNA by sequencing. To generate the fusion between the GAL4 activation domain (a.a. 768-881) and the N-terminus domain of CBP, the CBP cDNA (a.a. 1-450) was inserted into the pGAD424 yeast expression plasmid (Clontech).

Cotransfection assay. CV-1 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) were plated into 24-well plates. Cells were transfected with 0.2 μg of GAL4-PPARγ chimera receptor expression vector and 0.2 μg of luciferase reporter plasmid using Lipofectamine (Life Technologies,

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Inc.) according to the manufacturer's instructions. After 24hr, the liposomes were removed and cells were treated for 24hr with fresh DMEM containing 10% charcoal-treated FCS and various concentrations of T-174. Cell extracts were then prepared and assayed for luciferase activity using the PicaGene luciferase assay system (Wako).

Adipocyte differentiation assay. 3T3-L1 cells in DMEM supplemented with 10% FCS were plated into 24-well plates. At confluence, the cells were treated for 40 hours with various concentrations of T-174. Glycerophosphate dehydrogenase activity was measured 10 days later by a method of Wise and Green (22).

RT-PCR. RT-PCR were performed using Ready-To-Go You-Prime First-Strand Beads (Pharmacia Biotech) according to manufacturer's instruction. The PCR primer sequences were 5′-ATGGCCGAGAAC-TTGCTGGACGGAC-3′ (CBP a.a. 1-9) and 5′-TCTGTTGCCCTG-CACCAACAGAACC-3′ (CBP a.a. 464-457), and conditions were 95°C for 1 min, 75°C for 2 min: 35 cycles. Products were visualized with ethidium bromide on 1.0% agarose gel.

Yeast two-hybrid assay. Yeast strain SFY526 was cotransformed with the plasmids indicated by a lithium acetate method (23). Isolated yeast colonies were cultured with various concentrations of T-174 for five hours. The yeast cell extracts were assayed for β-galactosidase activity as described previously (23).

RESULTS AND DISCUSSION

Thiazolidinediones (TZDs) are a new class of synthetic drugs with increased efficacy in patients resistant to insulin. Since insulin resistance is a major component of non-insulin-dependent diabetes mellitus (NIDDM), these drugs are expected to be of clinical importance. Several TZDs are shown to have marked antidiabetic effets in animal models of NIDDM (24-26). A new TZD compound, named T-174 (Fig. 1A), has been developed based on insulin-sensitizing activity in an animal model of NIDDM, the Yellow *KK* mouse (21). Administration of T-174 to the genetically obese mice decreased their hyperglycemia (21).

Although the TZD drugs have similar structures, they vary in their potency as PPARγ activators (8,10). To gain better understanding of the actions of T-174, we carried out transient transfection assays to determine whether PPARγ could be transcriptionally activated by T-174. We used an established chimera system, in which the ligand-binding domain of PPARγ was fused to the DNA-binding domain of the yeast transcription factor GAL4. Expression plasmids for the GAL4-PPARγ chimera were transfected into CV-1 cells with a luciferase reporter construct containing six copies of the GAL4 binding site (6xUAS-luc). The ability of T-174 to regulate reporter gene expression was determined. The PPARγ chimera was activated by T-174 in a dose-dependent manner (Fig. 1B). The concentration of T-174 required to produce a half-maximal effect (ED_{50}) was approximately 1 μM. This ED_{50} value of T-174 is comparable to those reported previously in similar assays for the TZDs BRL49653 (1 μM) and pioglitazone (5 μM), which are known as potent activators of PPARγ (8). The DNA binding domain of GAL4 itself did not affect the basal levels of reporter gene expression in the presence or absence of T-174 (data not shown).

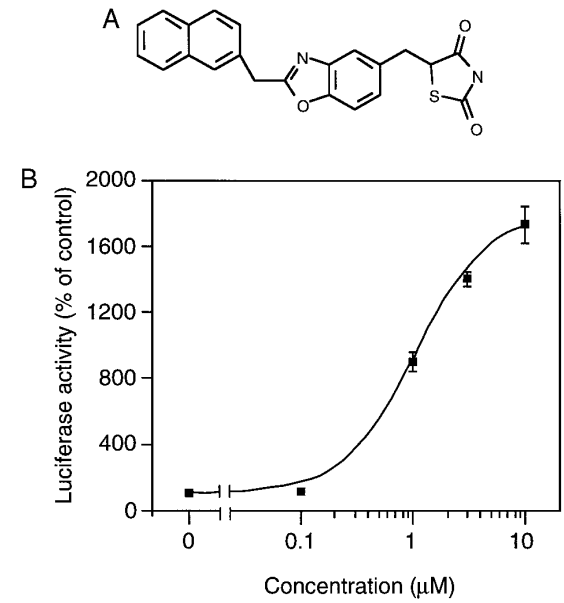

FIG. 1. A. Chemical structure of T-174. **B.** Activation of the GAL4-PPARγ fusion protein by T-174. CV-1 cells were transfected with expression plasmids for the GAL4-PPARγ chimera and the reporter plasmid 6xUAS-luc, before being treated with the indicated concentrations of T-174. Cell extracts were then assayed for luciferase activity. Luciferase activity was plotted as a percentage of activation relative to untreated cells. The bars represent the standard error of the mean luciferase activities on quadruplicate determinations for each point.

Since PPARγ functions as a key regulator of adipogenesis, we examined the effects of T-174 on adipocyte differentiation. 3T3-L1 cells, cloned from mouse Swiss 3T3 fibroblasts, differentiate in culture to develop the morphological and biochemical characteristics of mature adipocytes under the appropriate conditions (27). The differentiation of 3T3-L1 preadipocytes to adipocytes is a useful model for adipocyte differentiation. To assess the ability of T-174 to stimulate the differentiation of preadipose cells, T-174 was titrated into the cultures. Confluent 3T3-L1 cells were treated with DMEM containing 10% FCS and various concentrations of T-174. After 40 hours, the medium was replaced with fresh DMEM containing 10% FCS. Glycerophosphate dehydrogenase (GPDH) activity, which is used as a marker of adipocyte differentiation, was then measured 10 days later. The values for GPDH activity agreed well with the lipid droplet content of 3T3-L1 cells, as checked by oil red O staining (data not shown). Under these conditions, T-174 did not affect the viability of 3T3-L1 cells, as determined by microscopy. As shown in Fig. 2, addition of T-174 to the medium markedly enhanced the GPDH activity in a dose-dependent manner. The ED_{50} value for the effect of T-174 on adipocyte differentiation (approximately 0.7 μM) was similar to that observed for T-174-dependent activation

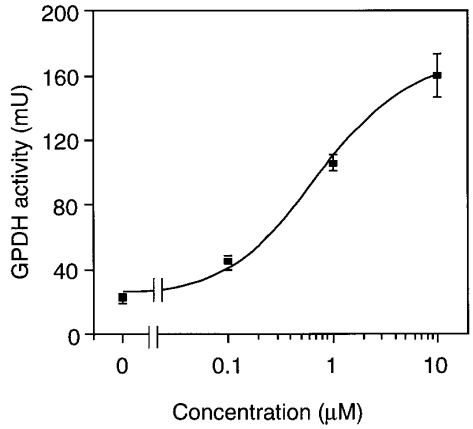

FIG. 2. Dose-dependent effects of T-174 on the adipocyte differentiation. 3T3-L1 cells were allowed to reach confluency and then treated with DMEM containing 10% FCS and various concentrations of T-174. After 40 hours, the medium was replaced with fresh DMEM containing 10% FCS, and GPDH activity measured 10 days later. Bars represent standard error from four independent cultures.

of PPARγ (1 μM) in transient transfection assays (Fig. 1B).

Recently, CBP was reported to interact with several members of the nuclear receptor superfamily and enhance RAR and T_3R transcriptional activity (19). To determine whether CBP might also contribute to the PPARγ pathway, the following experiments were performed. Since PPARγ is predominantly expressed in adipocytes (4-6), we examined the expression of CBP in adipocytes by RT-PCR analysis. Poly $(A)^+$ RNA was isolated from 3T3-L1 cells allowed to reach confluency and from differentiated adipocytes. Oligo-dT primed cDNA was prepared from the RNA, which served as the PCR template. DNA amplification with a set of PCR primers corresponding the mouse CBP cDNA (a.a. 1-464) was performed. An amplified fragment with an expected size of 1.4 kb was identified on the gel (Fig. 3), and verified as CBP cDNA by sequencing. RT-PCR analysis revealed that CBP mRNA was expressed in both preadipocytes and adipocytes. The physiological role of CBP as a co-factor involved in PPARγ signaling would require the proper interaction between PPARγ and CBP. To examine whether CBP could associate with PPARγ *in vivo*, we used a yeast two-hybrid system. The yeast strain SFY526 was cotransformed with PPARγ bait (expressing the GAL4 DNA-binding domain and PPARγ fusion protein) and CBP prey (GAL4 activating domain and CBP N-terminus fusion protein). The yeast was cultured in the absence or presence of T-174. The yeast cell extracts were then assayed for β-galactosidase activity. T-174 markedly stimulated the specific interaction of PPARγ and CBP (Fig. 4A) with an ED_{50} value of about 0.1 μM (Fig. 4B). There

FIG. 3. Detection of CBP mRNA by RT-PCR on RNAs from preadipocytes and adipocytes. Poly $(A)^+$ RNA was isolated from 3T3-L1 cells allowed to reach confluency (Conf.) and from differentiated (Dif.) adipocytes. RNAs which were incubated either in the presence (+) or absence (−) of reverse transcriptase (RT) served as the PCR templates. Aliquots of the PCR products were analyzed in each lane. The approximate size of the band was 1.4kb.

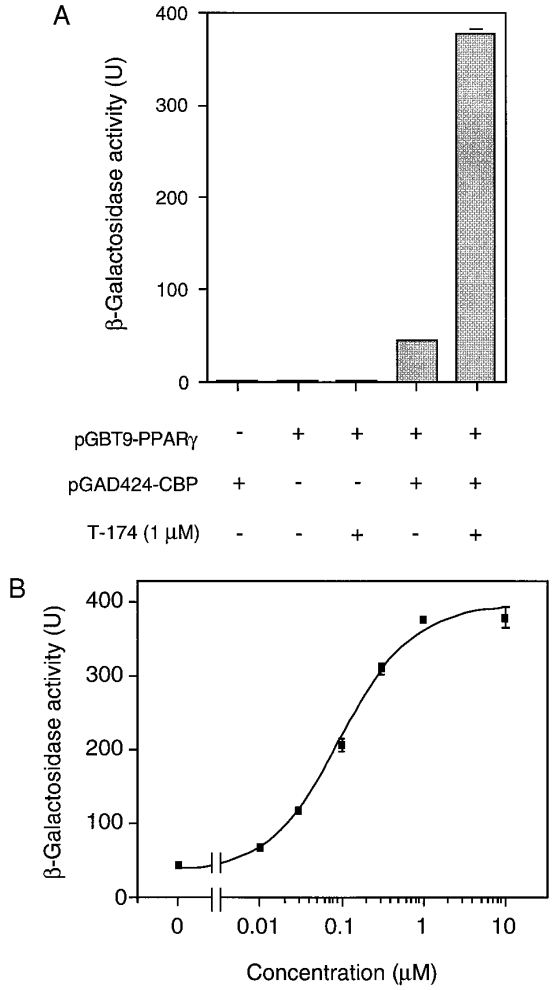

FIG. 4. A, B. Ligand-dependent interaction between PPARγ and CBP in a yeast two-hybrid system. CBP N-terminus (a.a. 1-450) was used as prey (pGAD424-CBP). Interaction of the GAL4 fusion protein encompassing the C-terminal ligand binding domain of PPARγ (bait, pGBT9-PPARγ) was measured in the presence of T-174. Bars represent standard error of the mean of β-galactosidase activities from four independent cultures.

were correlations between the ability of T-174 to activate PPARγ-mediated transcription, stimulate adipocyte differentiation, and induce the interaction of PPARγ and CBP. These findings suggest that the PPARγ signaling pathway activated by TZD, including adipogenesis, involves the recruitment of CBP. The function of CBP would be important for key aspects of adipocyte differentiation.

CBP interacts with various transcription factors and signaling molecules including CREB, AP1, Myb, SREBP, STAT1,2, NF-κB, pp90^{RSK}, and also nuclear receptors including PPARγ (16-20, 28-34). Various hormones and reagents affect adipocyte differentiation; positively such as glucocorticoid, thyroid hormone, insulin, cAMP, and TZDs; and negatively retinoic acid, phorbol ester, TNFα, and interferonγ (11-15, and reviewed in 35). Interestingly, CBP seems to provide a molecular platform for these signals by interacting with their downstream nuclear molecules: i.e., simplistically, the glucocorticoid receptor for glucocorticoid, T_3R for thyroid hormone, pp90^{RSK} for insulin, CREB for cAMP, RAR for retinoic acid, AP-1 and/or NF-κB for phorbol ester and/or TNFα, and STAT1 for interferonγ. CBP may integrate the signals of diverse growth factors, hormones, and intracellular ligands which act together to modulate adipocyte differentiation.

The observation that PPARγ is a receptor for antidiabetic TZDs suggests that defects in the PPARγ signaling pathway contribute to the pathogenesis of NIDDM. Thus, screening for potent PPARγ agonists represents a logical and potentially rapid approach toward the development of novel therapeutic agents for NIDDM. Our yeast two-hybrid system (PPARγ bait and CBP prey) will be useful for the screening of antidiabetic PPARγ agonists.

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