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Research Article

Thiazolidinediones, a class of anti-diabetic drugs, inhibit Id2 expression through a PPARγ-independent pathway in human aortic smooth muscle cells

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Abstract. Inhibitor of DNA binding (Id2) is a member of the helix-loop-helix family of transcription regulators that is known to play important roles in the proliferation and differentiation of many cell types. Overexpression of Id2 has been reported to result in significant enhancement of vascular smooth muscle cell growth via increased S phase entry. We hypothesized that downregulation of Id2 gene expression by thiazolidinediones (TZDs), a class of anti-diabetic drugs and peroxisome proliferator-activated receptor γ (PPARγ) activators, might contribute to the anti-atherosclerotic and anti-hypertensive effects of the PPARγ. Here we document that TZDs, including troglitazone and ciglitazone, repress Id2 gene expression in a doses- and time-dependent manner. However, GW7845, a high-affinity and non-TZD PPARγ activator, had no inhibitory effect on Id2 gene expression. In addition, PPARγ antagonist GW9662 did not rescue TZD-induced Id2 repression. Taken together, our data suggest that TZDs repress Id2 expression through a PPARγ-independent pathway.

Key word. Diabetes; atherosclerosis,; vascular smooth muscle cell; proliferation; gene expression.

Thiazolidenediones (TZDs), including troglitazone and ciglitazone, are a class of novel anti-diabetic drugs that lower the blood glucose level by enhancing peripheral insulin sensitivity [1]. Increasing evidence supports the concept that these actions are mediated by peroxisome proliferator-activated receptor γ (PPARγ), a member of the ligand-activated nuclear transcription factors [2, 3]. In addition to a glucose-lowering effect, TZDs have other beneficial effects for diabetic patients such as redistribution of central body fat stores, lowering of triglycerides and blood pressure and the increase of high-density lipoprotein (HDL).

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Recent studies have documented that the expression of PPARγ is upregulated in intimal vascular smooth muscle cells (VSMCs) [4]. Moreover, TZDs have been reported to inhibit neointima formation after balloon injury in association with decreased DNA synthesis [5]. However, a recent series of reports have documented that the administration of high doses of synthetic ligands in the TZD class can induce biological responses that are PPARγ independent [6–8].

To explore the mechanisms involved in the TZD anti-proliferative effect in VSMCs, we performed the filter-based microarrays (cat. no. GF-211) from Invitrogen to identify the PPARγ-regulated genes in VSMCs [9]. We documented that more than 50 genes are either up- or down-regulated at least two fold after PPARγ activator ciglita-

zone treatment. Id2 was one of the genes most downregulated by ciglitazone treatment (–4.5-fold). This led us to hypothesize that TZDs inhibit Id2 gene expression via PPARγ activation.

Inhibitor of DNA-binding proteins (Ids) include four members (Id1–Id4) and are a family of helix-loop-helix (HLH) transcription factors which are important regulators of cellular differentiation and proliferation [10]. Id proteins lacking the basic DNA-binding domain are capable of forming inactive heterodimers with basic HLH transcription factors such as MyoD [11]. These heterodimers cannot bind to DNA and function in a dominant-negative manner of inhibiting cell differentiation. In addition, accumulating evidence suggests that Id2 enhances cell proliferation [12]. Id2 binds to the retinoblastoma protein (Rb) family members and abolishes their growth-suppressing function [13]. Interestingly, overexpression of Id2 resulted in significant enhancement of VSMC growth [14]. Taken together, these findings suggest that Id2 plays an important role in the development of vascular diseases.

In this study, we used a pharmacological approach to investigate the mechanism of TZD-induced Id2 suppression in VSMCs. Our data documented that TZDs repress Id2 expression through a PPARγ-independent pathway.

Materials and methods

Materials

TZDs including troglitazone and ciglitazone (EC_{50} = ~0.5 μM by in vitro transient transfect report assay) were purchased from BioMol Research Laboratories (Plymouth Meeting, Pa.). GW7845, a high-affinity (EC_{50} = ~0.7 nM by in vitro transient transfect report assay) and non-TZD PPARγ agonist, and GW9662 (a PPARγ antagonist) were obtained from GlaxoSmithKline (Research Triangle Park, N. C.) [15]. Id2 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.).

Plasmids

Human Id2 promoter luciferase reporter construct was generated for this study. Briefly, a 641-bp human Id2 cDNA (GenBank number M97796) was used to search the human genome database and found to match an ~5000-kb DNA fragment (GenBank number NT_022194.9) from chromosome 2, the map position of Id2. Based on this ~5000-kb DNA sequence, we designed two primers to amplify an ~1-kb Id2 promoter by polymerase chain reaction. The Id2 promoter (nt –979 to +25) was cloned into a luciferase reporter plasmid (pGL3-Basic; Promega, Madison, Wisc.). We designated this Id2 promoter/luciferase reporter plasmid pId2-Luc. A human PPARγ expression vector (pcDNA3-hPPARγ) was constructed by our laboratory as described previously [9].

Cell culture

Human aortic smooth muscle cells (HASMCs) were purchased from Bio-Whittaker (San Diego, Calif.) and cultured in smooth muscle cell growth medium-2 containing 5% FBS, 2 ng/ml human basic fibroblast growth factor, 0.5 ng/ml human epidermal growth factor, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, and 5 μg/ml bovine insulin. For all experiments, early passages (5–7) of HASMCs were grown to 85–95% confluence and made quiescent by Opti-MEM (Invitrogen, Calsbad, Calif.) for 24 h. The rat aortic smooth muscle cell line (A7r5) was purchased from the American Type Culture Collection (Manassas, Va.) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS. All cells were grown in a 5% CO_2-humidified atmosphere at 37 °C.

Northern blotting analysis

Total RNA was isolated from cultured cells using an RNeasy mini kit (Qiagen, Valencia, Calif.). Total RNA (15 μg) was separated on 1% formaldehyde-agarose gels. After transferring to nylon membranes (Perkin Elmer Life Sciences, Boston, Mass.), the RNA was cross-linked to the membrane by a UV cross-linker (Bio-Rad, Hercules, Calif.). ^{32}P-labeled cDNA probes were generated using the random primer labeling system (Invitrogen). Blots were pre-hybridized and hybridized for 1 h at 65 °C, and then washed once with 1× SSC, and once with 0.1× SSC, 1% SDS at 65 °C for 15 min. The phosphor image Typhoon 9200 was used to detect the radioactivity of ^{32}P of each band, and the density of each band was quantified by ImageQuant Software (Amersham Life Science, Piscataway, N. J.). Lane loading differences were normalized using GAPDH expression levels.

Western-blotting analysis

The total cell proteins were extracted with the mammalian protein extraction kit (Pierce, Rockford, Ill.). Eighty micrograms of protein was subjected to SDS-PAGE and electrotransferred to nitrocellulose membrane (Bio-Rad). After blocking in 20 mM Tris-HCl, pH 7.6, containing 150 mM NaCl, 0.1% Tween 20, and 5% (w/v) non-fat dry milk, membranes were probed with specific antibodies against Id2 or β-actin overnight at 4 °C. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). The immunoactivity was visualized by the enhanced chemiluminescence detection system (Amersham) according to the manufacturer's instructions. The density of each band was quantified by ImageQuant Software. Lane loading differences were normalized using β-actin expression levels.

Transient transfection and luciferase assays

A7r5 cells were grown to 85~95% confluence in DMEM supplemented with 10% FBS before transfec-

tion. The Id2 promoter construct (pId2-Luc) was transfected together with a pcDNA3-hPPARγ plasmid using LipofectAMINE2000 (Invitrogen). Green fluorescence protein (GFP) expression plasmid was co-transfected as the control for transfection efficiency. The total amount of transfected DNA was kept constant by using corresponding vector DNA. Twenty-four hours after transfection, cells were cultured for 12 h in Optium-MEM and then treated with vehicle (DMSO), 10 µmol/l ciglitazone or 1 µmol/l GW7845 for 24 h. Luciferase activity was measured by a luminometer (Victor II; Perkin Elmer) using the reporter luciferase assay kit (Promega). Luciferase activity was normalized by GFP units.

Statistical analysis

Each experiment was repeated a minimum of three times. Statistical analyses were performed by ANOVA or unpaired Student t-test. Data are presented as mean ± SD.

Results

Ciglitazone and troglitazone inhibit Id2 expression in HASMCs

To determine whether TZDs inhibit Id2 expression, we first performed Northern blot analyses to study Id2 mRNA expression in HASMCs by ciglitazone stimulation. As shown in figure 1, abundant Id2 expression was observed in HASMCs before ciglitazone treatment. However, Id2 mRNA expression was dramatically reduced by about ten-fold after incubation with 10 µM ciglitazone for 24 h in HASMC. An increasing concentration of ciglitazone (0, 1, 5, and 10 µmol/l) decreased Id2 mRNA expression in a dose-dependent manner (fig. 1 A). In addition, cells treated with 10 µmol/l ciglitazone for different times as indicated resulted in the suppression of Id2 mRNA expression in a time-dependent manner (fig. 1 B). The significant inhibition of Id2 mRNA expression started at 6 h and reached a maximum at 24 h. Using Western blot analysis, we further confirmed the signi-

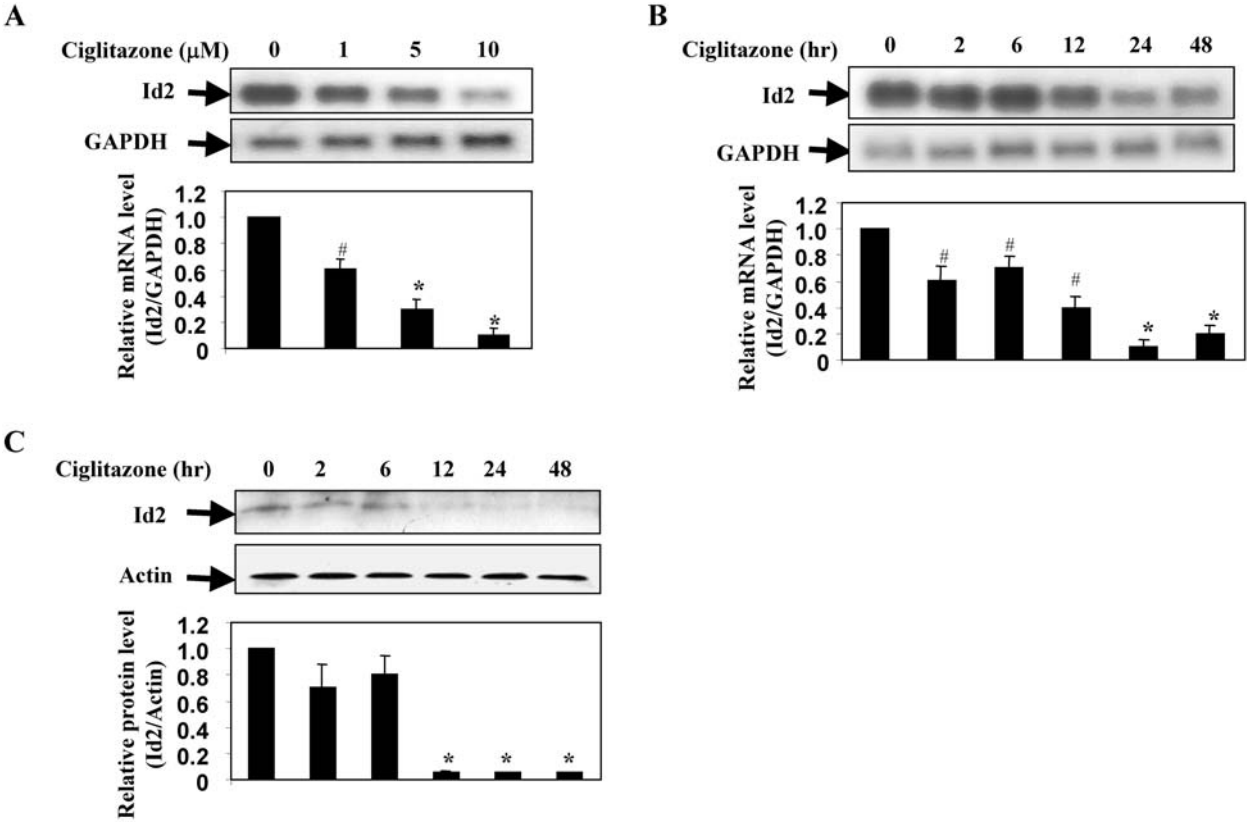

Figure 1. Ciglitazone inhibits Id2 expression in HASMCs. (*A*) Cells were made quiescent by Opti-MEM treatment for 24 h and then treated with increasing concentrations of ciglitazone (0, 1, 5, and 10 µmol/l) for 24 h. (*B*) Quiescent cells were treated with 10 µmol/l ciglitazone for different times as indicated. Northern blot analyses were performed to measure the Id2 mRNA levels in each group. The Id2 mRNA levels normalized by GAPDH level were expressed in relative units to no ciglitazone stimulation (control) as 1. Values for Id2 mRNA are expressed as mean ± SD (n = 3, #p < 0.05 vs control, *p < 0.01 vs control) in the lower panels of A and B. (*C*) Levels of Id2 protein were examined by Western blot analyses. Total protein was extracted from the cells treated with ciglitazone at 10 µmol/l for various periods as indicated. The Id2 protein levels normalized by β-actin were expressed in relative units to no ciglitazone stimulation (control) as 1. Values for Id2 protein are expressed as mean ± SD (n = 3, *p < 0.01 vs control).

ficant inhibition of Id2 protein by 10 µmol/l of ciglitazone stimulation in HASMCs (fig. 1C). In addition, we confirmed that both cliglitazone and troglitazone up to 20 µmol/l are not toxic to VSMCs (data not shown). Taken together, our data documented that ciglitazone suppresses Id2 expression in a dose- and time-dependent manner.

In parallel experiments, we also documented that troglitazone suppresses Id2 gene expression in a dose- and time-dependent manner (fig. 2).

Ciglitazone stimulation does not affect Id2 mRNA stability in HASMC

To evaluate whether Id2 mRNA stability contributes to TZD-induced Id2 downregulation, we examined the half-life of Id2 mRNA in HASMCs. Northern blotting analyses were performed with the addition of actinomycin D (5 µg/ml) after 6 h of ciglitazone (10 µmol/l) stimulation. In HASMCs, the half-life of Id2 mRNA was ~1.5 h. There were no significant differences between ciglitazone-treated and untreated cells (fig. 3). The data suggested that ciglitazone does not affect Id2 mRNA stability and that TZD-induced Id2 downregulation is mainly at the transcriptional level.

Ciglitazone and troglitazone inhibit Id2 expression through a PPARγ-independent pathway

Recent reports suggest that PPARγ agonists such as ciglitazone, troglitazone, and 15d-PGJ_2 have various PPARγ-independent effects in many cell types [6–8, 16]. To determine if the suppression of Id2 expression is mediated by PPARγ in HASMCs, the cells were pretreated with or without GW9662, a PPARγ-specific antagonist at 1 µmol/l for 30 min prior to the addition of GW7845, a high-affinity and non-TZD PPARγ agonist (1 µmol/l) and ciglitazone (10 µmol/l) for 24 h. As shown in figure 4, Northern blotting analyses show that the GW7845 did not affect Id2 mRNA expression in HASMCs. In addition, ciglitazone-induced Id2 repression was not rescued by GW9662. Similar results were obtained in HASMCs with GW9662 and troglitazone treatment (data not shown). Taken together, these data indicated that the effect of TZDs on Id2 expression was independent of PPARγ.

To further confirm that ciglitazone-induced Id2 suppression was through a PPARγ-independent pathway, we examined the regulation of the Id2 gene promoter in VSMCs by transit transfection reporter assays. As shown in figure 5, ciglitazone (10 µmol/l) strongly inhibited Id2 (eight- to nine-fold) promoter activation, but GW7845 (10 µmol/l) did not significantly affect Id2 promoter activation with or without PPARγ overexpression. These results suggested that ciglitazone inhibited Id2 expression at the transcriptional level through a PPARγ-independent mechanism.

Discussion

VSMC proliferation is the key event in vascular proliferative disorders including atherosclerosis and restenosis. Since are TZDs generally believed to inhibit VSMC growth through the PPARγ-mediated pathway, the identification of TZD-induced Id2 repression in VSMCs led us to hypothesize that Id2 downregulation by TZDs might contribute to the anti-atherosclerotic and anti-hypertensive effects of PPARγ. However, our data in the present study clearly documented that TZDs repress Id2

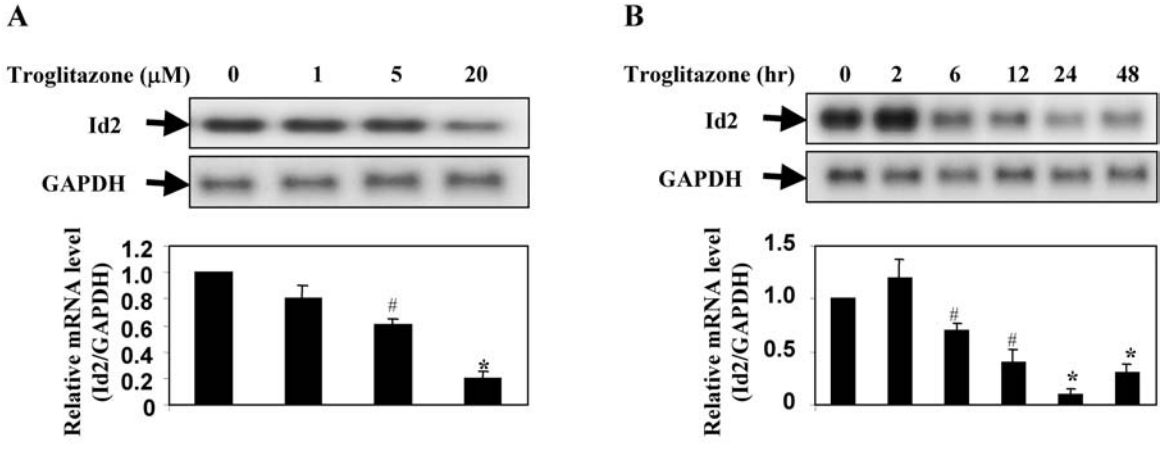

Figure 2. Troglitazone inhibits Id2 expression in HASMC. (*A*) Cells were made quiescent by Opti-MEM treatment for 24 h and then treated with increasing concentrations of troglitazone (0, 1, 5, and 20 µmol/l) for 24 h. (*B*) Quiescent cells were treated with 20 µmol/l troglitazone for different times as indicated. Northern blot analyses were performed to measure the Id2 mRNA levels in each group. Id2 mRNA levels normalized by GAPDH level were expressed in relative units to no troglitazone stimulation (control) as 1. Values for Id2 mRNA are expressed as the mean ± SD ($n = 3$, #$p < 0.05$ vs control, *$p < 0.01$ vs control) in the lower of panels of *A* and *B*.

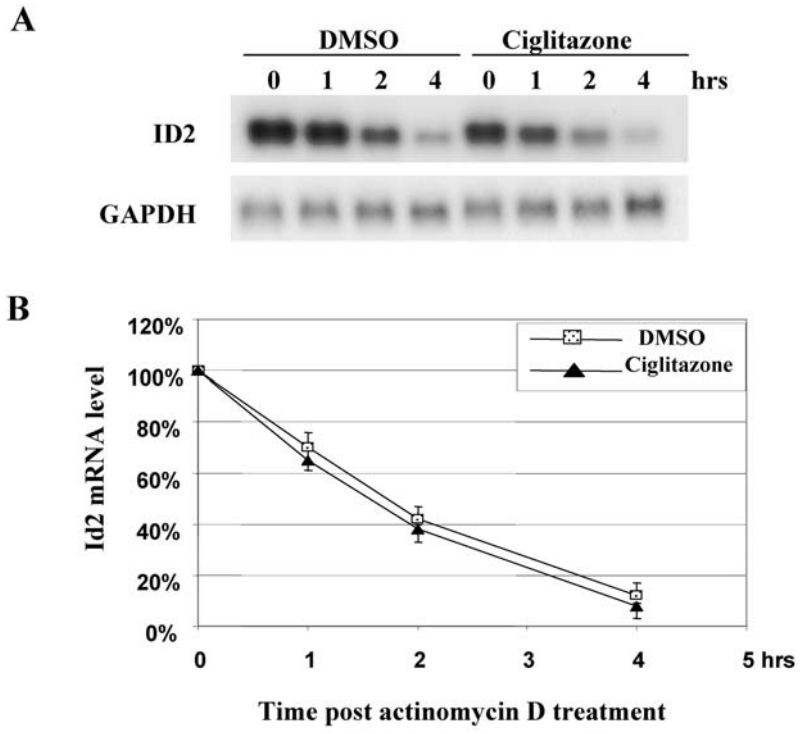

Figure 3. Ciglitazone-induced Id2 downregulation does not affect Id2 mRNA stability in HASMCs. HASMCs were incubated with or without 10 μmol/l of ciglitazone for 8 h and de novo mRNA transcription was inhibited by addition of actinomycin D (5 μg/ml). Total RNA was extracted at 0, 1, 2, and 4 h after administration of actinomycin D for Northern blot analyses. A representative Northern blot is shown in the top panel. The Id2 mRNA levels normalized by GAPDH level were expressed in relative units to no actinomycin D treatment as 100%. Values for Id2 mRNA are expressed as mean ± SD (n = 3) in the bottom panel.

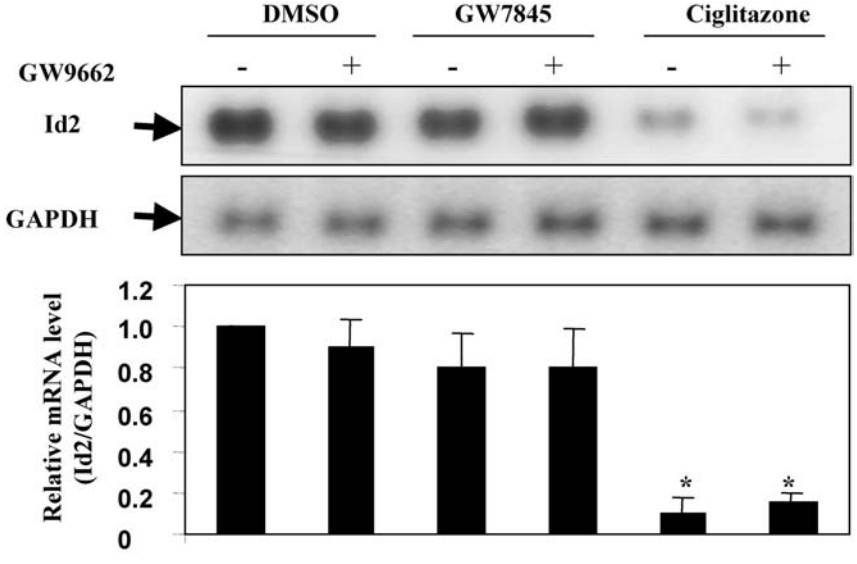

Figure 4. Ciglitazone-induced Id2 repression is independent of PPARγ in HASMCs. Quiescent cells were treated with a PPARγ antagonist GW9662 (1 μmol/l) for 30 min before the treatment with ciglitazone (10 μmol/l) or the high-affinity PPARγ agonist GW7845 (1 μmol/l) for 24 h. Total RNA was extracted and the levels of Id2 mRNA were determined by Northern blot analyses. Id2 mRNA levels normalized by GAPDH level were expressed in relative units to DMSO without GW9662 treatment (control) as 1. Values for Id2 mRNA are expressed as mean ± SD (n = 3, *p < 0.01 vs control) in the lower panel.

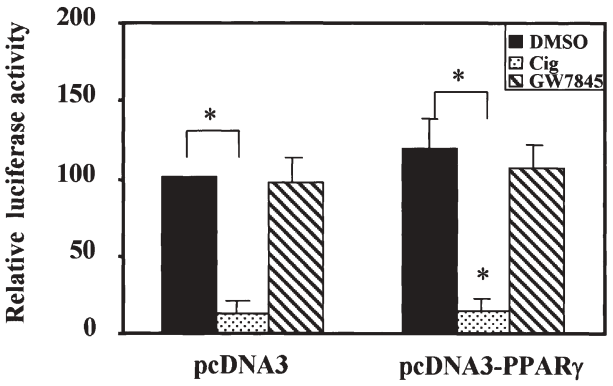

Figure 5. Ciglitazone inhibits Id2 gene promoter activity. A pId2-Luc reporter construct was co-transfected with or without a PPARγ expression plasmid in rat VSMCs. A GFP reporter plasmid was used as the control for transfection efficiency. Quiescent transfected cells were treated with vehicle (DMSO), 10 µmol/l ciglitazone or 1 µmol/l GW7845 for 24 h. Luciferase activity normalized by GFP is expressed in relative units to pcDNA3 with DMSO treatment (mean ± SD, n = 6, *p < 0.01).

expression through one PPARγ-independent pathway, suggesting that TZDs might inhibit VSMC growth via Id2 downregulation, which is independent from PPARγ activation.

TZDs are a novel class of anti-hyperglycemic drugs used in the treatment of type II diabetes. Mounting data have documented that binding and activating of PPARγ by TZDs correlate well with their anti-diabetic potency. TZDs also have other beneficial effects for diabetes such as the lowering of triglycerides and blood pressure. Although the emerging evidence supports the mediation of these TZD actions by PPARγ, a recent series of reports documented that the administration of TZDs can induce biological responses that are PPARγ independent [6–8]. Moreover, the side effects of increasing fluid retention or swelling which could lead to congestive heart failure, and promote liver problems, have been observed in TZD-treated type II diabetic patients. Therefore, the dissection of TZD PPARγ-dependent and PPARγ-independent actions will provide new insights into the development of better drugs that specifically target PPARγ.

The Id class of HLH proteins is involved in cell differentiation and proliferation in a variety of cell types [10]. Id2 was reported to promote VSMC growth by increasing S phase entry via downregulation of the $p21^{Cip1}$, a cyclin-dependent kinase inhibitor [14]. Id3 was documented to mediate angiotensin II-induced VSMC growth through depressing $p21^{Cip1}$, $p27^{kip1}$, and p53 [17]. In addition, upregulation of Id2 and Id3 in vessel walls was recently reported during vascular lesion formation [14, 18]. Taken together, these data suggest that Id proteins are a class of novel molecular determinants in vascular diseases.

The mechanisms underlying the inhibitory effects of TZDs on Id2 gene expression remain to be explored. Interestingly, troglitazone is less potent at repressing Id2 expression than ciglitazone (fig. 2 vs. fig. 1). This difference may be generated from different chemical structures. Troglitazone contains a vitamin E moiety. Of interest will be to examine whether vitamin E has any effect on Id2 expression in VSMCs. A recent paper documented that ciglitazone activates the three MAP kinase families through PPARγ-independent mechanisms involving reactive oxygenated species [8]. Moreover, troglitazone was reported to repress phosphoenolpyruvate carboxykinase gene expression via a PPARγ-independent antioxidant-related mechanism [19]. In addition, our data in this study suggests that the TZD-induced Id2 repression is mainly at the transcriptional level in VSMCs. Searching for the TZD-responsive element in the Id2 promoter by systematic deletion mapping analysis may provide clues and a better understanding of TZD-induced Id2 repression. Further studies on TZD-induced Id2 suppression may lead us to identify novel molecular mechanisms for the inhibition of VSMC growth by TZDs.

In conclusion, our results provide the first evidence for TZD-induced Id2 repression by the PPARγ-independent pathway. This study is thus important for understanding the pharmacological roles of TZDs that are widely used for the treatment of type II diabetes.

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1 Olefsky J. M. and Saltiel A. R. (2000) PPAR gamma and the treatment of insulin resistance. Trends Endocrinol. Metab. **11:** 362–368
2 Kersten S., Desvergne B. and Wahli W. (2000) Roles of PPARs in health and disease. Nature **405:** 421–424
3 Olefsky J. M. (2000) Treatment of insulin resistance with peroxisome proliferator-activated receptor gamma agonists. J. Clin. Invest. **106:** 467–472
4 Bishop-Bailey D., Hla T. and Warner T. D. (2002) Intimal smooth muscle cells as a target for peroxisome proliferator-activated receptor-gamma ligand therapy. Circ. Res. **91:** 210–217
5 Law R. E., Goetze S., Xi X. P., Jackson S., Kawano Y., Demer L. et al. (2000) Expression and function of PPARgamma in rat and human vascular smooth muscle cells. Circulation **101:** 1311–1318
6 Moore K. J., Rosen E. D., Fitzgerald M. L., Randow F., Andersson L. P., Altshuler D. et al. (2001) The role of PPAR-gamma in macrophage differentiation and cholesterol uptake. Nat. Med. **7:** 41–747
7 Chawla A., Barak Y., Nagy L., Liao D., Tontonoz P., Evans R. M. et al. (2001) PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. Nat. Med. **7:** 48–52
8 Lennon A. M., Ramauge M., Dessouroux A. and Pierre M. (2002) MAP kinase cascades are activated in astrocytes and preadipocytes by 15-deoxy-delta(12-14)-prostaglandin J(2)

and the thiazolidinedione ciglitazone through peroxisome proliferator activator receptor gamma-independent mechanisms involving reactive oxygenated species. J. Biol. Chem. 2002 **277:** 29681–29685
9 Fu M., Zhang J., Zhu X., Myles D. E., Willson T. M., Liu X. et al. (2001) Peroxisome proliferator-activated receptor gamma inhibits transforming growth factor beta-induced connective tissue growth factor expression in human aortic smooth muscle cells by interfering with Smad3. J. Biol. Chem. **276:** 45888–45894
10 Yokota Y. and Mori S. (2002) Role of Id family proteins in growth control. J. Cell Physiol. **190:** 21–28
11 Norton J.D., Deed R.W., Craggs G. and Sablitzky F. (1998) Id helix-loop-helix proteins in cell growth and differentiation. Trends Cell Biol. **8:** 58–65
12 Norton J. D. (2000) ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. J. Cell Sci. **113:** 3897–3905
13 Iavarone A., Garg P., Lasorella A., Hsu J. and Israel M. A. (1994) The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. Genes Dev. **8:** 1270–1284
14 Matsumura M. E., Lobe D. R. and McNamara C. A. (2002) Contribution of the helix-loop-helix factor Id2 to regulation of vascular smooth muscle cell proliferation. J. Biol. Chem. **277:** 7293–7297
15 Willson T. M., Brown P. J., Sternbach D. D. and Henke B. R. (2000) The PPARs: from orphan receptors to drug discovery. J. Med. Chem. **43:** 527–550
16 Zhang J., Fu M., Zhao L., and Chen Y. E. (2002) 15-deoxy-prostaglandin J_2 inhibits PDGF-A and -B chain expression in human vascular endothelial cells independent of PPARγ. Biochem. Biophys. Res. Commun. **298:** 128–132
17 Mueller C., Baudler S., Welzel H., Bohm M. and Nickenig G. (2002) Identification of a novel redox-sensitive gene, Id3, which mediates angiotensin II-induced cell growth. Circulation **105:** 2423–2428
18 Matsumura M. E., Li F., Berthoux L., Wei B., Lobe D. R., Jeon C. et al. (2001) Vascular injury induces posttranscriptional regulation of the Id3 gene: cloning of a novel Id3 isoform expressed during vascular lesion formation in rat and human atherosclerosis. Arterioscler. Thromb. Vasc. Biol. **21:** 752–758
19 Davies G. F., Khandelwal R. L., Wu L., Juurlink B. H. and Roesler W. J. (2001) Inhibition of phosphoenolpyruvate carboxykinase (PEPCK) gene expression by troglitazone: a peroxisome proliferator-activated receptor-gamma (PPARgamma)-independent, antioxidant-related mechanism. Biochem. Pharmacol. **62:** 1071–1079