

Phosphorylated Troglitazone Activates PPAR γ and Inhibits Vascular Smooth Muscle Cell Proliferation and Proteoglycan Synthesis

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Abstract: Phosphorylation of α -tocopherol produces an entity with enhanced antiatherogenic properties. Troglitazone, an α -tocopherol derivative of a 2,4-thiazolidinedione nucleus, is an antidiabetic agent that shows fatal idiosyncratic hepatotoxicity, a property not shared by later agents. We investigated the effects of phosphorylation of troglitazone (to yield “phosphoglitazone”) on the biochemical pharmacologic properties of troglitazone. We investigated its ability to act as a PPAR γ agonist and to inhibit 2 atherogenic properties of vascular smooth muscle cells (vSMC)—proliferation and proteoglycan synthesis. PPAR γ activity was assessed in a transfection assay. Proliferation was assessed by [3 H]-thymidine incorporation and cell counting and proteoglycan synthesis by [35 S]-sulfate incorporation using human vSMCs stimulated with platelet-derived growth factor (PDGF; 50 ng/mL) and transforming growth factor (TGF)- β (2 ng/mL). Phosphoglitazone was a full agonist for PPAR γ with a potency and efficacy similar to troglitazone. Phosphoglitazone also inhibited cell proliferation and proteoglycan synthesis with potency similar to troglitazone. We conclude that phosphorylation retains the pharmacologic activity of troglitazone while decreasing its lipophilicity and therefore potentially its toxicity. A phosphorylated derivative of a 2, 4-thiazolidinedione warrants further investigation as a potential new therapeutic agent for the treatment of insulin resistance and Type 2 diabetes.

Key Words: PPAR γ , troglitazone, phosphorylated troglitazone, vascular smooth muscle, proliferation, proteoglycan synthesis

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INTRODUCTION

The newest class of oral antihyperglycemic agents for the treatment of insulin-resistant states, such as Type 2 diabetes, is the thiazolidinediones (TZDs).^{1,2} TZDs act as PPAR γ ligands to modify gene transcription in insulin-responsive tissues such as skeletal muscle, liver, and fat, resulting in increased insulin sensitivity and a slow onset and prolonged antihyperglycemic action.^{3,4} As the ultimate efficacy of these drugs is judged on the prevention of cardiovascular disease the most successful agents targeting cardiovascular risk factors also have beneficial so-called “pleiotropic” actions directed to cells of the cardiovascular system. The first clinical TZD was troglitazone, which has been followed by rosiglitazone and pioglitazone. Troglitazone was withdrawn from the market shortly after its introduction because of a rare but fatal hepatotoxicity.⁵ Troglitazone appeared to be highly efficacious in reducing hyperglycemia and also in preventing diabetes based on data from the Diabetes Prevention Program obtained before the drug was withdrawn.⁶ Troglitazone was designed with a 2,4-thiazolidinedione nucleus to which a hydrophobic α -tocopherol moiety was added to confer antioxidant properties.⁷ Troglitazone is thus highly hydrophobic and the toxicity may reside in a hydrophobic metabolite because the less hydrophobic entities, rosiglitazone and pioglitazone, have not displayed similar toxicity.⁸

Phosphorylation is a chemical and biochemical modification that has the potential to greatly alter the biological or pharmacologic activity of target molecules. Phosphorylation greatly increases the solubility of molecules and the amphiphilic nature and increases the ability to cross cell membranes. New drug molecules may manifest toxicity because of inherent high lipophilicity or toxicity of lipophilic metabolites. Phosphorylation has the potential to favorably modify the properties and actions of lipophilic drugs.^{9,10} The critical question is whether the pharmacologic and therapeutic activity is maintained in the phosphorylated derivative.

The concept that phosphorylation can maintain pharmacologic activity has been demonstrated successfully for the in vitro properties of phosphorylated α -tocopherol.⁹ Because troglitazone contains the α -tocopherol moiety in the orientation that allows for phosphorylation, we have used it as the basis to further evaluate if phosphorylation can yield active drugs that maintain their pharmacologic activity.

We have prepared phosphorylated troglitazone (which we have termed phosphoglitazone) and evaluated its primary actions as a PPAR- γ ligand able to modify gene transcription and 2 antiatherogenic activities—the inhibition of vascular smooth muscle cell (vSMC) proliferation and proteoglycan synthesis.

MATERIALS AND METHODS

Materials

Troglitazone was a gift from Parke Davis Pharmaceutical Research (Ann Arbor, Michigan). Platelet-derived growth factor (PDGF) and α -tocopherol (α -T) were from Sigma Chemical Co. (St. Louis, Missouri). Phosphorylated α -tocopherol mixture (TPm) containing 55% phosphorylated α -T, 31% phosphorylated di α -T, and 5% α -T was synthesized and provided by Phosphagenics Ltd (Melbourne, Australia). Phosphoglitazone containing a minimum of 90% of the phosphorylated form of troglitazone [high-performance liquid chromatography (HPLC) assay] was synthesized and provided by Phosphagenics Ltd (Melbourne, Australia). Rosiglitazone was kindly provided by GlaxoSmithKline Australia (Boronia, VIC). Transforming Growth Factor- β (TGF- β) was from R and D Systems (Minneapolis, Minnesota).

Synthesis of Phosphorylated Troglitazone

Phosphoric anhydride was reacted with hexamethyldisiloxane in a weight ratio of 0.8:1 for >4 hours at 85°C. The resulting silyl phosphate intermediate was reacted with troglitazone in a weight ratio of 9:1 for 5.5 hours using stirring and heat (87°C). Hydrolysis of pyrophosphates was effected at this temperature by stirring the product with milli-Q water in a molar ratio of 1:2 water:phosphorus for 1.5 hours. The solid product was dissolved in methanol and dried on a rotary evaporator, followed by drying under vacuum for 6 hours. Phosphoglitazone was isolated on a flash column packed with Merck silica gel 60, with dichloromethane/methanol as the eluting solvents (yield 0.31g, 42%; phosphoglitazone assay: HPLC peak area >98%; mass spectrometry, negative mode, single peak cluster at 520.1, 521.1, and 522.1 m/z; ¹H NMR (CD₃OD) δ 1.40 (s, 3H), 1.90 (m, 1H), 2.05 (s, 3H), 2.12 (m, 1H), 2.21 (s, 3H), 2.23 (s, 3H), 2.67 (m, 2H), 3.10 (dd, 1H, J = 8.8, 14 Hz), 3.38 (dd, 1H, J = 4, 14.8 Hz), 3.96 (AB, 2H), 4.68 (dd, 1H, J = 3.6, 8.8 Hz), 6.88 (d, 2H, J = 8.4 Hz), 7.17 (d, 2H, J = 8.4 Hz); ³¹P NMR (CD₃OD), 32 scans, 2 dummy scans, delay between scans of 4 seconds, single peak at -5 ppm).

Culture of Vascular Cells

Human vSMCs were grown from human saphenous veins otherwise discarded from cardiac surgery at the Alfred Hospital (Melbourne, Australia) using the explant technique.¹¹ Rat aortic smooth muscle cells were prepared and treated as previously described.¹²

PPAR γ Transactivation Assay

PPAR γ transactivation was analyzed as previously described in detail.¹² Briefly, rat aortic vSMC were grown to 70%–80% confluency in 6-well plates and placed in OPTI-MEM

medium. DNA (400 ng) of a full-length PPAR γ 1 expression vector was cotransfected with 200 ng of a luciferase reporter construct driven by 3 peroxisome proliferator-response element (PPRE) copies from the acyl-CoA (coenzyme A) oxidase gene linked to the minimal thymidine kinase promoter using LipofectAMINE 2000. Twelve hours after the transfection, cells were serum starved in Dulbecco's modified eagle medium (DMEM) containing 0.4% fetal bovine serum (FBS) for 24 hours and stimulated for 24 hours with the PPAR γ 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 at least 3 times with different cell preparations.

Analysis of vSMC Proliferation by Cell Counting

vSMCs were seeded at 50–100 \times 10³ cells per well in 6-well plates (Becton Dickinson) in DMEM containing 10% FBS and 5 mM glucose; the medium changed to DMEM containing 0.1% FBS the following day. After 48 hours, vSMCs were treated with control medium containing 5% FBS/0.1% dimethyl sulfoxide (DMSO), 5% FBS/troglitazone (1–10 μ M), or 5% FBS/phosphoglitazone (1–10 μ M). After 3 days, the cells were harvested using trypsin and counted on a Coulter counter. To determine the baseline count, cells from individual wells were counted before drug treatment.

Analysis of vSMC Proliferation by Reinitiation of DNA Synthesis

vSMCs were established as previously described.¹³ Confluent and serum-deprived cells were pretreated with 0.1% FBS/0.1% DMSO, 0.1% FBS/troglitazone (1–10 μ M), or 0.1% FBS/phosphoglitazone (1–10 μ M) for 6 hours. The treatment media were removed and replaced with fresh treatment media with PDGF (50 ng/mL). After 16 hours, the treatment media were removed and replaced with DMEM containing 0.1% FBS and [³H]-thymidine (1 μ Ci/mL) for 3 hours. Incorporated [³H]-thymidine into newly synthesized DNA was harvested and quantitated as previously described.¹³

Quantitation of Proteoglycans by CPC Precipitation Assay

Proteoglycan synthesis was quantitated as previously described.¹⁴ Briefly, cells were cultured at 5 \times 10⁴ cells per well in a 24-well plate and were established and treated in an identical manner to that described earlier. Cells were metabolically labeled with 50 μ Ci/mL [³⁵S] sulfate (16 hours). Parallel plates were established without [³⁵S] sulfate to count cells.¹³ Incorporation of [³⁵S] sulfate into secreted proteoglycans and cell-associated proteoglycans was quantitated using the CPC precipitation assay.¹³

Statistics

Data were analyzed by Student *t* test and analysis of variance (ANOVA). Dose-response data for PPAR γ gene activation studies were subjected to log transformation and analysis by 2-way ANOVA. Results were accepted as statistically significant at *P* < 0.05 or less.

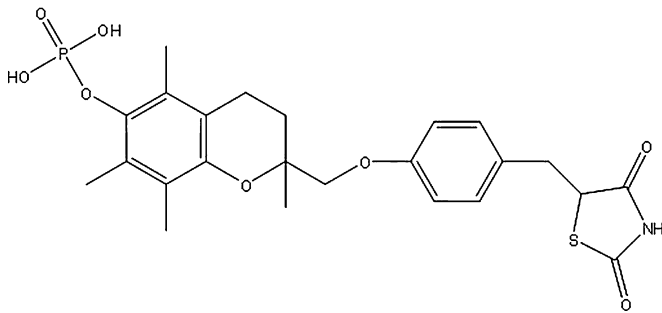


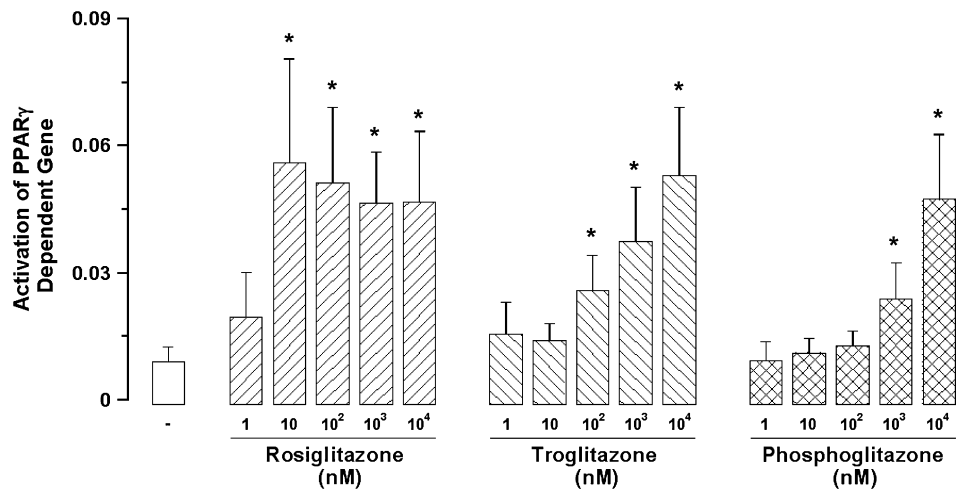
FIGURE 1. Chemical structure of phosphorylated troglitazone (“phosphoglitazone”). Synthesis and characterization are described in Materials and Methods.

RESULTS

Troglitazone was phosphorylated as described in Materials and Methods to produce phosphorylated troglitazone (“phosphoglitazone”), the structure of which is shown in Figure 1.

Activation of PPAR γ by phosphoglitazone was assessed by cotransfection of a full-length PPAR γ 1 expression vector with a luciferase reporter gene driven by 3 PPRE copies from the acyl-CoA oxidase gene linked to the minimal thymidine kinase promoter. In this transactivation assay, vSMC cells exhibited a strong ligand-dependent transcriptional response to increasing concentrations of the potent TZD full PPAR γ agonist rosiglitazone. Maximal PPAR γ activation by rosiglitazone was observed at 10 nM, consistent with our previous reports documenting an EC₅₀ for rosiglitazone in vSMC at nanomolar concentrations (Fig. 2).¹² Rosiglitazone elicits the maximum response attainable in this assay. We next compared this potent PPAR γ activation by the full agonist rosiglitazone to the activity of troglitazone. Troglitazone elicited a similar maximal response compared to rosiglitazone, but the half maximally effective concentration was approximately 100-fold higher for troglitazone. Phosphoglitazone activated PPAR γ to

FIGURE 2. Phosphoglitazone activates PPAR γ . Rat aortic vascular smooth muscle cells were transiently cotransfected with a PPAR γ 1 expression vector and a luciferase reporter gene driven by 3 PPRE copies from the acyl-CoA oxidase gene linked to a thymidine kinase promoter. Cells were treated with 0.1% DMSO (–) or stimulated with the indicated concentrations of the thiazolidinedione full PPAR γ agonist rosiglitazone (*left panel*), troglitazone (*middle panel*), or phosphoglitazone (*right panel*). 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 \pm standard error of the mean (SEM) from 3 separate experiments. There was no significant difference between troglitazone and phosphoglitazone ($P > 0.05$); phosphoglitazone showed a statistically significant ($P < 0.05$) linear dose response. * $P < 0.05$ versus control.



a similar level of efficacy as troglitazone and exhibited only a slightly higher half maximally effective concentration compared to troglitazone (Fig. 2). There was no statistically significant difference between the activity of troglitazone and phosphoglitazone ($P > 0.05$). Phosphoglitazone showed a statistically significant linear dose response relationship ($P < 0.05$). Thus, phosphorylation of troglitazone generates a molecule that retains activity to activate PPAR γ .

TZDs are known to inhibit the proliferation of human vSMCs,¹⁵ and this response was used to evaluate the effect of phosphorylation on a pharmacologic activity of troglitazone. We first sought to test if the original finding that phosphorylation of α -tocopherol maintained or even increased the inhibitory action of α -tocopherol toward vSMC proliferation.^{9,10} We used reinitiation of DNA synthesis following the stimulation of vSMCs with PDGF and assessed by [³H]-thymidine incorporation into DNA to assess activity. The parent compound, α -tocopherol, at the concentration used did not inhibit reinitiation of DNA synthesis (Fig. 3A). However, phosphorylated α -tocopherol (TPm) inhibited reinitiation of DNA synthesis in a concentration-dependent manner (Fig. 3A).

Troglitazone inhibited [³H]-thymidine incorporation into vSMCs as previously reported.¹⁶ Phosphoglitazone inhibited reinitiation of DNA synthesis with a potency slightly, but not significantly, greater than troglitazone at each of the concentrations tested up to 10 μ M, but troglitazone was more potent than phosphoglitazone at 30 μ M (Fig. 3B). Both troglitazone and phosphoglitazone inhibit the increases in vSMC numbers over 3 days in the presence of 10% serum with no difference in the antiproliferative activity between the 2 agents (Fig. 3C). Phosphoglitazone had no apparent toxicity (assessed by morphologic examination) toward these cells but reduced cell numbers consistent with its inhibitory action (Fig. 3D).

We have previously demonstrated that troglitazone inhibits vSMC proteoglycan synthesis and reduces LDL binding.^{17,18} We evaluated the effect of phosphoglitazone,

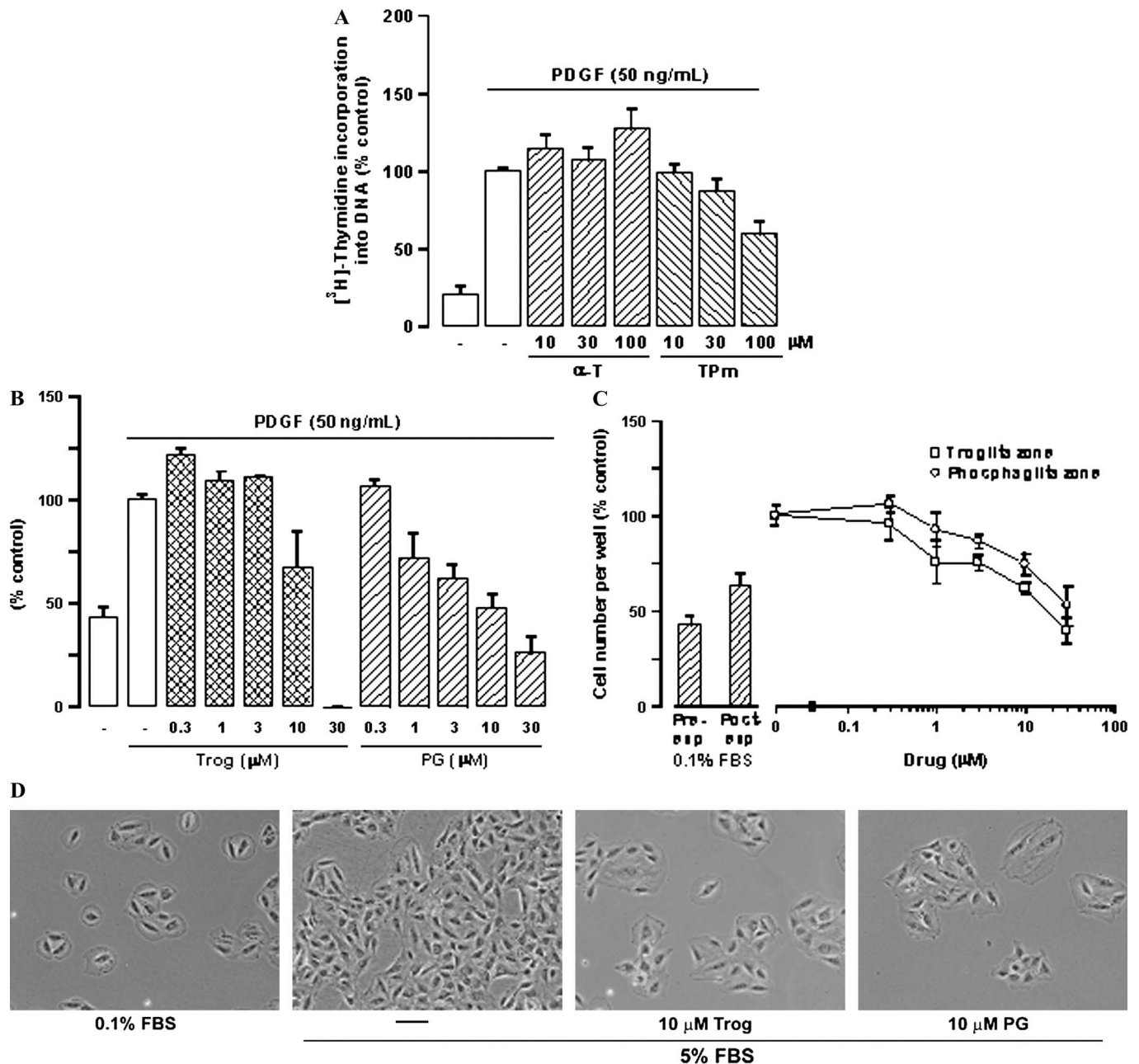


FIGURE 3. Phosphorylation of α -tocopherol and troglitazone maintains the biological activity to inhibit vSMC proliferation. A, Human vSMCs were treated with 0.1% DMSO (–), α -tocopherol (1–100 μ M), or a mixture of phosphorylated α -tocopherol (1–100 μ M) and assessed for incorporation of [3 H]-thymidine into newly synthesized DNA (data represent 2 experiments in duplicate, mean \pm SEM). B, Human vSMCs were treated with 0.1% DMSO (–) in the absence and presence of PDGF or troglitazone (1–30 μ M) and phosphoglitazone (1–30 μ M) in the presence of PDGF and assessed for incorporation of [3 H]-thymidine into newly synthesized DNA (data represent 2 experiments in duplicate, mean \pm SEM). C, Cell number by cell counting after human vSMCs were treated with 0.1% FBS and counted on day 0 (preexperiment), on day 3 after treatment with 0.1% FBS/0.1% DMSO (postexperiment), and on day 3 after treatment with troglitazone (1–30 μ M) and phosphoglitazone (1–30 μ M) in the presence of 5% serum (data represent 2 experiments in duplicate, mean \pm SEM). D, Phase contrast microscopy of vSMCs treated with 0.1% FBS, 5% FBS (–), and 5% FBS in the presence of troglitazone (Trogl, 10 μ M) and phosphoglitazone (PG, 10 μ M). * P < 0.05 and ** P < 0.01 versus control.

compared to troglitazone, to inhibit basal proteoglycan synthesis and synthesis activated by 2 atherogenic growth factors, TGF- β and PDGF.¹⁹ Troglitazone caused

concentration-dependent inhibition of [35 S]-sulfate incorporation into CPC precipitable material under all 3 conditions (Fig. 4A, B, and C). Treatment of human vSMCs with

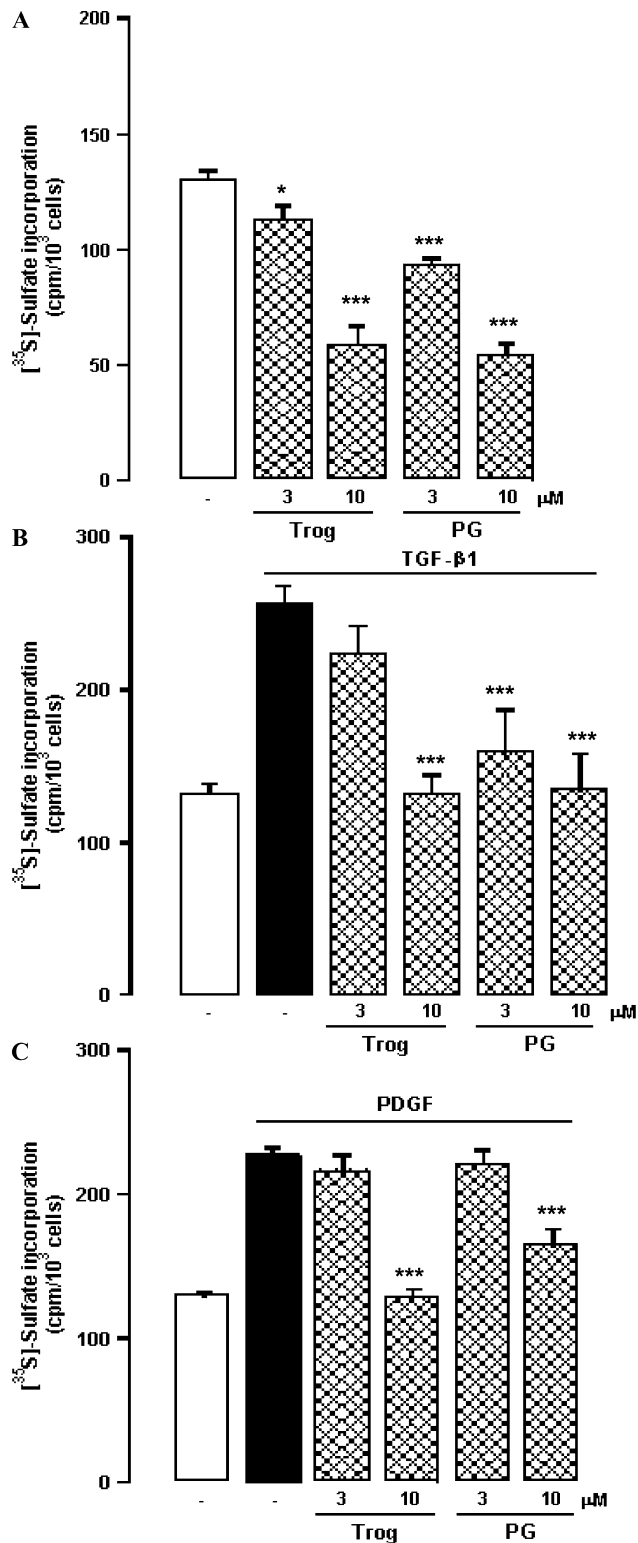


FIGURE 4. Phosphoglitzazone inhibits vascular smooth muscle cell proteoglycan synthesis. Human vSMCs were treated with 0.1% DMSO (–), troglitazone (Trog, 3, 10 μM), or phosphoglitzazone (PG, 3, 10 μM) under basal conditions (A), in the presence of TGF-β1 (2 ng/mL) (B), and in the presence of PDGF (50 ng/mL) and radiolabelled with [³⁵S]-sulfate (C). Histograms

phosphoglitzazone caused concentration-dependent inhibition of [³⁵S]-sulfate incorporation into CPC precipitable material with activity very similar to troglitazone (Fig. 4A, B, C).

DISCUSSION

We evaluated the potential of phosphorylation to favorably alter the chemical properties while retaining pharmacologic activity using the α-tocopherol derivative of a 2,4-thiazolidinedione, troglitazone, which is an insulin-sensitizing PPARγ ligand and antidiabetic drug, as a model compound.⁷ We confirmed earlier findings that phosphorylation of α-tocopherol enhances the inhibitory activity toward vSMC mitogenesis.^{9,10} We have extended this observation by demonstrating phosphorylation of the α-tocopherol moiety of troglitazone yields a molecule that activates PPARγ and shows direct antiatherogenic activities toward vSMC, being inhibition of cell proliferation and inhibition of proteoglycan synthesis.

TZDs have been extensively studied in animal models of atherosclerosis,^{16,20} and recently results of clinical trials such as PROactive (PROspective Pioglitazone Clinical Trial in Macrovascular Events)²¹ and DREAM (Diabetes REDuction Approaches with Ramipril and Rosiglitazone Medications)²² have been reported. These drugs act as insulin sensitizers with a slow onset and prolonged antihyperglycemic action. There are indications, not yet confirmed, that TZDs may be the first class of oral hypoglycemic agents capable of reducing cardiovascular disease by mechanisms that are glucose dependent and also via direct actions on the cardiovascular system.^{21,23} TZDs are the first class of antidiabetic drugs to show appreciable direct actions on cardiovascular cells.¹⁸ TZDs inhibit vSMC proliferation by mechanisms that are dependent and independent of PPARγ.¹²

We recently investigated the action of 3 TZDs on the proliferation of vSMCs from 3 different vessels used for coronary artery bypass surgery and reported that the potency was dependent on the drug and not the cell origin.¹⁵ Moreover, the relative potency of troglitazone, rosiglitazone, and pioglitazone correlates more closely with the lipophilicity of the agent than with the PPARγ binding affinity.¹⁵ Troglitazone is the most lipophilic and the most potent inhibitor of vSMC proliferation.¹⁵ Phosphorylation modifies the lipophilicity of molecules and the Log *P* value of troglitazone is decreased from 6.1 to <5.7 by phosphorylation (data provided by Dr Chris Burns, Cytopia Ltd, Melbourne, Australia). Thus, reducing the lipophilicity would be expected to reduce the potency of phosphoglitzazone toward the vascular actions investigated in this work, but the potency of phosphoglitzazone compared to troglitazone was maintained. This suggests in accord with the effect of phosphorylation on α-tocopherol that phosphorylation per se increases potency, but this is negated

show the incorporation of [³⁵S]-sulfate into proteoglycans assessed by the CPC precipitation assay. Data are expressed as cpm [³⁵S]-sulfate/10³ cells and represent the mean ± SEM from 2 separate experiments. A: **P* < 0.05 and ****P* < 0.001 versus control. B: ****P* < 0.001 versus TGF-β1, ****P* < 0.001 versus PDGF.

by the overall decrease in the lipophilicity of the derivative. It is unknown how lipophilicity influences the activity of TZDs and thus whether the passage of the drug through the cell to the target or the interaction with the target, PPAR γ , is altered.

Many drugs in the PPAR (α , γ and α/γ) classes have not progressed to implementation as human therapeutic agents for a variety of reasons associated with various toxicities.⁷ Only a small number of antihyperglycemic drug classes are in clinical use and new agents would be beneficial. It is unlikely that phosphoglitzazone as a derivative of a withdrawn entity would be developed for human therapeutic use. However, the structure (Fig. 1) indicates that it would be possible to have derivatives prepared that retain the ability to be phosphorylated and these would be potentially interesting new agents. For further proof of concept in relation to the efficacy it would be interesting to determine if phosphoglitzazone exhibits glucose-lowering activity in animal models of insulin resistance and Type 2 diabetes. It also would be interesting to use the surrogate assays of toxicity,—for example, gene expression studies of TZD and derivative effects on HepG2 cells—to assess if phosphoglitzazone exhibits reduced toxicity in relation to troglitazone.

In conclusion, phosphorylation of the 2,4-thiazolidinedione derivative, troglitazone, maintains its pharmacologic properties as a PPAR- γ agonist and as an inhibitor of human vSMC proliferation and proteoglycan synthesis. The implication of these results is that such an agent, or an analog thereof, warrants further investigation of its toxicity and potential clinical utility for the treatment of insulin resistance and the prevention of cardiovascular disease.

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