Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 466 (2003) 225–234
www.elsevier.com/locate/ejphar

# A non-thiazolidinedione partial peroxisome proliferator-activated receptor γ ligand inhibits vascular smooth muscle cell growth

Dennis Bruemmer[a,b], Joel P. Berger[c], Joey Liu[a], Ulrich Kintscher[b], Shu Wakino[a], Eckart Fleck[b], David E. Moller[c], Ronald E. Law[a,*]

[a] *Division of Endocrinology, Diabetes and Hypertension and The Gonda (Goldschmied) Diabetes Center, David Geffen School of Medicine, University of California, Los Angeles, Warren Hall, Suite 24-130, 900 Veteran Avenue, Los Angeles, CA-90095-7073, USA*
[b] *Department of Medicine/Cardiology, German Heart Institute, 13353 Berlin, Germany*
[c] *Merck Research Laboratories, Rahway, NJ 07065, USA*

Received 27 January 2003; received in revised form 21 February 2003; accepted 28 February 2003

**Abstract**

Several peroxisome proliferator-activated receptor γ (PPARγ) agonists of the thiazolidinedione class inhibit vascular smooth muscle cell proliferation. It is not known whether the antiproliferative activity of PPARγ agonists is limited to the thiazolidinedione class and/or is directly mediated through PPARγ-dependent transactivation of target genes. We report here that a novel non-thiazolidinedione partial PPARγ agonist (nTZDpa) attenuates rat aortic vascular smooth muscle cell proliferation. In a transfection assay for PPARγ transcriptional activation, the non-thiazolidinedione partial PPARγ agonist elicited ~25% of the maximal efficacy of the full PPARγ agonist rosiglitazone. In the presence of the non-thiazolidinedione partial PPARγ agonist, the transcriptional activity of the full agonist, rosiglitazone, was blunted, indicating that the non-thiazolidinedione partial PPARγ agonist inhibits rosiglitazone-induced PPARγ activity. The non-thiazolidinedione partial PPARγ agonist (0.1–10 μM) inhibited vascular smooth muscle cell growth which was accompanied by an inhibition of retinoblastoma protein phosphorylation. Mitogen-induced downregulation of the cyclin-dependent kinase (CDK) inhibitor $p27^{kip1}$, and induction of the G1 cyclins cyclin D1, cyclin A, and cyclin E were also attenuated by the non-thiazolidinedione partial PPARγ agonist. Maximal antiproliferative activity of the non-thiazolidinedione partial PPARγ agonist required functional PPARγ as adenovirus-mediated overexpression of a dominant-negative PPARγ mutant partially reversed its inhibition of vascular smooth muscle cell growth. In contrast, overexpression of dominant-negative PPARγ did not reverse the inhibitory effect of the non-thiazolidinedione partial PPARγ agonist on cyclin D1. As the full PPARγ agonist rosiglitazone exhibited no effect on cyclin D1, inhibition of that G1 cyclin by the non-thiazolidinedione partial PPARγ agonist likely occurred through a PPARγ-independent mechanism. These data demonstrate that a non-thiazolidinedione partial PPARγ agonist may constitute a novel therapeutic for proliferative vascular diseases and could provide additional evidence for the important role of PPARγ in regulating vascular smooth muscle cell proliferation.


*Keywords:* PPARγ (Peroxisome proliferator-activated receptor γ); Smooth muscle cell, vascular; Cell cycle; Cyclin D1

## 1. Introduction

Vascular smooth muscle cells within the media of adult arteries are normally quiescent, proliferate at low frequency and are arrested in the G0 phase of the cell cycle. Reentry of vascular smooth muscle cells into and progression through the cell cycle plays a pivotal role in the pathogenesis of proliferative vascular diseases such as restenosis, transplant vasculopathy, vein graft disease, and atherosclerosis (Ross, 1995). After arterial injury, growth factors and proinflammatory cytokines released from endothelial cells, monocytes, lymphocytes, and platelets induce vascular smooth muscle cells to migrate to the arterial intima, where they proliferate and subsequently form the neointima (Schwartz, 1997). Vascular growth factors which include basic fibroblast growth factor, platelet-derived growth factor (PDGF), transforming growth factor-ß1, angiotensin II, and insulin-

* Corresponding author. Tel.: +1-310-794-7555; fax: +1-310-794-7654.
*E-mail address:* rlaw@mednet.ucla.edu (R.E. Law).

0014-2999/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0014-2999(03)01556-5

like growth factor, utilize distinct signaling pathways to promote vascular smooth muscle cell proliferation and DNA synthesis. However, these diverse signaling pathways must converge upon common regulators of the cell cycle (Lukas et al., 1996).

Mitogens trigger a series of biochemical events in G1 that permit cells to progress through a cell cycle checkpoint to enter S phase and to replicate their DNA. This G1 → S phase transition requires increased Rb phosphorylation which in a hypophosphorylated state sequesters the S phase transcription factor, E2F, and blocks expression of E2F-regulated genes encoding the enzymatic machinery for DNA synthesis (Weinberg, 1996). Phosphorylation of Rb and E2F release is regulated by the formation and activation of cyclin and cyclin-dependent kinase (CDK) complexes (Morgan, 1995). CDKs become activated by their association with regulatory subunits; cyclin D1, E, and A (Sherr, 1996). Activity of cyclin–CDK complexes can be attenuated by association with negative regulatory subunits, the CDK inhibitors (CDKI) (Morgan, 1995). A number of CDKIs, including $p21^{cip1}$, $p27^{kip1}$, p15, and p16, regulate CDK activity during G1 in vascular smooth muscle cells (Braun-Dullaeus et al., 1999; Tanner et al., 2000). These CDKIs are important negative regulators of cell growth by inhibiting cyclin/CDK activity and Rb phosphorylation, resulting in G1 arrest (Schulze et al., 1995).

Peroxisome proliferator-activated receptor γ (PPARγ) is expressed in all cell types participating in vascular injury: endothelial cells, macrophages, and vascular smooth muscle cells (Law et al., 2000; Marx et al., 1998; Ricote et al., 1998). There is growing evidence that PPARγ agonists may influence cardiovascular disease by directly modulating vessel wall function. Thiazolidinediones are high-affinity PPARγ ligands and are commonly used as insulin-sensitizing agents in the treatment of patients with type II diabetes. We have recently reported that the PPARγ agonists of the thiazolidinedione class, rosiglitazone and troglitazone, inhibit vascular smooth muscle cell proliferation by attenuating the activity of key cell cycle regulators that control G1 → S progression (Wakino et al., 2000).

It is not known whether the antiproliferative properties of PPARγ agonists are limited to the thiazolidinedione class and/or are directly mediated through PPARγ-dependent regulation of target genes. We report here that a novel non-thiazolidinedione partial PPARγ agonist (nTZDpa) attenuates vascular smooth muscle cell proliferation by targeting important cell cycle regulators. Using adenovirus-mediated overexpression of a dominant-negative PPARγ, we also find that inhibition of vascular smooth muscle cell growth by this PPARγ ligand is partially mediated through a PPARγ-dependent mechanism. In contrast, inhibition of cyclin D1 expression by the non-thiazolidinedione partial PPARγ agonist likely occurs through a mechanism that does not involve PPARγ.

## 2. Materials and methods

### 2.1. Materials

Materials were obtained from the following suppliers: Dulbecco's modified Eagle's medium (DMEM), L-glutamine and OPTI-MEM® were purchased from GIBCO BRL (Gaithersburg, MD, USA). Antibiotics and rat recombinant PDGF-BB were from Sigma (St. Louis, MO, USA), insulin was from Eli Lilly (Indianapolis, IN, USA). Fetal bovine serum was purchased from Irvine Scientific (Santa Ana, CA, USA). Antibodies were obtained from the following suppliers: $p27^{kip1}$ (sc-1641), cyclin A (sc-751), cyclin E (sc-481), and PPARγ (sc-7196) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); cyclin D1 (05-362) was from Upstate (Lake Placid, NY, USA); and the phospho-Rb Ser 807/811 (no. 9308) was obtained from Cell Signaling Technology (Beverley, MA, USA). Horseradish peroxidase-linked anti-mouse antibody, peroxidase-conjugated anti-rabbit immunoglobulin G (IgG), and enhanced chemiluminescence Western blotting detection reagents were from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

The full-length PPARγ1 expression vector was obtained from Alex Elbrecht (Merck Research Laboratories, Rahway, NJ) (Elbrecht et al., 1996). The luciferase reporter construct driven by three copies of the peroxisome proliferator-response element (PPRE) from the acyl-CoA oxidase gene linked to the thymidine kinase promotor was kindly provided by Peter Tontonoz (University of California, Los Angeles, CA) (Chawla et al., 2001). The Quick Change site-directed mutagenesis kit for construction of the dominant-negative PPARγ and the Adeno-X Expression System were from Clontech (Palo Alto, CA, USA). LipofectAMINE 2000 was purchased from Life Technologies (Rockville, MN, USA). The Dual Luciferase Reporter Assay System and pRL-CMV were purchased from Promega (Madison, MA, USA). The non-thiazolidinedione partial PPARγ agonist [nTZDpa, 1-(*p*-chlorobenzyl)-5-chloro-3-phenylthiobenzyl-2-yl carboxylic acid] was a generous gift from Merck (Berger et al., 2003), and rosiglitazone was from Smith Kline Beecham (King of Prussia, PA, USA).

### 2.2. Cell culture

Rat aortic vascular smooth muscle cells were prepared from the thoracic aorta of 2-to 3-month-old Sprague–Dawley rats by using the explant technique. Relatively pure (>98%) rat aortic vascular smooth muscle cells were confirmed by specific α-actin staining (data not shown). The cells were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. For all experiments, early passaged (five to eight) rat aortic vascular smooth muscle cells were grown to 60–70% confluency and made quiescent by serum starvation (0.4% fetal bovine serum) for at least 24 h. Each

reagent examined was added 30 min before the addition of rat recombinant PDGF-BB and insulin at the final concentration of 20 ng/ml and 1 μM, respectively. For all data shown, each individual experiment was performed using an independent preparation of rat aortic vascular smooth muscle cells.

### 2.3. Cell growth assay

Rat aortic vascular smooth muscle cells were plated at $1.0 \times 10^6$ cells on 60-mm plates and maintained under starvation in DMEM containing 0.4% fetal bovine serum. After 48 h, cells were pretreated with the non-thiazolidinedione partial PPARγ agonist for 30 min and were stimulated with growth factors (PDGF-BB 20 ng/ml + insulin 1 μM) for 48 h. Cells were harvested and cell proliferation was measured by counting the cells using a hematocytometer. Data were based on three different experiments from three different preparations of rat aortic vascular smooth muscle cells.

### 2.4. Western immunoblotting

Cells were harvested at the indicated time after the addition of growth factors and sonicated in solubilization buffer (20 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA, 1% Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM sodium vanadate; 10 μg/ml each aprotinin and leupeptin; 1 mM phenylmethylsulfonyl fluoride). Cell lysates were cleared by centrifugation and protein concentrations were determined by Lowry assay. Cell lysates containing equal amount of protein were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Protein was transferred electrophoretically to a nitrocellulose membrane. After blocking in 20 mM Tris–HCl (pH 7.6) containing 150 mM NaCl, 0.1% Tween-20, and 2% (w/v) nonfat dry milk, blots were incubated with specific primary antibodies. Immunoreactive bands were visualized by incubation with peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG antibody. The antigen–antibody complex were detected using enhanced chemiluminescence Western blotting detection reagents. Quantification of the Western blots was performed by densitometry.

### 2.5. Transient transfection and luciferase assay

Rat aortic vascular smooth muscle cells were grown to 70–80% confluency in 6-well plates and placed in OPTI-MEM medium. 200 ng DNA of the acyl-CoA oxidase PPRE-Tk-luciferase reporter construct and 400 ng of the full-length PPARγ1 expression vector were cotransfected using LipofectAMINE 2000. Twelve hours after the transfection, cells were starved in DMEM medium containing 0.4% fetal bovine serum for 24 h and were stimulated for 24 h with the PPARγ ligands. Luciferase activity was assayed using a Dual Luciferase Reporter Assay System according to the manufacturer's instructions. Transfection efficiency was adjusted by normalizing firefly luciferase activities to the renilla luciferase activities generated by cotransfection with 10 ng pRL-CMV. All experiments were repeated al least three times with different cell preparations.

### 2.6. Construction of a dominant-negative PPARγ and adenoviral infection of rat aortic vascular smooth muscle cells

A dominant-negative form of PPARγ was constructed by mutating the $Leu^{468}$ and $Glu^{471}$ of the full-length PPARγ1 into Ala. Mutations at these sites create a dominant-negative form of PPARγ (Gurnell et al., 2000). This dominant-negative PPARγ was subcloned into recombinant type 5 adenovirus and designated as Adx-D/N-PPARγ. Recombinant type 5 adenovirus expressing the LacZ gene was generated similarly and used as a control vector (Adx-LacZ). Rat aortic vascular smooth muscle cells were infected with 20 plaque forming units (PFU)/cell in DMEM containing 0.4% fetal bovine serum for 24 h. After further starvation for 24 h, cells were pretreated with the non-thiazolidinedione partial PPARγ agonist for 30 min and were stimulated with PDGF-BB (20 ng/ml) + insulin (1 μM) for 24 h for analysis of cyclin D1 expression and for 48 h for cell growth assays.

### 2.7. Statistical analysis

Data were expressed as mean ± standard of the mean (S.E.M.). Statistical significance was determined using the Student's *t*-test. Values of $P < 0.05$ were considered as statistically significant.

## 3. Results

### 3.1. A non-thiazolidinedione PPARγ ligand serves as a partial agonist for PPARγ

Activation of PPARγ by the non-thiazolidinedione partial PPARγ agonist was assayed by cotransfection of a full-length PPARγ1 expression vector with a luciferase reporter gene driven by three PPRE copies from the acyl-CoA oxidase gene linked to the minimal thymidine kinase promotor. Ligand-induced transcriptional activation of endogenous PPARγ in vascular smooth muscle cells was modest; thus, overexpression by transient transfection was employed to determine more accurately the $EC_{50}$ and the $IC_{50}$ of the non-thiazolidinedione partial PPARγ agonist. Transfected vascular smooth muscle cells exhibited a strong ligand-dependent transcriptional response to increasing concentrations of the thiazolidinedione rosiglitazone with an $EC_{50}$ of 26 nM (Fig. 1A). The non-thiazolidinedione PPARγ ligand functioned as a partial agonist ($EC_{50}$ 48 nM) eliciting ~25% of the maximum efficacy of the full

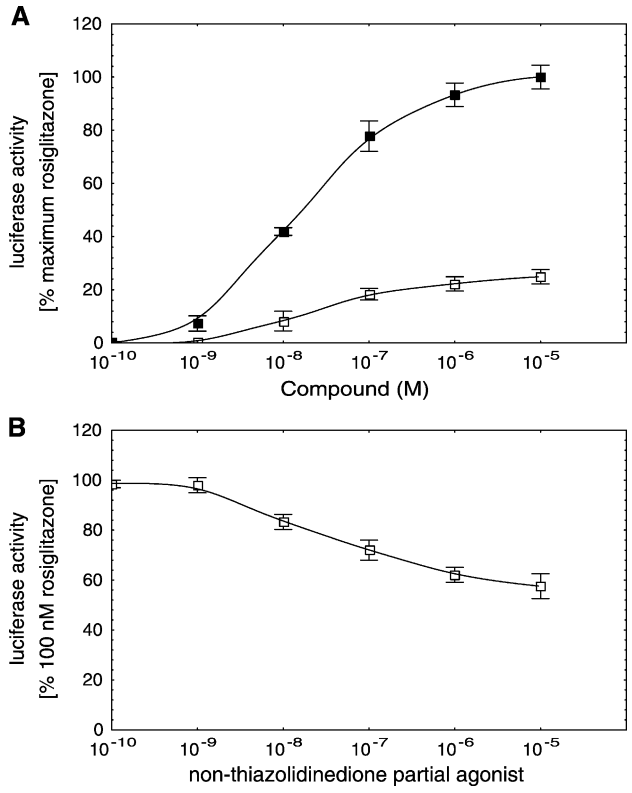


Fig. 1. A non-thiazolidinedione PPARγ ligand seves as partial agonist for PPARγ. Rat aortic vascular smooth muscle cells were transiently cotransfected with a PPARγ1 expression vector and a luciferase reporter gene driven by three PPRE copies from the acyl-CoA oxidase gene linked to a thymidine kinase promotor. Cells were stimulated with the indicated concentrations of the thiazolidinedione full PPARγ agonist rosiglitazone (black squares) or the non-thiazolidinedione partial PPARγ agonist (white squares) (panel A) or with rosiglitazone (100 nM) and the indicated concentration of the non-thiazolidinedione partial PPARγ agonist (panel B). Forty-eight hours after stimulation, firefly luciferase activity was measured and normalized to renilla luciferase activity from cotransfected pRL-CMV. The results represent the mean ± S.E.M from three separate experiments.

PPARγ agonist rosiglitazone. To determine whether the non-thiazolidinedione partial PPARγ agonist competes with a PPARγ full agonist, experiments were performed using a fixed concentration of rosiglitazone (100 nM) and increasing concentrations of the non-thiazolidinedione partial PPARγ agonist (100 pM–10 μM). In the presence of the non-thiazolidinedione partial PPARγ agonist, rosiglitazone-induced PPARγ transcriptional activity was attenuated by ~ 43% ($IC_{50}$ of 32 nM), indicating that the non-thiazolidinedione partial PPARγ agonist effectively inhibits a PPARγ full agonist (Fig. 1B).

### *3.2. The non-thiazolidinedione partial PPARγ agonist inhibits mitogen-induced vascular smooth muscle cell growth and Rb phosphorylation*

To determine the effects of the non-thiazolidinedione partial PPARγ agonist on mitogen-stimulated vascular smooth muscle cell growth, quiescent cells were treated with increasing concentrations of the non-thiazolidinedione partial PPARγ agonist (0.1–10 μM) and were stimulated with PDGF + insulin for 48 h. At these concentrations, no cytotoxic effects were observed, as determined by uptake of the vital dye, trypan blue, and lack of cell detachment. The non-thiazolidinedione partial PPARγ agonist inhibited PDGF + insulin-stimulated vascular smooth muscle cell proliferation by 95.4 ± 8.1% (10 μM, $n = 3$, $P < 0.05$) in a dose-dependent manner (Fig. 2A). This observed inhibition of

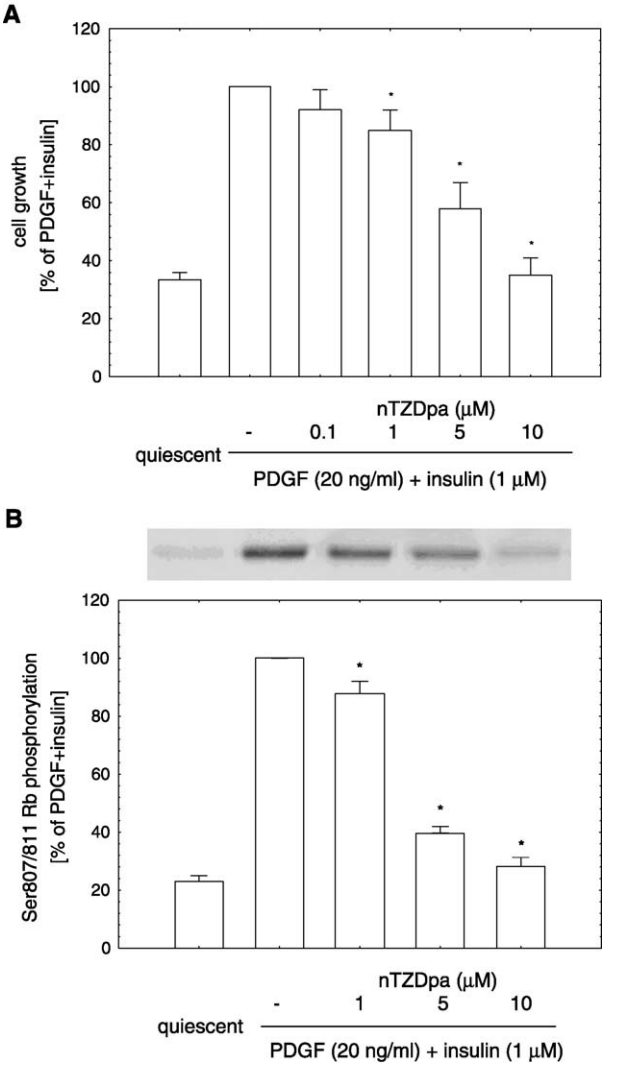


Fig. 2. The non-thiazolidinedione partial PPARγ agonist inhibits cell growth and phosphorylation of the retinoblastoma protein. (A) Quiescent vascular smooth muscle cells were preincubated with the indicated concentrations of the non-thiazolidinedione partial PPARγ agonist (nTZDpa) for 30 min prior to stimulation with PDGF (20 ng/ml) + insulin (1 μM). After 48 h, cells were harvested and cell counts were performed using a hematocytometer. Results are expressed as percent of the maximal mitogenic induction of cell proliferation from three separate experiments. (B) Twenty-four hours after stimulation, whole cell lysates (75 μg) were assayed by Western immunoblotting using an anti-phospho-Rb Ser807/811 antibody. The autoradiogram is representative of three separate experiments. Results are expressed as mean ± S.E.M from three separate experiments (*$P < 0.05$ vs. PDGF + insulin).

vascular smooth muscle cell proliferation correlated with a concentration-dependent decrease in mitogen-induced Rb phosphorylation at Ser807/811 (Fig. 2B). Quiescent vascular smooth muscle cells exhibited low levels of phosphorylated Ser807/811. Phosphorylation of Ser807/811 increased substantially after 24 h stimulation with growth factors (PDGF + insulin). The non-thiazolidinedione partial PPARγ agonist inhibited mitogen-induced phosphorylation of Ser807/811 by 88.7 ± 4.3% ($n = 3$, $P < 0.05$). In combination, these findings suggest that the non-thiazolidinedione partial PPARγ agonist prevents DNA synthesis, at least in part, by inhibition of Rb phosphorylation at specific phosphorylation sites, such as Ser807/811, which mediate CDK-dependent regulation of Rb function and are important for cells to exit G1 and enter S phase (Connell-Crowley et al., 1997).

### 3.3. The non-thiazolidinedione partial PPARγ agonist prevents mitogen-induced downregulation of $p27^{kip1}$

The CDK inhibitor $p27^{kip1}$ is an important negative regulator of Rb phosphorylation, and we have recently reported that PPARγ ligands inhibit mitogen-induced degradation of $p27^{kip1}$ in vascular smooth muscle cells (Wakino et al., 2000). Therefore, we investigated the effects of the non-thiazolidinedione partial PPARγ agonist on $p27^{kip1}$ levels after mitogenic stimulation (Fig. 3).

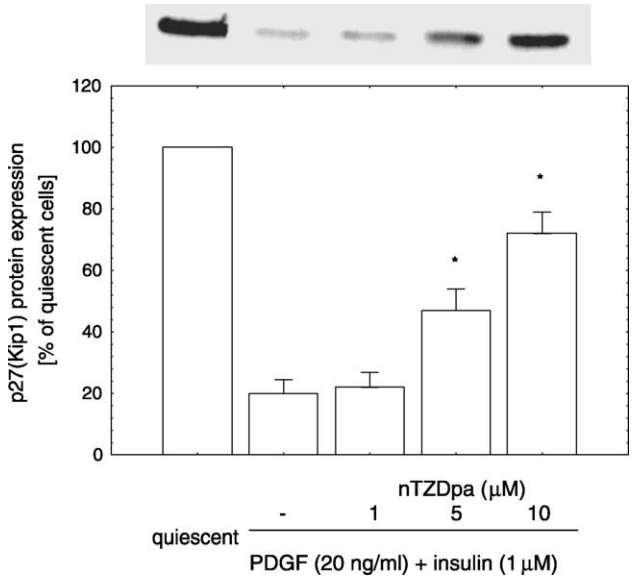


Fig. 3. The non-thiazolidinedione partial PPARγ agonist prevents mitogen-induced downregulation of $p27^{kip1}$. Quiescent rat aortic vascular smooth muscle cells were stimulated with PDGF (20 ng/ml) and insulin (1 μM) for 24 h. Cells were preincubated with the indicated concentrations of the non-thiazolidinedione partial PPARγ agonist (nTZDpa) for 30 min prior to addition of mitogens. Whole cell proteins (30 μg) were assayed by Western immunoblotting using an anti-$p27^{kip1}$ antibody. The autoradiogram is representative of three separate experiments. Results are expressed as mean ± S.E.M. (*$P < 0.05$ vs. PDGF + insulin).

Quiescent vascular smooth muscle cells express substantial $p27^{kip1}$ protein as determined by Western analysis. Mitogenic stimulation (PDGF + insulin) for 24 h resulted in a marked decrease of $p27^{kip1}$ expression (20.6 ± 3.8% of quiescent cells, $n = 3$, $P < 0.05$). Addition of the non-thiazolidinedione partial PPARγ agonist significantly attenuated mitogen-induced downregulation of $p27^{kip1}$ in a dose-dependent manner (3.3 ± 0.2 fold increase vs. PDGF + insulin, $n = 3$, $P < 0.05$). The non-thiazolidinedione partial PPARγ agonist-mediated inhibition of vascular smooth muscle cell DNA synthesis and Rb phosphorylation results, at least in part, through the prevention of mitogen-induced downregulation of $p27^{kip1}$.

### 3.4. The non-thiazolidinedione partial PPARγ agonist inhibits mitogen-induced expression of early G1 cyclins (cyclin D1, cyclin E) and late G1/S phase cyclin A

To examine additional mechanisms by which the partial PPARγ agonist inhibits DNA synthesis and Rb hyperphosphorylation, we next investigated its effects on the expression of the G1 phase cyclins D1 and cyclin E. Western analysis revealed that cyclin D1 was expressed at low levels in quiescent rat aortic vascular smooth muscle cells and substantially increased in response to a 24-h mitogenic stimulation. The non-thiazolidinedione partial PPARγ agonist potently inhibited PDGF + insulin-induced expression of cyclin D1 (Fig. 4A, 83.4 ± 3.4% inhibition, $n = 3$, $P < 0.05$) in a dose-dependent manner. Rosiglitazone at a concentration of 10 μM exhibited no effect on mitogen-induced cyclin D1 expression (Fig. 4B). Data in Fig. 4C show that the non-thiazolidinedione partial PPARγ agonist also substantially inhibited PDGF + insulin-induced cyclin E expression (Fig. 4C, 58.4 ± 4.1% inhibition, $n = 3$, $P < 0.05$).

Cyclin A is essential for cell cycle G1 → S transition and its induction late in G1 and early in S phase is contingent on Rb phosphorylation (Schulze et al., 1995). Therefore, we investigated the effect of the non-thiazolidinedione partial PPARγ agonist on cyclin A expression (Fig. 4D). Mitogenic induction of cyclin A by PDGF + insulin was attenuated by the non-thiazolidinedione partial PPARγ agonist (71.5 ± 4.7% inhibition, $n = 3$, $P < 0.05$). In concert, these findings suggest that inhibition of cell growth and Rb phosphorylation by this partial PPARγ agonist may result from maintaining elevated levels of negative regulators of Rb phosphorylation ($p27^{kip1}$) and blocking the expression of pivotal G1 cyclins necessary for activating CDKs to phosphorylate Rb. The effects of non-thiazolidinedione partial PPARγ agonist on cell cycle regulation were qualitatively similar to those previously reported for rosiglitazone, except for a marked inhibition of mitogen-induced cyclin D1 expression (Wakino et al., 2000).

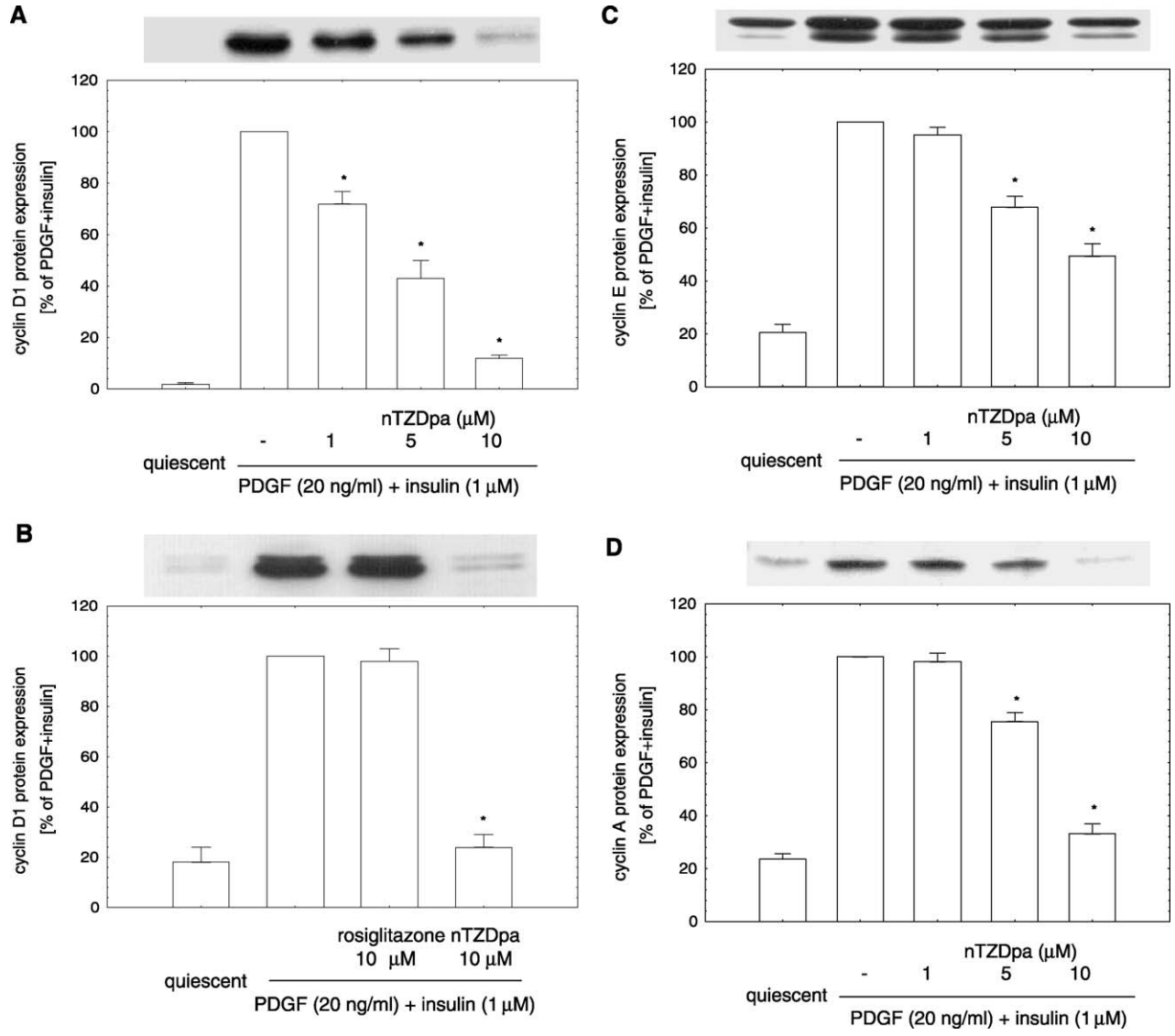


Fig. 4. The non-thiazolidinedione partial PPARγ agonist inhibits mitogenic induction of G1 cyclins. Quiescent rat aortic vascular smooth muscle cells were stimulated with PDGF (20 ng/ml) and insulin (1 μM) for 24 h. Cells were preincubated with the indicated concentrations of the non-thiazolidinedione partial PPARγ agonist (nTZDpa) or rosiglitazone 30 min prior to the addition of mitogens. Whole cell proteins (30 μg) were assayed by Western immunoblotting using an anti-cyclin D1 antibody (panels A and B), anti-cyclin E antibody (panel C), or anti-cyclin A antibody (panel D). The autoradiograms are representative of three separate experiments. Results are expressed as mean ± S.E.M. (*$P < 0.05$ vs. PDGF + insulin).

### *3.5. Adenovirus-mediated overexpression of a dominant-negative PPARγ partially reverses the inhibitory effect of the non-thiazolidinedione partial PPARγ agonist on vascular smooth muscle cell growth*

To determine whether the effect of the non-thiazolidinedione partial PPARγ agonist on vascular smooth muscle cell growth was mediated through a PPARγ-dependent mechanism, we employed an adenovirus to overexpress the dominant-negative $L^{468}A/E^{471}A$ PPARγ mutant (Adx-D/N-PPARγ) to block the function of endogenous wild-type PPARγ. Ligand-induced transcriptional activation of PPARγ in cells overexpressing this compound mutant is severely impaired (Gurnell et al., 2000). Infection of vascular smooth muscle cells with the control Adx-LacZ and stimulation with PDGF + insulin resulted in a 2.49 ± 0.33-fold increase in cell growth and a potent inhibition by the non-thiazolidinedione partial PPARγ agonist (Fig. 5, 100% inhibition at 10 μM, $n = 3$, $P < 0.05$). In contrast, when the dominant-negative PPARγ was overexpressed, the inhibitory effect of the non-thiazolidinedione partial PPARγ agonist on vascular smooth muscle cell growth was significantly attenuated (55.1 ± 6.3% inhibition, $n = 3$, $P < 0.05$). Taken together, these results indicate that inhibition of vascular smooth muscle cell proliferation by the non-thiazolidinedione partial PPARγ agonist is partially reversed by dominant-negative PPARγ. Therefore, inhibition of vascular smooth muscle cell growth by this partial PPARγ ligand is,

at least in part, mediated through a PPARγ-dependent pathway.

### 3.6. The non-thiazolidinedione partial PPARγ agonist-mediated inhibition of mitogen-induced cyclin D1 upregulation may be PPARγ-independent

As the full PPARγ agonist rosiglitazone exhibited no effect on mitogen-induced cyclin D1 expression, we next investigated whether the non-thiazolidinedione partial PPARγ agonist blocked cyclin D1 expression through a PPARγ-dependent pathway. As shown in Fig. 6A, we observed faint expression of endogenous PPARγ in whole cell extracts of rat aortic vascular smooth muscle cells, consistent with our previous findings that detection of endogenous PPARγ protein was detectable only in nuclear fractions (Law et al., 2000). Adenoviral-mediated infection of rat aortic vascular smooth muscle cells resulted in marked overexpression of dominant-negative PPARγ. Infection of rat aortic vascular smooth muscle cells with Adx-D/N-PPARγ had no effect on the activity of the non-thiazolidinedione partial PPARγ agonist to inhibit mitogen-induced cyclin D1 upregulation compared to control Adx-LacZ infected cells (Fig. 6B). In concert, these data suggest that the inhibition of cyclin D1 by the non-thiazolidinedione partial PPARγ agonist likely occurs through a PPARγ-independent mechanism.

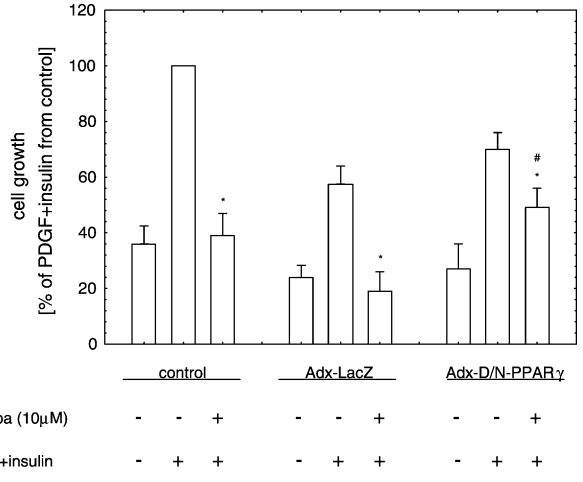


Fig. 5. Adenovirus-mediated overexpression of a dominant-negative PPARγ partially reverses the inhibitory effect of the non-thiazolidinedione partial PPARγ agonist on vascular smooth muscle cell proliferation. Rat aortic vascular smooth muscle cells were infected with 20 PFU/cell recombinant adenovirus overexpressing dominant-negative PPARγ (Adx-D/N-PPARγ) or the LacZ gene as a control vector (Adx-LacZ). After starvation for 24 h, cells were pretreated with the indicated concentrations of the non-thiazolidinedione partial PPARγ agonist (nTZDpa) for 30 min and stimulated with PDGF (20 ng/ml) + insulin (1 μM). After 48 h, cells were harvested and counted using a hematocytometer. Results are expressed as percent of the maximal mitogenic induction of cell proliferation in uninfected control rat aortic vascular smooth muscle cells. Data represent mean ± S.E.M from three separate experiments (*$P < 0.05$ vs. PDGF + insulin, #$P < 0.05$ vs. PDGF + insulin and Adx-LacZ-infected cells treated with the non-thiazolidinedione partial PPARγ agonist).

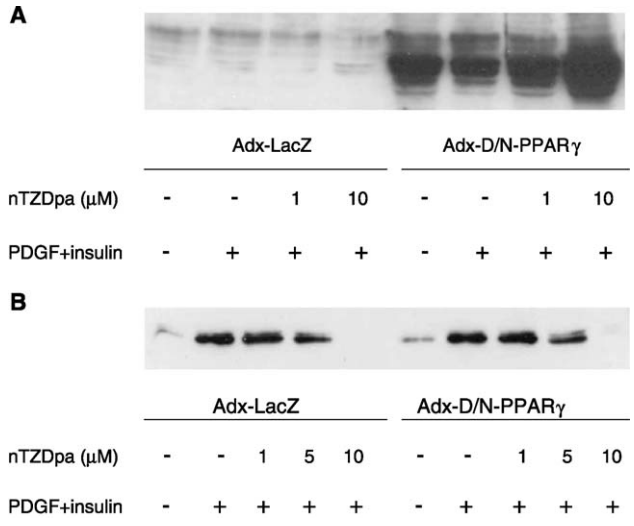


Fig. 6. The non-thiazolidinedione partial PPARγ agonist-mediated inhibition of mitogen-induced cyclin D1 upregulation may be PPARγ-independent. Vascular smooth muscle cells were infected with 20 PFU/cell adenovirus overexpressing dominant-negative PPARγ (Adx-D/N-PPARγ) or the LacZ gene as a control vector (Adx-LacZ). After starvation for 24 h, cells were pretreated with the indicated concentrations of the non-thiazolidinedione partial PPARγ agonist (nTZDpa) for 30 min and stimulated with PDGF (20 ng/ml) + insulin (1 M) for 24 h. Whole cell proteins (30 μg) were assayed by Western immunoblotting for expression of PPARγ using an anti-PPARγ antibody (panel A) or anti-cyclin D1 antibody (panel B). The autoradiograms are representative of three separate experiments.

## 4. Discussion

In the present investigation, we report that a novel non-thiazolidinedione partial PPARγ agonist inhibits vascular smooth muscle cell proliferation. The antiproliferative effect of this partial PPARγ agonist results from its activity to modulate several key cell cycle regulators that control G1 → S phase progression. The non-thiazolidinedione partial PPARγ agonist prevented Rb phosphorylation by inhibiting cyclin (D1, E, and A) expression and by preventing the mitogen-induced downregulation of $p27^{kip1}$. Inhibition of vascular smooth muscle cell growth by the non-thiazolidinedione partial PPARγ agonist was partially reversed by adenovirus-mediated overexpression of dominant-negative PPARγ. Effects of this partial PPARγ agonist on cyclin D1 were not mimicked by the full PPARγ agonist rosiglitazone. Inhibition of cyclin D1 expression in G1 by the non-thiazolidinedione partial PPARγ agonist likely occurs independent of its binding to PPARγ as it persisted in vascular smooth muscle cells overexpressing a dominant-negative PPARγ mutant.

We have previously reported that the classical thiazolidinediones, rosiglitazone and troglitazone, inhibited Rb phosphorylation and G1 → S phase transition by blocking

mitogen-induced downregulation of $p27^{kip1}$ (Wakino et al., 2000). Downregulation of $p27^{kip1}$ during G1 phase in response to mitogens is important for maximal activation of G1 cyclin/CDK complexes, particularly cyclin E-CDK2 (Rao, 1999). In the present study, we also observed that the non-thiazolidinedione partial PPARγ agonist is a potent inhibitor of $p27^{kip1}$ downregulation induced by PDGF + insulin, underscoring the importance of this CDKI as a target for both thiazolidinedione and non-thiazolidinedione PPARγ ligands as well as for full vs. partial PPARγ agonists.

Using antisense oligonucleotides to $p21^{cip1}$ and $p27^{kip1}$, other investigators have suggested that the inhibition of cell cycle progression by thiazolidinediones occurs independently of these CDKIs (Hupfeld and Weiss, 2001). Despite this controversy concerning the role of $p27^{kip1}$ in in vitro studies, accumulating evidence implicates $p27^{kip1}$ as an important regulator of vascular smooth muscle cell growth in vivo. Neointima formation after mechanical injury of vessels triggers a dramatic decrease of $p27^{kip1}$ protein levels (Wei et al., 1997). Adenoviral overexpression of $p27^{kip1}$ in cultured vascular smooth muscle cells inactivated CDK2 and CDK4 activity and in vivo gene transfer of $p27^{kip1}$ significantly inhibited neointimal cell proliferation (Tanner et al., 2000).

In addition to its effect on $p27^{kip1}$, modulation of the cyclin/CDK system by the non-thiazolidinedione partial PPARγ agonist further contributes to cell growth inhibition. Previous studies have shown that a variety of growth factors induce expression of cyclin D1, which partners with CDK4 or CDK6 to phosphorylate Rb early in G1 (reviewed by Roovers and Assoian, 2000; Sherr, 1996). In our previous studies, rosiglitazone did not affect levels of cyclin D1 or cyclin E in mitogen-stimulated rat aortic vascular smooth muscle cells (Wakino et al., 2000). In marked contrast, we find that the non-thiazolidinedione partial PPARγ agonist potently inhibits expression of these G1 cyclins. These findings demonstrate a differential activity of the non-thiazolidinedione partial PPARγ agonist vs. thiazolidinedione PPARγ ligands to affect G1 cyclin levels. However, downregulation of cyclin D1 by the non-thiazolidinedione partial PPARγ agonist may be of particular importance for its activity to inhibit vascular smooth muscle cell growth. Induction of G1 arrest in endothelial cells by the antiangiogenic factor endostatin has been recently shown to result singularly from an inhibition of cyclin D1 transcription (Hanai et al., 2002). Overexpression of cyclin D1 rendered endothelial cells refractory to cell cycle inhibition by endostatin. Additional studies are required to determine whether selective targeting of cyclin D1 by the non-thiazolidinedione partial PPARγ agonist enhances its antiproliferative activity in vivo after arterial injury compared to thiazolidinedione full PPARγ agonists that target only $p27^{kip1}$.

Although the ability of thiazolidinediones to induce PPARγ-mediated adipose cell differentiation has been clearly demonstrated, it is unclear whether the cell cycle-inhibitory effects of thiazolidinediones occur through this nuclear receptor. To specifically address this question, we employed a dominant-negative PPARγ mutant. Using adenovirus-mediated infection of vascular smooth muscle cells, we find that in cells overexpressing dominant-negative PPARγ, the inhibitory effect of the non-thiazolidinedione partial PPARγ agonist on vascular smooth muscle cell growth was partially attenuated. Therefore, the antiproliferative activity of this PPARγ ligand against vascular smooth muscle cells stems in part from its being a ligand for PPARγ. In our studies, the non-thiazolidinedione partial PPARγ agonist potently suppressed mitogen-induced cyclin D1 expression, while the full PPARγ agonist rosiglitazone did not affect cyclin D1 expression. Moreover, we observed that overexpression of the dominant-negative PPARγ had no effect to suppress the non-thiazolidinedione partial PPARγ agonist-mediated inhibition of mitogen-induced cyclin D1 expression, suggesting that cyclin D1 may not be a direct target for PPARγ. Thiazolidinediones were recently shown to inhibit cyclin D1 expression and cell proliferation in PPARγ null embryonic stem cells which provides strong evidence that this effect occurs independent of their binding to and activating PPARγ (Palakurthi et al., 2001). The precise mechanism by which the non-thiazolidinedione partial PPARγ agonist and thiazolidinediones inhibit cell growth and differentially target cell cycle regulators remains to be elucidated.

Although thiazolidinediones and the non-thiazolidinedione partial PPARγ agonist belong to distinct chemical classes, they share a common biological activity as PPARγ ligands. All members of the PPAR family (α, δ, and γ) possess unusually large binding pockets, compared to other nuclear receptors, which accommodate a diverse set of lipophilic acids as ligands (Nolte et al., 1998). Antiproliferative effects of PPARγ ligands are observed at concentrations considerably higher than their $EC_{50}$ for transcriptional activation in cell-based transfection assays or in in vitro binding assays with isolated ligand-binding domain fragments (Berger et al., 1999). At high concentrations, spillover of PPARγ-selective ligands to PPARα and/or PPARδ is quite possible. The antiproliferative activity of thiazolidinediones observed in PPARγ null cells, or for the non-thiazolidinedione partial PPARγ agonist in the presence of a dominant-negative PPARγ mutant, could be explained by their binding to and activation of PPARα or PPARδ. Activation of PPARδ has been reported to stimulate rather than inhibit growth of vascular smooth muscle cells (Zhang et al., 2002) and keratinocytes (Tan et al., 2001) and no studies have reported an antiproliferative effect for PPARα ligands. Bishop-Bailey et al. (2002) have recently reported that rosiglitazone induced apoptosis in intimal vascular smooth muscle cells through a PPARγ-dependent pathway although effective concentrations of the thiazolidinediones were 2–3 logs higher than its $EC_{50}$ for receptor binding. Our data on the antiproliferative effect of the non-thiazolidinedione partial PPARγ agonist leads us to a similar

conclusion that biological activity observed at supraphar-macologic concentrations can be still mediated by PPARγ.

At present, pioglitazone and rosiglitazone are in clinical use as insulin sensitizers for the treatment of type II diabetes. Patients with type II diabetes are at increased risk for the development and progression of atherosclerotic disease (Hsueh and Law, 1998). This population also responds more poorly to intervention for their coronary artery disease by exhibiting a higher frequency of restenosis compared to nondiabetic subjects (The BARI investigators, 2000). Thiazolidinedione PPARγ ligands have been shown to inhibit vascular lesion formation in a variety of animal models of atherosclerosis and restenosis (Collins et al., 2001; Koshiyama et al., 2001; Law et al., 1996; Li et al., 2000). The clinical use of full PPARγ agonists is sometimes accompanied by undesirable side effects including edema, slight reductions in hemoglobin and hematocrit (due to hemodilution), and weight gain (Lebovitz, 2002). Therefore, great effort is being expended to generate new PPARγ ligands with lesser side effects. Partial agonists for PPARγ that regulate only a subset of genes modulated by more potent full agonists are promising candidates for minimizing side effects while preserving clinical benefits of the class.

Thiazolidinediones exert their antidiabetic effects as insulin sensitizers through a mechanism that involves PPARγ-mediated alteration of expression of multiple genes involved in glucose and lipid metabolism in adipose tissue, muscle and liver (Olefsky and Saltiel, 2000; Moller, 2001). Inhibition of cell proliferation by thiazolidinediones and the non-thiazolidinedione partial PPARγ agonist is more complex and likely involves a combination of PPARγ-dependent and PPARγ-independent mechanisms. Therefore, antiproliferative activity of PPARγ ligands in vascular cells may be readily differentiated from their metabolic effects in peripheral tissues. This may ultimately lead to the development of new agents selected primarily for their vascular, rather than metabolic, activity.

## Acknowledgements

This study was supported by a National Institutes of Health Grant HL 58328 to Willa A. Hsueh. Dennis Bruemmer was supported by a grant from MSD Sharp & Dohme (Acute Coronary Syndrome 2000) and by a research fellowship from the Gonda (Goldschmied) Diabetes Center, University of California, Los Angeles.

## References

Berger, J.P., Petro, A.E., Macnaul, K.L., Kelly, L.J., Zhang, B.B., Richards, K., Elbrecht, A., Johnson, B.A., Zhou, G., Doebber, T.W., Biswas, C., Parikh, M., Sharma, N., Tanen, M.R., Thompson, G.M., Ventre, J., Adams, A.D., Mosley, R., Surwit, R.S., Moller, D.E., 2003. Distinct properties and advantages of a novel PPAR{gamma} selective modulator. Mol. Endocrinol., 2002–2217.

Berger, J., Leibowitz, M.D., Doebber, T.W., Elbrecht, A., Zhang, B., Zhou, G., Biswas, C., Cullinan, C.A., Hayes, N.S., Li, Y., Tanen, M., Ventre, J., Wu, M.S., Berger, G.D., Mosley, R., Marquis, R., Santini, C., Sahoo, S.P., Tolman, R.L., Smith, R.G., Moller, D.E., 1999. Novel peroxisome proliferator-activated receptor (PPAR) gamma and PPARdelta ligands produce distinct biological effects. J. Biol. Chem. 274, 6718–6725.

Bishop-Bailey, D., Hla, T., Warner, T.D., 2002. Intimal smooth muscle cells as a target for peroxisome proliferator-activated receptor-gamma ligand therapy. Circ. Res. 91, 210–217.

Braun-Dullaeus, R.C., Mann, M.J., Ziegler, A., von der Leyen, H.E., Dzau, V.J., 1999. A role for the cyclin-dependent kinase inhibitor p27(Kip1) in angiotensin II-stimulated vascular smooth muscle cell hypertrophy. J. Clin. Invest. 104, 815–823.

Chawla, A., Boisvert, W.A., Lee, C.H., Laffitte, B.A., Barak, Y., Joseph, S.B., Liao, D., Nagy, L., Edwards, P.A., Curtiss, L.K., Evans, R.M., Tontonoz, P., 2001. A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. Mol. Cell. 7, 161–171.

Collins, A.R., Meehan, W.P., Kintscher, U., Jackson, S., Wakino, S., Noh, G., Palinski, W., Hsueh, W.A., Law, R.E., 2001. Troglitazone inhibits formation of early atherosclerotic lesions in diabetic and nondiabetic low density lipoprotein receptor-deficient mice. Arterioscler. Thromb. Vasc. Biol. 21, 365–371.

Connell-Crowley, L., Harper, J.W., Goodrich, D.W., 1997. Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Mol. Cell. Biol. 8, 287–301.

Elbrecht, A., Chen, Y., Cullinan, C.A., Hayes, N., Leibowitz, M., Moller, D.E., Berger, J., 1996. Molecular cloning, expression and characterization of human peroxisome proliferator activated receptors gamma 1 and gamma 2. Biochem. Biophys. Res. Commun. 224, 431–437.

Gurnell, M., Wentworth, J.M., Agostini, M., Adams, M., Collingwood, T.N., Provenzano, C., Browne, P.O., Rajanayagam, O., Burris, T.P., Schwabe, J., Lazar, M.A., Chatterjee, V.K.K., 2000. A dominant-negative peroxisome proliferator-activated receptor gamma (PPARgamma) mutant is a constitutive repressor and inhibits PPARgamma-mediated adipogenesis. J. Biol. Chem. 275, 5754–5759.

Hanai, J., Dhanabal, M., Karumanchi, S.A., Albanese, C., Waterman, M., Chan, B., Ramchandran, R., Pestell, R., Sukhatme, V.P., 2002. Endostatin causes G1 arrest of endothelial cells through inhibition of cyclin D1. J. Biol. Chem. 277, 16464–16469.

Hsueh, W.A., Law, R.E., 1998. Diabetes is a vascular disease. J. Investig. Med. 46, 387–390.

Hupfeld, C.J., Weiss, R.H., 2001. TZDs inhibit vascular smooth muscle cell growth independently of the cyclin kinase inhibitors p21 and p27. Am. J. Physiol: Endocrinol. Metab. 281, E207–E216.

Koshiyama, H., Shimono, D., Kuwamura, N., Minamikawa, J., Nakamura, Y., 2001. Inhibitory effect of pioglitazone on carotid arterial wall thickness in type 2 diabetes. J. Clin. Endocrinol. Metab. 86, 3452–3457.

Law, R.E., Meehan, W.P., Xi, X.P., Graf, K., Wuthrich, D.A., Coats, W., Faxon, D., Hsueh, W.A., 1996. Troglitazone inhibits vascular smooth muscle cell growth and intimal hyperplasia. J. Clin. Invest. 98, 1897–1905.

Law, R.E., Goetze, S., Xi, X.P., Jackson, S., Kawano, Y., Demer, L., Fishbein, M.C., Meehan, W.P., Hsueh, W.A., 2000. Expression and function of PPARγ in rat and human vascular smooth muscle cells. Circulation 101, 1311–1318.

Lebovitz, H.E., 2002. Differentiating members of the thiazolidinedione class: a focus on safety. Diabetes Metab. Res. Rev. 18, S23–S29.

Li, A.C., Brown, K.K., Silvestre, M.J., Willson, T.M., Palinski, W., Glass, C., 2000. Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. J. Clin. Invest. 106, 523–531.

Lukas, J., Bartkova, J., Bartek, J., 1996. Convergence of mitogenic signalling cascades from diverse classes of receptors at the cyclin D-cyclin-dependent kinase-pRb controlled G1 checkpoint. Mol. Cell. Biol. 16, 6917–6925.

Marx, N., Schonbeck, U., Lazar, M.A., Libby, P., Plutzky, J., 1998. Per-

oxisome proliferator-activated receptor gamma activators inhibit gene expression and migration in human vascular smooth muscle cells. Circ. Res. 83, 1097–1103.

Moller, D.E., 2001. New drug targets for type 2 diabetes and the metabolic syndrome. Nature 414, 821–827.

Morgan, D.O., 1995. Principles of CDK regulation. Nature 374, 131–134.

Nolte, R.T., Wisely, G.B., Westin, S., Cobb, J.E., Lambert, M.H., Kurokawa, R., Rosenfeld, M.G., Willson, T.M., Glass, C.K., Milburn, M.V., 1998. Ligand binding and co-activator assembly of the peroxisome proliferator activated receptor-gamma. Nature 395, 137–143.

Olefsky, J.M., Saltiel, A.R., 2000. PPAR gamma and the treatment of insulin resistance. Trends Endocrinol. Metab. 11, 362–368.

Palakurthi, S.S., Aktas, H., Grubissich, L.M., Mortensen, R.M., Halperin, J.A., 2001. Anticancer effects of thiazolidinediones are independent of peroxisome proliferator-activated receptor gamma and mediated by inhibition of translation initiation. Cancer Res. 61, 6213–6218.

Rao, G.N., 1999. Differential regulation of p27Kip1 levels and CDK activities by hypertrophic and hyperplastic agents in vascular smooth muscle cells. Biochim. Biophys. Acta 1448, 525–532.

Ricote, M., Huang, J., Fajas, L., Li, A., Welch, J., Najib, J., Witzum, J.L., Auwerx, J., Palinski, W., Glass, C.K., 1998. Expression of the peroxisome proliferator-activated receptor gamma (PPAR-γ) in human atherosclerosis and regulation in macrophages by colony stimulating factors and oxidized low-density lipoprotein. Proc. Natl. Acad. Sci. U. S. A. 23, 7614–7619.

Roovers, K., Assoian, R.K., 2000. Integrating the MAP kinase into the G1 phase cell cycle machinery. BioEssays 22, 816–826.

Ross, R., 1995. Cell biology of atherosclerosis. Annu. Rev. Physiol. 57, 791–804.

Schulze, A., Zerfass, K., Spitkovsky, D., Middendorp, S., Berges, J., Helin, K., Jansen-Durr, P., Henglein, B., 1995. Cell cycle regulation of the cyclin A gene promotor is mediated by a variant E2F site. Proc. Natl. Acad. Sci. U. S. A. 92, 11264–11268.

Schwartz, S.M., 1997. Perspectives series: cell adhesion in vascular biology. Smooth muscle migration in atherosclerosis and restenosis. J. Clin. Invest. 99, 2814–2816.

Sherr, C.J., 1996. Cancer cell cycles. Science 274, 1672–1677.

Tan, N.S., Michalik, L., Noy, N., Yasmin, R., Pacot, C., Heim, M., Fluhmann, B., Desvergne, B., Wahli, W., 2001. Critical roles of PPAR beta/delta in keratinocyte response to inflammation. Genes Dev. 15, 3263–3277.

Tanner, F.C., Boehm, M., Akyurek, L.M., San, H., Yang, Z.Y., Tashiro, J., Nabel, G.J., Nabel, E.G., 2000. Differential effects of the cyclin-dependent kinase inhibitors p27(KIP1), p21(CIP1), and 16(Ink4) on vascular smooth muscle cell proliferation. Circulation 101, 2022–20225.

The BARI Investigators. National Heart, Lung and Blood Institute, Bethesda, Maryland, USA, 2000. Seven year outcome in the bypass angioplasty revascularization investigation (BARI) by treatment and diabetic status. J. Am. Coll. Cardiol. 35, 1122–1129.

Wakino, S., Kintscher, U., Kim, S., Yin, F., Hsueh, W.A., Law, R.E., 2000. Peroxisome proliferator-activated receptor γ ligands inhibit retinoblastoma phosphorylation and G1 → S phase transition in vascular smooth muscle cells. J. Biol. Chem. 275, 22435–22441.

Wei, G.L., Krasinski, K., Kearney, M., Isner, J.M., Walsh, K., Andres, V., 1997. Temporally and spatially coordinated expression of cell cycle regulatory factors after angioplasty. Circ. Res. 80, 418–426.

Weinberg, R.A., 1996. E2F and cell proliferation: a world turned upside down. Cell. 85, 457–459.

Zhang, J., Fu, M., Zhu, X., Xiao, Y., Mou, Y., Zheng, H., Akinbami, M.A., Wang, Q., Chen, Y.E., 2002. Peroxisome proliferator-activated receptor delta is upregulated during vascular lesion formation and promotes post confluent cell proliferation in vascular smooth muscle cells. J. Biol. Chem. 277, 11505–11512.