TZDs inhibit vascular smooth muscle cell growth independently of the cyclin kinase inhibitors p21 and p27

CHRISTOPHER J. HUPFELD1 AND ROBERT H. WEISS2,3,4
Divisions of 1Endocrinology and 2Nephrology, Department of Internal Medicine, and 3Cell and Developmental Biology Graduate Group, University of California, Davis 95616; and 4Department of Veterans Affairs Northern California Health Care System, Mather, California 95655

Received 18 December 2000; accepted in final form 16 March 2001.

Hupfeld, Christopher J., and Robert H. Weiss. TZDs inhibit vascular smooth muscle cell growth independently of the cyclin kinase inhibitors p21 and p27. Am J Physiol Endocrinol Metab 281: E207–E216, 2001.—The thiazolidinediones (TZDs) are commonly used to treat hyperglycemia in type 2 diabetes. Diabetes is associated with macrovascular disease, leading to accelerated atherosclerosis caused by aberrant vascular smooth muscle (VSM) cell proliferation. Although VSM cell proliferation is inhibited by the TZDs, the mechanism of this effect has not been established. Because of reports that the cyclin kinase inhibitors (CKIs) p21Waf1/Cip1 and p27Kip1 can exhibit both growth-inhibitory and growth-permissive effects in VSM cells, we asked whether alterations in these cell cycle regulatory proteins are the mechanism by which the TZDs inhibit VSM cell growth. We show that platelet-derived growth factor-BB increases p21 and p27 and that this increase is attenuated by TZDs. Surprisingly, when VSM cells were transfected with antisense oligodeoxynucleotides to p21 and p27, inhibition of DNA synthesis by TZDs occurred to the same degree as in control cells. Furthermore, the TZDs have inhibitory effects on cyclin D1 and cyclin E levels, suggesting another mechanism by which these drugs decrease VSM cell growth. These data suggest that the TZD-mediated reduction in CKI levels is not the sole mechanism for their antiproliferative effects. The observed decrease in levels of the G1 cyclins by the TZDs suggests a possible mechanism of VSM cell growth inhibition.

atherosclerosis, a disease characterized by aberrant vascular smooth muscle (VSM) cell proliferation, is the most common cause of mortality in the Western world and is highly prevalent among diabetic patients. These individuals develop an accelerated form of atherosclerosis, which, when occurring concomitantly with insulin resistance, obesity, hyperlipidemia, and hypertension, has been labeled the “insulin resistance syndrome.” The significance of atherosclerosis in diabetes is underscored by findings that 80% of deaths in the diabetic population are related to atherosclerotic diseases such as stroke, myocardial infarction, and peripheral vascular disease (15). Many pathological conditions common to diabetes, including hyperglycemia, presence of advanced glycation end products, oxidative stress, and chronic inflammation, contribute to enhanced VSM cell proliferation and, thus, accelerated atherosclerosis, such that research on the mechanism leading to pharmacological attenuation of such proliferation is of paramount importance in clinical medicine.

The VSM cell normally resides in the arterial media, where it performs several important functions, including maintenance of vascular tone and synthesis of structural proteins. After arterial injury, growth factors and cytokines released from endothelial cells, monocytes, lymphocytes, and platelets induce VSM cells to migrate to the arterial intima, where they proliferate and secrete various chemotactic and inflammatory factors. If the mitogenic signal to the VSM cell is persistent, migration and proliferation continue unabated and lead to formation of an atherosclerotic plaque (35).

The G1-to-S phase transition in VSM and other cells is accompanied by phosphorylation of the retinoblastoma protein (Rb), releasing its inhibitory effect on the S phase transcription factors, E2F, and resulting in transcription of early genes required for mitosis (16). The cyclin-dependent kinases (CDK2, CDK4, and CDK6), in complex with the G1 cyclins (cyclin D1, cyclin E), phosphorylate Rb during G1 and thus set into motion events of cell cycle transit. The cyclin kinase inhibitors (CKIs) have been shown to regulate the activity of cyclin/CDK complexes and thus can have a profound effect on G1-to-S phase progression. The Cip/Kip family of CKIs, which includes p21Waf1/Cip1 and p27Kip1, are capable of inhibiting cyclin/CDK complex activity in G1 phase (36), yet recent work on these molecules has shown that they are, under some conditions, required for assembly of cyclin D- and cyclin E-dependent kinases (7, 11, 44).

The thiazolidinediones (TZDs) are a class of pharmaceuticals which are commonly used to treat hyperglycemia in patients with type 2 diabetes mellitus. They act as high-affinity ligands for the peroxisome proliferator-activated receptor-γ; cell cycle; proliferation; atherosclerosis; diabetes

Address for reprint requests and other correspondence: R. H. Weiss, Division of Nephrology, TB 136, Dept. of Internal Medicine, Univ. of California, Davis, CA 95616 (E-mail: rweiss@ucdavis.edu).

http://www.ajpendo.org 0193-1849/01 $5.00 Copyright © 2001 the American Physiological Society E207
proliferator-activated receptor-γ (PPARγ), a nuclear receptor and transcription factor involved in energy metabolism (31). When used in diabetic patients, these medications lower blood glucose levels by enhancing peripheral insulin sensitivity (10). In addition, they have clinically beneficial effects on many aspects of the insulin resistance syndrome, including redistribution of central body fat stores, lowering of triglycerides, raising of high-density lipoprotein cholesterol, and lowering of blood pressure (2). Recent studies have shown that TZDs also convey anti-inflammatory (17) and antiproliferative (21) effects in vascular tissues, in some cases correlating with changes in CKI levels (43). However, the mechanism of the antiproliferative effect in VSM cells, although postulated to involve CKI modulation, has yet to be established. We now show that, although the TZDs ciglitazone and rosiglitazone inhibit both platelet-derived growth factor (PDGF)-induced DNA synthesis and upregulation of p21 and p27, attenuation of these CKIs is not the mechanism by which these medications attenuate VSM cell growth. Our work suggests that, rather than conveying a causal effect on cell proliferation, CKI attenuation serves to mediate another, as-yet-undetermined effect of these medications in vascular, and perhaps other, tissues. Our findings that the G1 cyclins are decreased by the TZDs suggests a possible mechanism for VSM cell growth inhibition by these medications.

EXPERIMENTAL PROCEDURES

Materials. Ciglitazone was purchased from Sigma (St. Louis, MO). Rosiglitazone was a gift from SmithKlineBeecham Pharmaceuticals (Exxen, UK). Human recombinant PDGF-BB was obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal PPARγ and cyclin E antibodies; mouse monoclonal p21Waf1/Cip1, p27Kip1, and cyclin D1 antibodies; and goat polyclonal CDK2 and CDK4 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectin was obtained from Life Technologies (Rockville, MD). [3H]thymidine and [3H]leucine were obtained from Amersham (Arlington Heights, IL). All other reagents, including mouse monoclonal α-actin antibody, were obtained from Sigma.

Cell culture. Cultures of A10 rat aortic VSM cells were obtained from American Type Culture Collection (Rockville, MD) and were used between passages 15 and 24. The cells were maintained at 37°C, 5% CO2, in media consisting of DMEM, 20 mM HEPES (pH 7.4), 5 mg/ml transferrin, 0.5 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). After a 20-min incubation, cells were scraped, passed through a 25-gauge needle three times, and centrifuged for 10 min at 10,000 rpm and 4°C. Supernatant was saved, and protein concentration was determined using Bio-Rad protein concentration reagent and 4°C. Supernatant was saved, and protein concentration was determined using Bio-Rad protein concentration reagent.

Antisense transfections. Phosphorothioate antisense oligodeoxynucleotides were synthesized by Oligos Etc. (Wilsonville, OR). The p21Waf1/Cip1 antisense vector was designed around the start codon of rat p21Waf1/Cip1, with the sequence 5′-TGG CTC TCC GCC TGC-3′. The random sequence control oligodeoxynucleotide was 5′-TGG ATC CGA CAT GTC AGA-3′. Cells to be transfected were grown to 80% confluence in complete media. They were then washed once with sterile phosphate-buffered saline (PBS) and then exposed to a mixture of Opti-MEM medium, Lipofectin (6.6 μl/ml Opti-MEM), and 400 nM oligodeoxynucleotide for 4 h at 37°C. Medium was then changed to quiescence medium for 20 h.

[3H]thymidine incorporation. Cells were grown to 80% confluence on 6-cm tissue culture plates and then placed in quiescence medium for 24 h. PDGF-BB and/or TZDs were then added for an additional 24 h. After the various treatments, cells were placed on ice, washed three times in cold PBS, and lysed with 200 μl of cold lysis buffer (50 mM HEPES, 1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). After a 20-min incubation, cells were scraped, passed through a 25-gauge needle three times, and centrifuged for 10 min at 10,000 rpm and 4°C. Supernatant was saved, and protein concentration was determined using Bio-Rad protein concentration reagent and a spectrophotometer. Gel electrophoresis was then performed as described previously (45), and density of the bands was determined by the ImageQuant software.

Cell viability studies. [3H]leucine incorporation was determined to assess for cell toxicity in the presence of ciglitazone and rosiglitazone. Procedures follow the same protocol as that described above for [3H]thymidine incorporation, except that 1 μCi/ml of [3H]leucine was used in place of thymidine.

Statistics. Unpaired and ratio-paired t-tests were performed for statistical analysis, as indicated. Data are expressed as means ± SD.

RESULTS

PPARγ protein is present in VSM cells, and its level is not altered by serum removal. PPARγ is abundant in adipose tissue, consistent with its role in controlling a variety of genes involved in fatty acid metabolism and adipocyte differentiation (40), and thus studies on the mechanisms of action of the PPARγ agonists have generally focused on these cell lines. The expression of PPARγ has also been studied in mesenchymal cell lines; however, there exist descriptions of its presence (20) as well as its absence (39) in various tissues, including VSM cells. To determine whether PPARγ is present in the A10 VSM cell line used in this study, we performed immunoblots using mouse monoclonal PPARγ antibody, demonstrating the presence of PPARγ protein at similar levels in both serum-starved and serum-stimulated VSM cells (Fig. 1).

Both ciglitazone and rosiglitazone decrease PDGF-BB-induced DNA synthesis in VSM cells. Several TZDs have been shown to inhibit DNA synthesis in primary
VSM cells, as well as in endothelial (46) and renal mesangial (1) cells and B lymphocytes (32). To determine whether the growth-inhibitory effect of the TZDs is present in A10 VSM cells, we examined [3H]thymidine incorporation by these cells in culture after stimulation with the mitogen PDGF-BB. To assess progression through the cell cycle by PDGF, we first serum starved the cells; this procedure has been demonstrated in this and other VSM cell lines to synchronize the cells and cause their arrest in G0/G1 phase of the cell cycle (9a, 29), such that the effect of addition of a growth factor can be evaluated. After the addition of serum or growth factor, synchronized A10 cells progress into S phase, such that 80% are in S phase 18 h after serum repletion (9a). Addition of PDGF-BB (30 ng/ml) caused an increase in [3H]thymidine incorporation in serum-starved VSM cells, an effect that we have shown to correlate with an increase in cell number (44). Pretreatment of these cells with either ciglitazone (Fig. 2A) or rosiglitazone (Fig. 2B) caused a dose-dependent reduction in PDGF-BB-induced [3H]thymidine incorporation when added at concentrations ranging from 1 to 10 μM.

The TZDs attenuate PDGF-BB-mediated induction of p21 and p27 at 24 h. The Cip/Kip CKIs, originally shown to exert inhibitory effects on growth in a variety of cell lines (38), have recently been demonstrated to have pleiotropic effects on cell cycle events in VSM cells (20). We (44) and others (7, 11) have shown that attenuation of p21 levels is, under some conditions and with some cell types, associated with growth inhibition. This “assembly factor” role of p21 in VSM cell CDK-mediated growth events may explain why some mitogens increase CKI expression early after stimulation. To determine whether mitogenic concentrations of PDGF-BB caused an increase in CKI expression in VSM cells, we examined levels of these proteins after PDGF stimulation. PDGF-BB (30 ng/ml) caused an increase in both p21 and p27 proteins that persisted for ≥24 h (column 2, Figs. 3 and 4).

It has been postulated that TZD-mediated growth attenuation after costimulation with PDGF-BB and insulin may involve inhibition of PDGF-induced changes in levels of p21 and p27 (43). To ascertain whether the TZDs may be interfering with the assembly factor role of the CKIs, we first examined the effect of both TZDs on the PDGF-BB-mediated increase in CKI levels seen in VSM cells. Serum-starved VSM cells were pretreated with either ciglitazone or rosiglitazone at antimitogenic concentrations before the addition of PDGF-BB. Compared with cells treated with PDGF alone, the addition of ciglitazone (Fig. 3) or rosiglitazone (Fig. 4) caused a dose-dependent reduction in p21 and p27 protein levels, with the effect being maximal at 10 μM, of the concentrations tested, in all cases.

Inhibition of p21 and p27 levels does not abolish the antiproliferative actions of the TZDs. The findings that both p21 and p27 are decreased in VSM cells exposed to the TZDs led us to ask whether this attenuation is the mechanism of TZD-mediated growth inhibition in these cells. This result would be consistent with the previously reported assembly factor role of p21 and would be supported by reports from other investigators of a reduction in p21 levels after incubation of VSM cells with PDGF and insulin (43). Transfection of VSM cells with antisense oligodeoxynucleotides (oligos) to p21 caused near total reduction of cellular p21 and p27.
levels (Fig. 5), whereas transfection of cells with anti-sense oligodeoxynucleotides to p27 showed little effect on p21 levels but near total reduction of p27 levels (Fig. 6). We have previously shown that, upon examination by fluorescence microscopy of FITC-labeled oligos, these cells demonstrate 100% transfection efficiency of both the p21 antisense and control oligos after lipofection (44); similar results have been reported using this technique in other cells (9). Specificity of these antisen-
ses oligos toward the CKIs is confirmed by the lack of an effect of both antisense oligos on levels of the smooth muscle cell protein α-actin (Fig. 7A) and by the lack of effect of the random sequence control oligo on p21 and p27 levels (Fig. 7B), as we have reported in the case of p21 (44).

To determine whether altered expression of p21 and p27 is required for the growth inhibitory effects of the TZDs, we examined VSM cells transfected with antisense oligos to p21 or p27; as shown above, these transfections result in a substantial decrease, and even in some cases

Fig. 3. Ciglitazone (Cig) inhibits PDGF-BB induction of p21 and p27. VSM cells were grown to confluence and serum starved as in Fig. 2. Cells were stimulated with PDGF-BB in the presence of Cig (at concentrations indicated) or DMSO vehicle for 24 h. Equal protein quantities of cell lysate were electrophoresed and immunoblotted with p21 (A) or p27 (B) antibodies. The density of the p21 (C) and p27 (D) bands was determined and expressed as degree of change from control ("fold change"). Experiment shown is representative of 3 separate experiments.
abolition of CKI translation. PDGF-BB-induced DNA synthesis was significantly reduced in cells treated with antisense p21 or p27, as we have previously reported (44); (see left columns in Fig. 8, A and B). Surprisingly, however, despite significant reduction of the CKIs by the p21 and p27 antisense oligos, there was no change in proportional decrease in [3H]thymidine incorporation caused by incubation of the cells with either ciglitazone (Fig. 8A) or rosiglitazone (Fig. 8B), demonstrating that these CKIs are not required for this effect. In these experiments, the differences in CKI effects in the absence of the TZDs are the result of varying confluence of the cells used in each experiment.

**The TZDs have variable effects on cyclins D and E and CDKs 2 and 4.** To further investigate the mechanism by which the TZDs cause CKI-independent VSM cell growth inhibition, we examined levels of early (G1 phase) cyclin/CDK pairs. These molecules control cell

![Fig. 4. Rosiglitazone (Ros) inhibits PDGF-BB induction of p21 and p27. VSM cells were grown to confluence and serum starved as in Fig. 2. Cells were stimulated with PDGF-BB in the presence of Ros (at concentrations indicated) or DMSO vehicle for 24 h. Equal protein quantities of cell lysate were electrophoresed and immunoblotted with p21 (A) or p27 (B) antibodies. Density of the p21 (C) and p27 (D) bands was determined and expressed as degree of change ("fold control"). Experiment shown is representative of 3 separate experiments.](http://ajpendo.physiology.org/)

Abnormal calcium homeostasis, of CKI translation.
cycle transit at specific phases of the cycle (4), with the earliest growth factor-mediated events occurring after cyclin D1/CDK4 interaction (18). In VSM cells treated with PDGF, ciglitazone (Fig. 9A) led to a reduction in cyclin D1 but had no effect on cyclin E, CDK2, or CDK4. Treatment with rosiglitazone (Fig. 9B) led to a reduction in cyclin D1 and cyclin E protein but had no effect on CDK2 or CDK4 levels. Thus, although p21 and p27 are not required, other alterations in the cyclin/CDK system, such as reduction of cyclin D1 or cyclin E protein, may be important in TZD-mediated VSM cell growth inhibition.

TZD treatment at the concentrations used lacks toxicity in VSM cells. To determine whether the antiproliferative effect of the TZDs at the concentrations used in this study was a byproduct of generalized cell toxicity, we measured [3H]leucine incorporation, a measure of cellular protein synthesis and a surrogate for cellular toxicity, in the presence and absence of the TZDs at the concentrations used in the experiments just described. Although cells treated with PDGF showed a slight increase in [3H]leucine incorporation, there was no decrement in [3H]leucine incorporation after incubation of the cells with 10 μM of either ciglitazone or rosiglitazone, the maximum concentration used in this study (Fig. 10). Therefore, TZDs added to VSM cells at the concentrations used in this study do not cause generalized toxicity to these cells.

DISCUSSION

The role of PPARγ and its ligands in cell cycle withdrawal and growth arrest was first described in studies of adipocyte transformation in fibroblast cell lines (41) and has since been described in cultures of VSM cells (3, 21, 43, 47), fibroblasts (25), aortic endothelial cells (13), choroidal endothelial cells (27), and several cancer cell lines (5, 26, 42). Activation of PPARγ directly controls the expression of many genes involved in adipocyte differentiation; however, in nonadipocyte cell lines, such as VSM cells, the mechanism of cell cycle withdrawal associated with PPARγ activation remains to be determined. Understanding this mechanism, and the role of PPARγ agonists in mediating VSM cell proliferation in general, would be highly significant, because VSM cell proliferation plays a major role in many diabetic macrovascular complications (28).

In light of our recent work demonstrating that p21 plays an assembly factor role in VSM cell proliferation (44), we asked whether suppression of levels of the CKIs p21 and p27 is required for the growth-suppressive effect of the TZDs. In the rat aorta VSM line used in this study, serum-starved cells, which have been growth arrested in G0, display low levels of both p21 and p27. Upon stimulation with PDGF-BB, p21 and p27 proteins are both increased, an effect that is at least partially attenuated by the TZDs. Unexpectedly, when VSM cells were treated with TZDs in the pres-
ence of both PDGF-BB and antisense oligos to p21 or p27 such that levels of these CKIs were significantly reduced, the TZDs caused the same relative inhibition of new DNA synthesis as they did in the absence of antisense oligo transfection. Thus the TZDs are fully capable of inhibiting VSM cell proliferation independently of their modulating effect on p21 and p27.

Other investigators have shown that PPARγ agonists inhibit both VSM cell proliferation and intimal hyperplasia (21) and that this effect is associated with alterations in p21 and p27 (43). The predominant finding in the latter study was that p27, in contrast to our findings, was upregulated by PPARγ agonists, leading to the hypothesis that it was the cell cycle inhibitory effect of high p27 levels that was responsible for cell cycle arrest induced by the TZDs. More consistent with our results were their finding that PPARγ agonists attenuated p21 induction; however, this did not occur with all PPARγ agonists studied. We now show attenuation, by two different TZDs, of the induction of both p21 and p27 caused by PDGF-BB in VSM cells. Therefore, a growth-inhibitory effect of p27 induction in VSM cells as a response to TZD treatment is not, we believe, a viable mechanistic explanation for the growth arrest seen after treatment with these drugs.

The disparity between our results and those of Wakino et al. (43) may be due to differences between A10 rat VSM cells and the earlier-passage VSM cells used in that study. The relationship between p27 protein, cyclin/CDK complex activity, and growth factors is not consistent among all cell types, nor among similar cell types under varying experimental conditions, suggesting a complex interaction between growth-stimulatory and growth-inhibitory signals and the cell cycle machinery. Contrary to the common paradigm that p27 levels fall after mitogen exposure, p27 levels are low in quiescent primary rat hepatocytes, begin to accumulate in mid-G1 phase, and are maximal during...
Furthermore, in VSMs from spontaneously hypertensive rats, high levels of p27 protein are associated with high cyclin E/CDK2 complex activity, accompanied by a high proportion of cells in S phase. Inhibition of VSM cell proliferation, reduction of p27 protein levels, and reduction of cyclin E/CDK2 activity were achieved with angiotensin II receptor antagonists (19).

It is also likely that incubation of VSM cells with PDGF-BB alone (12). Our results, we believe, are more representative of the effect on the CKIs of the TZDs in the presence of a single tyrosine kinase-type growth factor. It is conceivable that activation of tetrameric receptor tyrosine kinases, such as the insulin receptor, and activation of downstream elements, such as the insulin receptor substrate-1, have complex effects on CKI function.

Whether TZD-induced changes in cyclin D1 and cyclin E levels are critical for the growth-inhibitory effects of the TZDs is not known, but significant correlations are beginning to appear. Guan et al. (14) showed that troglitazone inhibits cyclin D1 expression in transitional cell bladder cancer and arrests cell growth. Others have reported downregulation of cyclin D1 with two separate PPARγ ligands and subsequent Rb hypophosphorylation in a lung cancer cell line (6). In VSM cells exposed to both PDGF and insulin, Wakino et al. (43) reported that the natural ligand for PPARγ, 15-deoxy-12,14Δ-prostaglandin J2, decreased cyclin D1 levels but that troglitazone and rosiglitazone did not. We have shown that, in VSM cells exposed to PDGF-BB alone, rosiglitazone decreases both cyclin D1 and cyclin E, whereas ciglitazone decreases cyclin D1 only. This finding suggests that different TZDs have different abilities to affect G₁ cyclin levels but that a decrement in the G₁ cyclin D1 by the TZDs may well be important in the mechanism of TZD-mediated growth inhibition. Consistent with these data, it has been demonstrated that overexpression of both cyclin D1 and cyclin E significantly shortens G₁ phase (33), such that a decrement in these cyclins may result in lengthening G₁ and the subsequent cell cycle inhibition that we observe. Furthermore, the G₁ cyclins are relatively unstable and require persistent mitogen stimulation to remain at high levels within the cell (37), and varia-

S phase (22). Furthermore, in VSMs from spontaneously hypertensive rats, high levels of p27 protein are associated with high cyclin E/CDK2 complex activity, accompanied by a high proportion of cells in S phase. Inhibition of VSM cell proliferation, reduction of p27 protein levels, and reduction of cyclin E/CDK2 activity were achieved with angiotensin II receptor antagonists (19).

It is also likely that incubation of VSM cells with PDGF-BB in the presence of insulin, the protocol used in the previously cited (43) study, has different effects on cell cycle protein expression and activity than does PDGF stimulation alone. Insulin is a potentiating factor for PDGF-BB and performs this function by increasing the amount of cellular Ras available, enhancing signaling through the mitogen-activated protein (MAP) kinase cascade. This leads to doubling of new DNA synthesis and five- to eightfold increases of growth factor-regulated gene expression compared with PDGF-BB alone (12). Our results, we believe, are more representative of the effect on the CKIs of the TZDs in the presence of a single tyrosine kinase-type growth factor. It is conceivable that activation of tetrameric receptor tyrosine kinases, such as the insulin receptor, and activation of downstream elements, such as the insulin receptor substrate-1, have complex effects on CKI function.

Whether TZD-induced changes in cyclin D1 and cyclin E levels are critical for the growth-inhibitory effects of the TZDs is not known, but significant correlations are beginning to appear. Guan et al. (14) showed that troglitazone inhibits cyclin D1 expression in transitional cell bladder cancer and arrests cell growth. Others have reported downregulation of cyclin D1 with two separate PPARγ ligands and subsequent Rb hypophosphorylation in a lung cancer cell line (6). In VSM cells exposed to both PDGF and insulin, Wakino et al. (43) reported that the natural ligand for PPARγ, 15-deoxy-12,14Δ-prostaglandin J2, decreased cyclin D1 levels but that troglitazone and rosiglitazone did not. We have shown that, in VSM cells exposed to PDGF-BB alone, rosiglitazone decreases both cyclin D1 and cyclin E, whereas ciglitazone decreases cyclin D1 only. This finding suggests that different TZDs have different abilities to affect G₁ cyclin levels but that a decrement in the G₁ cyclin D1 by the TZDs may well be important in the mechanism of TZD-mediated growth inhibition. Consistent with these data, it has been demonstrated that overexpression of both cyclin D1 and cyclin E significantly shortens G₁ phase (33), such that a decrement in these cyclins may result in lengthening G₁ and the subsequent cell cycle inhibition that we observe. Furthermore, the G₁ cyclins are relatively unstable and require persistent mitogen stimulation to remain at high levels within the cell (37), and varia-

Fig. 10. Thiazolidinediones (TZDs) at doses used do not impair protein synthesis in VSM cells. VSM cells were grown to confluence, serum starved for 24 h, and then incubated for 30 min with ciglitazone or rosiglitazone (10 μM) or DMSO vehicle before exposure to PDGF-BB (30 ng/ml) for an additional 24 h. [3H]leucine (1 μCi/ml) was added for the last 6 h, and incorporation into DNA was determined as described in EXPERIMENTAL PROCEDURES. Data are presented as means ± SD of 3 wells and are representative of 2 separate experiments.

Fig. 9. Cigiltazone and rosiglitazone have variable effects on G₁ cyclin and CDK levels. VSM cells were grown to confluence and serum starved as in Fig. 2. Cells were stimulated with PDGF-BB (30 ng/ml) where indicated in the presence of ciglitazone (A) or rosiglitazone (B), at concentrations indicated, or DMSO vehicle. Equal protein quantities of cell lysate were electrophoresed and immunblotted with the indicated cyclin or CDK antibody. Experiment shown is representative of 3 separate experiments.

Fig. 10. Thiazolidinediones (TZDs) at doses used do not impair protein synthesis in VSM cells. VSM cells were grown to confluence, serum starved for 24 h, and then incubated for 30 min with ciglitazone or rosiglitazone (10 μM) or DMSO vehicle before exposure to PDGF-BB (30 ng/ml) for an additional 24 h. [3H]leucine (1 μCi/ml) was added for the last 6 h, and incorporation into DNA was determined as described in EXPERIMENTAL PROCEDURES. Data are presented as means ± SD of 3 wells and are representative of 2 separate experiments.
tions in the ability of each TZD to inhibit MAP kinase signaling may explain some of these differences.

Other mechanisms could also be used to explain TZD-induced cell cycle withdrawal. PPARγ agonists induce apoptosis in a variety of cell types, including vascular endothelial cells (4), macrophages (8), and some cancer cell lines (6, 14, 32). A recent study showed that troglitazone caused apoptosis in VSM cells through the p53 and growth arrest and DNA damage-inducible pathways, whereas pioglitazone did not. The authors concluded that some PPARγ agonists may cause apoptosis by a PPAR-independent mechanism (30). PPARγ is also directly involved in adipocyte maturation and can induce immature cell lines, such as preadipocytes or fibroblasts, to assume a mature adipocyte phenotype and exit from the cell cycle (25).

Proliferating VSM cells are also considered phenotypically “immature,” and one study using PPARγ ligands induced a more “mature” VSM cell phenotype, as determined by α-actin levels, accompanied by reduced proliferation (24). Finally, PPARγ ligands can induce the VSM cell to assume a foam cell-like phenotype as well, also presumably less likely to progress through the cell cycle (22).

Although our p21 antisense oligo did cause crossover inhibition of p27, this does not alter our findings. If either TZD required the reduction of p21 to produce its antiproliferative effect, it should not have been capable of persistent inhibition of DNA synthesis in the presence of the p21 antisense, regardless of the effect of this oligo on p27. Thus attenuation of PDGF-induced up-regulation of p21 by the TZDs, although present, is not the sole mechanism of their antiproliferative effect.

The TZDs have become an important medication in the treatment of diabetes and its complications. That atherosclerosis and vascular complications are the major cause of mortality in diabetic patients underscores the importance of research into the effects of the commonly used hypoglycemic drugs on vascular cell proliferation. Our findings of G1 cyclin inhibition by the TZDs suggest further avenues of investigation of potential signaling pathways for TZD-mediated VSM cell growth inhibition. In addition, although our work shows that the CKIs are dispensable for the growth-inhibitory effect of the TZDs, whether these cell cycle proteins play a role in other effects of these drugs, such as phenotype alteration or glucose metabolism, is the subject of continued investigation in our laboratory.

We thank Amy Matayoshi and Laura Howard for technical assistance.

This work was supported by the Research Service of the US Department of Veterans Affairs, grants from the National Kidney Foundation and the UC Davis Health System, and by a gift from the Department of Veterans Affairs, grants from the National Kidney Foundation and the UC Davis Health System, and by a gift from Dialysis Clinics, Inc.

REFERENCES


13. Endothelial cell apoptosis induced by 10.220.32.246 on August 14, 2017 http://ajpendo.physiology.org/ Downloaded from

14. Endothelial cell apoptosis induced by 10.220.32.246 on August 14, 2017 http://ajpendo.physiology.org/ Downloaded from

15. Endothelial cell apoptosis induced by 10.220.32.246 on August 14, 2017 http://ajpendo.physiology.org/ Downloaded from

16. Endothelial cell apoptosis induced by 10.220.32.246 on August 14, 2017 http://ajpendo.physiology.org/ Downloaded from

17. Endothelial cell apoptosis induced by 10.220.32.246 on August 14, 2017 http://ajpendo.physiology.org/ Downloaded from

18. Endothelial cell apoptosis induced by 10.220.32.246 on August 14, 2017 http://ajpendo.physiology.org/ Downloaded from

19. Endothelial cell apoptosis induced by 10.220.32.246 on August 14, 2017 http://ajpendo.physiology.org/ Downloaded from

20. Endothelial cell apoptosis induced by 10.220.32.246 on August 14, 2017 http://ajpendo.physiology.org/ Downloaded from

21. Endothelial cell apoptosis induced by 10.220.32.246 on August 14, 2017 http://ajpendo.physiology.org/ Downloaded from

22. Endothelial cell apoptosis induced by 10.220.32.246 on August 14, 2017 http://ajpendo.physiology.org/ Downloaded from

23. Endothelial cell apoptosis induced by 10.220.32.246 on August 14, 2017 http://ajpendo.physiology.org/ Downloaded from


