Troglitazone Stimulates Repair of the Endothelium and Inhibits Neointimal Formation in Denuded Rat Aorta

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Objective—Vascular endothelium is emerging as a therapeutic target for atherosclerotic macrovascular disease in diabetes using oral hypoglycemic agents with pleiotropic actions. We have addressed whether the thiazolidinedione troglitazone has effects on the endothelial cell response to injury in rat aorta and its interaction with the growth response of underlying vascular smooth muscle.

Methods and Results—Repair of rat aorta after balloon catheter injury in troglitazone-treated (400 mg/kg per day by mouth) rats showed early acceleration of reendothelialization and late reduction in neointima formation. Complementary in vitro studies showed that troglitazone dose-dependently inhibited migration and proliferation of cultured macrovascular endothelial and vascular smooth muscle cells in low-glucose (5 mmol/L) and high-glucose (25 mmol/L) media. However, in endothelial cells, the inhibitory response at low (<3 μmol/L) troglitazone concentrations resulted from direct inhibition of proliferation, whereas inhibition at higher (10 μmol/L) concentrations was secondary to apoptosis and necrosis. Additional studies indicated a concentration-specific activity of troglitazone to protect endothelial cells from apoptosis.

Conclusions—Troglitazone had effects consistent with maintenance of vascular integrity and protection against mechanisms of atherosclerosis and restenosis, which may arise from a concentration-specific effect to reduce high rates of apoptosis occurring in cultured cells and repairing vessels. (Arterioscler Thromb Vasc Biol. 2003;23:

Key Words: troglitazone ■ endothelial cells ■ vascular smooth muscle cells ■ reendothelialization ■ apoptosis ■ proliferation ■ migration

Thiazolidinediones are the newest class of oral hypoglycemic agents available for the treatment of hyperglycemia present in insulin resistant states, particularly type 2 diabetes. These agents are peroxisome proliferator-activating receptor (PPAR-γ) ligands that act as insulin sensitizers to reduce glucose and consequently insulin levels in patients with diabetes. It has become apparent that agents directed at primary cardiovascular risk factors, blood pressure and hyperlipidemia, can have concurrent direct vascular actions to restrict the development of atherosclerosis.

The early oral hypoglycemic agents had neutral or negative actions on blood vessels. Initial reports indicate that thiazolidinediones have a multitude of vascular actions, which may indeed be proatherosclerotic or antiatherosclerotic. For example, troglitazone was initially shown to inhibit vascular smooth muscle cell proliferation in vitro and in vivo and the expression of plasminogen activator inhibitor type-1 in endothelial cells. In contrast, PPAR-γ ligands enhance CD36 expression of macrophages and stimulate oxidized LDL uptake, which may initiate atherosclerosis. Most importantly, it has recently been demonstrated that troglitazone inhibits the development of atherosclerotic lesions in male LDL receptor–deficient mice. Hence, the proatherosclerotic or antiatherosclerotic activity of PPAR-γ ligands in the setting of diabetes results from favorable systemic actions on glucose and lipoproteins and both favorable and unfavorable biochemical and cellular actions directly in the vessel wall. It is thus particularly pertinent for this new class of compound that a full understanding of the properties of each individual PPAR-γ ligand be established to characterize their impact on atherogenesis. Troglitazone has been removed from clinical usage because of rare lethal hepatotoxicity; however, it has been used in other vascular studies, and an analysis of the effects of troglitazone provides a basis for the consideration of the action of new and emerging agents that may target any or all of the PPAR isoforms.

Although the effects of troglitazone on some vascular cells have been described, the effects on endothelial properties are less well characterized. Two studies have looked at troglitazone in the setting of severe vascular injury and extensive regrowth of vascular smooth muscle cells. We focused on the endothelium by examining the effect of troglitazone on reendothelialization of a denuded aorta in a normoglycemic rat model, an in vivo model of endothelial cell migration,
proliferation, and apoptosis that also gives information on the growth response of the underlying medial tissue. To explore the changes in cellular properties underlying the in vivo response, we examined the effects of troglitazone on the properties of cultured macrovascular endothelial and vascular smooth muscle cells that contribute to vascular changes underlying the atherogenic process. We demonstrate that troglitazone stimulates early endothelial repair in vivo and secondarily or directly inhibits smooth muscle cell proliferation. Troglitazone has similar inhibitory effects toward endothelial and vascular smooth muscle cell migration and proliferation, but we detected a concentration-specific effect of troglitazone to partially reduce endothelial apoptosis, which may explain the enhanced endothelial repair in vivo.

Methods

Cell Culture

Bovine aortic endothelial cells (ECs) were isolated by collagenase treatment and maintained in standard RPMI (11 mmol/L glucose) containing 10% FBS at 37°C in 5% CO2. Cells were characterized by immunostaining for von Willebrand Factor or endothelial nitric oxide synthase and the absence of smooth muscle α-actin. Experiments were conducted in media with low (5 mmol/L) or high (25 mmol/L) glucose concentrations.

Rat aortic smooth muscle cells (SMCs) were cultured as previously described and used for proliferation experiments. Migration experiments were conducted with human vascular smooth muscle cells derived from internal mammary arteries obtained from cardiac theaters at the Alfred Hospital, Melbourne, and approved by the relevant ethics committee.

Proliferation and Apoptosis

Cells were seeded at 2×10^4 per 30-mm plate and after 2 days incubated in media containing low or high glucose with or without troglitazone (0.1 to 10 μmol/L). After 2 days of treatment, the cells were counted using a Coulter counter, as previously described. Cell detachment occurred in EC cultures under some conditions, so the medium was collected and these floating cells were also counted.

For analysis of apoptosis, ECs were seeded at 2.5×10^4 per 100-mm plate and after 2 days were treated with troglitazone as above. After 2 days of treatment, the cells floating in media or attached were harvested separately and incubated in annexin V/pro-oxidase synthase and the absence of smooth muscle theaters at the Alfred Hospital, Melbourne, and approved by the

Morphometry

Evans Blue dye (60 mg/kg, IV) was administered to rats 60 minutes before euthanasia. After perfusion with 0.9% saline and 4% p-formaldehyde in phosphate buffer (pH 7.4), the entire thoracic aorta was removed and fixed overnight. Two 1-cm-long segments of aorta were opened longitudinally and pinned flat on a silicon pad for digital photography. Surface area measurements of the white patches and total vessel surface area were made using image analysis. For neointima and media area measurements, aorta cross-sections were prepared and stained by Verhoeff’s elastic van Gieson method, and then 3 measurements were made from each animal.

Statistics

Data were subjected to ANOVA to establish the existence of significant differences. Individual comparisons of values were by Student’s t test, and the levels of significance are indicated in the individual figures and tables. Values are presented as mean±SEM.

Results

Troglitazone and Endothelial Cell Proliferation

The effect of troglitazone on EC proliferation was investigated at glucose concentrations that correspond to levels up to the highest that may occur (eg, postprandially) in subjects with poorly controlled diabetes. In low (5 mmol/L) and high (25 mmol/L) glucose media, troglitazone caused a concentration-dependent reduction in the number of attached cells, with significant inhibition apparent at 3 μmol/L and greater inhibition at 10 μmol/L (Figures 1A and 1B). As an indication of the potential maximal inhibitory efficacy, a line on the Figures indicates the number of cells present on the day that the drugs were introduced (Figures 1A and 1B, solid line).

3H-Thymidine incorporation data from confluent serum-deprived EC cultures showed very high basal rates of labeling (data not shown), inferring a high basal proliferation rate. Those same EC cultures had a substantial number of free-floating cells, representing ~10% of the cells present in the dish, but SMC cultures did not (Figures 1A and 1B versus 1C and 1D). The data shown indicate that the number of floating cells was subject to regulation by troglitazone and that the effect of troglitazone was biphasic (Figures 1C and 1D). At low concentrations (<1 μmol/L), troglitazone had no effect on cell detachment. At 3 μmol/L, troglitazone significantly reduced the free-floating cells, indicating some protection against detachment. At higher concentrations (10 μmol/L), troglitazone activated cell detachment, and concentrations higher again caused overt toxicity and cell detachment (data not shown). It is apparent that the established approach of
measuring only attached cells or the very simple chromogenic assays would misrepresent the action of troglitazone on the underlying cellular mechanisms of endothelial cell proliferation and apoptosis.

At the conclusion of proliferation experiments, unattached cells were quantitatively recovered and analyzed by annexin V and propidium iodide staining (Figure 2). There was a substantial decrease in apoptotic and necrotic cells from 74% to 43% in the presence of 10 $\mu$mol/L troglitazone. There was also an increase of viable cells floating in troglitazone-treated cultures (26% versus 57%), and these cells could be subcultured to attach and grow; however, the nature of these viable cells was not pursued additionally. The attached cells were also harvested by trypsinization and analyzed by annexin V and propidium iodide staining. The attached cell population showed very small numbers of apoptotic and necrotic cells, and the total of these 2 classifications never exceeded 5% of the total cell number under any condition.

**Effect of Troglitazone on Vascular SMC Proliferation**

The endothelial cell phenomena of apoptosis and detachment does not occur (to anywhere near the same extent) in vascular SMCs, with the unattached cell population never achieving >2% of the total cell population. Troglitazone caused concentration-dependent inhibition of vascular SMC proliferation with a concentration dependency not appreciably different from that observed for endothelial cell proliferation (Figures 3A and 3B).

**Effect of Trexitin on Endothelial and Vascular SMC Migration**

Migration of cells in vivo is an active phenomenon that contributes significantly to vascular maintenance and repair. Migratory activity can be modeled in vitro by examining the rate at which cells recover a denuded area of the culture plate, and in short experiments of 16 hours or less, the contribution

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**Figure 1.** Effect of troglitazone on endothelial cell proliferation under low-glucose (5 mmol/L) and high-glucose (25 mmol/L) conditions. Numbers of attached cells after 48 hours of exposure to troglitazone in low (A) or high (B) glucose and unattached cells (C, low; D, high) that were harvested from the same cultures. The solid line (A and B) indicates the number of cells present on the day that the experimental media were introduced. Results (mean ± SEM) are pooled from 3 or more experiments conducted in triplicate. *P<0.05, ** P<0.001, *** P<0.0001 from respective controls.

**Figure 2.** Characterization of apoptotic, necrotic, or viable state of detached endothelial cells. Endothelial cells were harvested and stained with annexin V and propidium iodide to indicate apoptotic (A) and necrotic (B) cells, respectively. Viable cells do not stain with either dye and are not shown. Cells from control and troglitazone-treated cultures were harvested, stained, and counted, and the results are given in the text.
of proliferation is minimal. For endothelial cells, there was a lower migratory response in high glucose media, but this was not statistically significant (Figure 4A). Troglitazone (10 μmol/L) inhibited endothelial cell migration by 16% under low-glucose conditions and 28% in high-glucose media, with only the latter result attaining statistical significance ($P < 0.05$) (Figure 4A), and this effect was not evident at lower troglitazone concentrations (1 and 3 μmol/L, data not shown). Troglitazone as previously demonstrated in rat aortic and human coronary SMCs,6,15 also inhibited the closure of the wound after the scrape of confluent SMC cultures. Troglitazone inhibited SMC migration by 28% under low-glucose conditions and 23% in high-glucose media (Figure 4C). The inhibitory potency of troglitazone was similar toward endothelial and vascular SMC migration.

In chemotaxis assays, the extent of migration is dependent on directed migration in the presence of added chemotactic factors. We found in this assay that high glucose significantly stimulated endothelial cell migration to PDGF by 43% but did not significantly affect migration in SMCs ($P = 0.37$). Troglitazone (10 μmol/L) inhibited endothelial cell migration by 43% in both low- and high-glucose media (Figure 4B).
Inhibitory activity was also observed at 3 μmol/L (P = 0.04) but not at 1 μmol/L (data not shown). Troglitazone (10 μmol/L) inhibited SMC migration by 17% in low-glucose conditions and 34% in high-glucose media (Figure 4D).

**Repair of the Endothelium After Balloon Catheter Injury to Rat Aorta**

We studied the ability of endothelial cells to reestablish luminal coverage of a denuded section of aorta in rats treated with troglitazone or the dosing vehicle. Vital staining with Evans blue dye resulted in blue staining of aortic wall denuded of endothelium, but not where endothelial regrowth occurred (Figure 5A). The mild ballooning technique used completely denudes the endothelium without disruption of the internal elastic lamella, and 2 hours after ballooning, the entire vessel was stained blue (results not shown). At 4 days after injury, patches of endothelium had grown from the intercostal branch arteries, producing a series of ovoid-shaped white areas with approximate dimensions of 2×1 mm. The average area of each endothelial patch was increased by 17% in troglitazone-treated rats (P < 0.05; Figure 5B), indicating increased luminal coverage.

The duration of the experiment was extended to examine the effect on extensive levels of aortic reendothelialization. By 14 days after injury, endothelial repair had progressed to the point where the individual endothelial patches had coalesced with luminal coverage of ≈60% of the aortic surface (Figure 5C). Despite the apparent early advancement of endothelial repair in troglitazone-treated rats, there was no significant difference between the vehicle and troglitazone-treated groups in the extent of reendothelialization by 14 days after injury (Figure 5D). The results indicated that the potent and efficacious action of troglitazone to inhibit vascular SMC migration and proliferation is not similarly directed to endothelial cells in vivo.

**Analysis of Aortic Neointimal Formation After Balloon Catheter Injury**

An important parameter in the repair process of an injured blood vessel is the development of a neointima resulting from vascular SMC migration and proliferation. To establish whether troglitazone treatment directly (pharmacologically) or indirectly (eg, secondary to advanced endothelial coverage) influenced the development of neointimal tissue, the ratio of neointima to media cross-sectional area measurements in the vessels of vehicle and troglitazone-treated groups at 14 days was determined. There was no effect on media area (data not shown), but there was an appreciable (22%) and statistically significant (P < 0.05) decrease in the neointima to media ratio in troglitazone-treated rats (vehicle, 0.308 ± 0.016; troglitazone-treated, 0.239 ± 0.021), indicating reduced accumulation of vascular SMC mass and confirming a pharmacological action of troglitazone in this model.

**Discussion**

In vivo studies of reendothelialization of denuded rat aorta in troglitazone-treated rats showed early stimulation of endothelial cell recovery and late reduction in SMC mass in the neointima, with the latter finding attributable either to the early endothelial cell enhancement or a direct inhibitory activity on vascular SMCs, as previously reported. Troglitazone dose-dependently inhibited proliferation of cultured macrovascular endothelial cells with very similar activity in low- and high-glucose media. Whereas an inhibitory response
at low concentrations resulted from direct inhibition of proliferation, by examining the cells released from the under-lying matrix, it was shown that apparent inhibition at higher concentrations was secondary to mechanisms compromising cell viability and leading to cell detachment. The actual direct inhibitory efficacy of troglitazone in vitro seemed to be less than that reported for vascular SMCs using different assa-
yses,6,11 but using identical methodologies, based on actual cell numbers, the inhibitory potency of troglitazone is similar toward endothelial and vascular SMCs. Troglitazone had an inhibitory tendency toward endothelial cell migration in a scrape/wound assay under low glucose conditions, which was greater and became significant in high glucose media; trogli-tazone showed similar inhibitory activity toward vascular SMC migration in this scrape/wound assay. Similarly, in chemotaxis assays, troglitazone showed a weak inhibitory effect on the movement of both endothelial and vascular SMCs.

The enhanced endothelial repair observed in vivo may result from increased migration or proliferation or decreased apoptosis. Our complementary in vitro data indicated that troglitazone had similar inhibitory effects on migration and proliferation of endothelial and vascular SMCs, but some data suggested a concentration-specific effect to reduce apoptosis. Because apoptosis is a marked endothelial event during reendothelialization and reendothelialization results from the balance of growth and apoptosis, we speculate that a subtle effect on reducing the high level of apoptosis may explain the enhanced reendothelialization that we observed. There have been several reports that glitazones inhibit endothelial cell proliferation.16–18 Two of these reports relate to retinal endothelial cells, which are known to be very long lived, suggesting a very low propensity to undergo apoptosis. It is our contention that the high rate of apoptosis of macrovascular endothelial cells in culture and in repairing vessels underlies the results observed here for the actions of trogli-tazone to accelerate endothelial coverage in vivo by reducing the contribution of apoptosis.

In the aortic reendothelialization protocol, troglitazone treatment resulted in an early (4-day) stimulation of endothelial recovery, observed as a significant increase in the size of the endothelial patches. Such a response must arise from increased migration and proliferation or decreased apoptosis or a combination of these responses, leading to an increase in the viable cell population and restoring the vascular endothelial integrity after injury. The importance of apoptosis is receiving increasing recognition, and it certainly occurs in vivo. Gibbons and colleagues19 have recently shown that decreased apoptosis secondary to increased glucose uptake occurs during intimal lesion formation after injury in rat and rabbit carotid arteries. A thiazolidinedione PPAR-γ ligand, ciglitazone, has been shown to initiate apoptosis in vascular endothelial cells.20 In vitro experiments reported here show that under both low and high glucose concentrations in the media, the initial response to low concentrations of troglita-
zeone (<3 μmol/L) is a substantial reduction in the cells releasing from the underlying matrix, consistent with protec-
tion from apoptosis. At higher concentrations, clear evidence of cellular release, presumably secondary to activation of apoptosis, is apparent. Hence, we can speculate that the accelerated reendothelialization apparent 4 days after deendothelialization results from modest reduction of apoptosis by low concentrations of troglitazone. Although the potency of thiazolidinediones varies markedly, the data with ciglitazone show that, although no protection from apoptosis was appar-
ent at low concentrations, the activation of apoptosis arises only at concentrations at or above 10 μmol/L, which is consistent with the results observed here for troglitazone.20 Under the low-glucose conditions of the in vitro experiments, which match the normoglycemic conditions of the animals used in our study, troglitazone caused a halving of the combined apoptotic and necrotic endothelial cells (74% versus 43%) present in treated cultures. We speculate at this stage that if this phenomena was occurring in vivo, then it would explain the enhanced reendothelialization observed, and it should serve as a focus for additional investigation both for troglitazone and new and emerging PPAR-γ and even PPAR-α/γ ligands.

The early increase in endothelial cell coverage was not maintained at a very advanced stage of reendothelialization (14 days). There was, however, a late reduction in SMC mass in the neointima, which may be attributable to either the early endothelial cell enhancement inhibiting the development of an underlying neointima, and thus this is a secondary effect of troglitazone, or to a direct inhibitory effect on vascular SMCs, as previously reported in a rat aortic balloon injury model.6 These two lines of evidence suggest that troglitazone had direct pharmacological actions in the in vivo model.

Migration was most likely a significant component in the reendothelialization process, and we examined the effects of troglitazone in vitro in a scrape/wound assay, which models the in vivo experiment. High glucose stimulates the migration of vascular SMCs but did not stimulate and tended to be inhibitory toward the migration of macrovascular endothelial cells in the present experiments. Only the high-glucose-stimulated component and not basal migration was inhibited by troglitazone in both rabbit coronary artery cells15 and rat aortic SMCs.5 In our experiments, the inhibitory efficacy of troglitazone was greater in a high-glucose media, notwith-
standing that high glucose did not stimulate migration of endothelial cells. Our in vivo experiments showed an en-
hanced ability of endothelial cells to cover a denuded surface, and, therefore, because these animals were normoglycemic, we would conclude that troglitazone does not have a net inhibitory effect on migration of endothelial cells in vivo. The potency of troglitazone in reducing neointimal formation in injured vessels would suggest that it may inhibit both migration and proliferation of vascular SMCs in vivo, but a concomitant reduced activity toward endothelial cell migra-
tion would certainly be favorable in the maintenance of vascular endothelial integrity, an important component of vascular quiescence and protection against atherosclerosis.

In conclusion, the development of drugs for the treatment of hypertension and hyperlipidemia has resulted in agents that reduce these cardiovascular risk factors and significantly improve outcomes in clinical studies.2,3,21 It is likely that oral hypoglycemic agents may follow this development path, with agents having direct actions to inhibit the enhanced macro-
vascular disease that accompanies diabetes. The present study demonstrates that troglitazone has multiple vascular actions capable of influencing the progress of vascular disease; however, additional studies and clinical trials are required to assess the long-term therapeutic impact of thiazolidinediones such as troglitazone, but now more likely pioglitazone, on the progress of vascular disease in diabetes.

Acknowledgments
This work was supported by the National Heart Foundation of Australia, the Alfred Hospital Foundation, and the National Health and Medical Research Council of Australia. Troglitazone (Rezulin) was provided as a gift from Parke Davis Pharmaceutical Research, and Medical Research Council of Australia. Troglitazone (Rezulin) demonstrates that troglitazone has multiple vascular actions capable of influencing the progress of vascular disease; however, additional studies and clinical trials are required to assess the long-term therapeutic impact of thiazolidinediones such as troglitazone, but now more likely pioglitazone, on the progress of vascular disease in diabetes.

References
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Arterioscler Thromb Vasc Biol. published online March 27, 2003;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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