Troglitazone Improves Blood Flow by Inhibiting Neointimal Formation After Balloon Injury in Otsuka Long-Evans Tokushima Fatty Rats

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Troglitazone (TGZ) is an antidiabetic agent of the thiazolidinedione (TZD) class that potentiates insulin action. In addition to its effects on insulin action, TGZ has an antiproliferative effect on vascular smooth muscle cells (VSMCs), of which proliferation is a prominent feature of retenosis after balloon injury, as well as atherosclerosis. Therefore, we investigated the effects of TGZ on intimal formation and blood flow after balloon injury in insulin-resistant Otsuka Long-Evans Tokushima Fatty (OLETF) rats to see whether the decrease in insulin resistance could minimize VSMC proliferation and could maintain blood flow. OLETF rats, an animal model of type 2 diabetes, develop spontaneous hyperglycemia after the age of 24 weeks. Balloon injury was applied to the left common carotid arteries of the rats with a 2F Fogarty catheter. Two weeks after the balloon injury, blood flow velocity was measured with Doppler ultrasonography, and histomorphometric analyses of the common carotid arteries were performed. The neointimal formation caused by VSMC proliferation was inhibited by TGZ treatment by as much as 80% (0.197 ± 0.013 mm^2 *v* 0.157 ± 0.011 mm^2, *P* < .05). The ratio of neointimal to medial area also decreased by 22% with TGZ treatment (1.651 ± 0.148 *v* 1.292 ± 0.083, *P* < .05). These effects of TGZ in OLETF rats were accompanied by alterations in plasma insulin, triglyceride, and total cholesterol levels. To look into the relationship between VSMC proliferation and hyperinsulinemia, we used a [^{3}H]-thymidine incorporation assay to investigate the effects of TGZ on VSMC proliferation. Insulin (at a concentration of 17.3 nmol/L) significantly stimulated DNA synthesis (236.6% ± 7.4%, *P* < .001), and TGZ significantly inhibited the insulin-induced DNA synthesis in VSMCs (106.43% ± 4.23%, *P* < .001) in a dose-dependent manner. In balloon-injured arteries of the untreated group, systolic blood flow velocity decreased by 61% compared with uninjured arteries (*P* < .05). However, there was no significant difference in systolic blood flow velocity between injured and uninjured arteries in the treated group (0.906 ± 0.043 *v* 0.991 ± 0.066 meters per second [m/s], *P* = not significant [NS]). The systolic blood flow of injured arteries was improved by 143% in the treated group (*P* < .01). These data suggest that TGZ is a potent inhibitor of VSMC proliferation both in vivo and in vitro through a direct effect on VSMCs, and that TZDs might be very useful in the treatment and prevention of restenosis after balloon injury.

TROGLITAZONE (TGZ) IS an oral antidiabetic agent of the thiazolidinedione (TZD) class. It has been shown to decrease insulin resistance, which results in a reduction in plasma glucose and insulin levels in both insulin-resistant diabetic animals and humans with type 2 diabetes.[1-3] As well as its action as an insulin-sensitizing agent, TGZ has been shown to affect the cardiovascular system. Recent studies have demonstrated that TGZ inhibits vascular smooth muscle cell (VSMC) proliferation and migration induced by platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) and attenuates neointimal hyperplasia in Sprague-Dawley (SD) rat carotid artery after balloon injury.[4,5]

Balloon injury to the arterial wall triggers a sequence of events: proliferation of medial SMC, migration into the intima, and proliferation of intimal cells to form neointimal hyperplasia.[6] Therefore, it is important to inhibit the growth and proliferation of VSMCs, which is potentiated by several growth factors and cytokines, to prevent restenosis after balloon injury. Hypertension in insulin-resistant states has been attributed to hyperinsulinemia[7-9] and may be the result of a decreased ability of insulin to attenuate vasoconstriction.[10,11] TGZ lowers elevated blood pressure in experimental models[1,12] and in humans[3] and has been shown to induce vasorelaxation.[13,14] This agent increases the skin blood flow in both dexamethasone-induced diabetic obese Zucker rats and control Wistar rats[15] and in streptozotocin-induced diabetic rats.[16]

The Otsuka Long-Evans Tokushima Fatty (OLETF) rats used in this experiment are an animal model of obese type 2 diabetes mellitus associated with insulin resistance, as evidenced by hyperinsulinemia and late-onset hyperglycemia with late complications.[17,18] As hyperglycemia develops later in the OLETF rats than in Zucker fatty rats, OLETF rats might be a useful experimental model to explore the relationship between hyperinsulinemia and cardiovascular diseases, such as restenosis after balloon injury and atherosclerosis. In insulin resistance, VSMC proliferation and migration in response to insulin may be accelerated,[5] and we can hypothesize that a decrease in insulin resistance by using insulin sensitizers, such as TGZ, could minimize VSMC proliferation and could maintain blood flow. In this study, we investigated the effects of TGZ on blood flow and VSMC proliferation after balloon injury to the carotid arteries in insulin-resistant OLETF rats.

MATERIALS AND METHODS

Balloon Injury

Male OLETF rats (Otsuka Pharmaceutical, Tokushima, Japan), aged 10 weeks (average weight, 300 g), were used. The rats were housed individually under controlled light (12 hour/12 hour) and temperature (22°C) conditions and had free access to food and water. After a

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1-week acclimation period, rats were randomly assigned either to treatment with TGZ (a gift from Sankyo, Tokyo, Japan) as a 0.15% food admixture (TGZ group) or to normal rat chow only (control group). There was no difference in food consumption between the 2 groups of animals. One week later, balloon injury to the left common carotid artery was induced according to the procedure of Clowes et al[19,20] with some modifications. Briefly, the rats were anesthetized intraperitoneally with xylazine (20 mg/kg) and ketamine (35 mg/kg), and a 2F Fogarty arterial embolectomy catheter (Baxter Healthcare, Irvine, CA) was introduced through the left external carotid artery and advanced into the left common carotid artery. The catheter was inflated with 0.5 mL saline and then withdrawn to the carotid bifurcation. This procedure was repeated 3 times. The catheter was removed, and the proximal external carotid artery was ligated. The right common carotid artery was not damaged and served as a control. Rats were fed their respective diets for 2 more weeks. Fourteen days after the operation, blood was collected in a tube containing EDTA to measure plasma glucose, insulin, free fatty acid (FFA), triglyceride, and total cholesterol levels. Each animal was then perfused with 4% paraformaldehyde solution for 10 minutes. After perfusion, the common carotid arteries were carefully removed and fixed in 4% paraformaldehyde. Blood flow velocity of the arteries was measured with Doppler ultrasonography (15L8, Sequoia System; Acuson, Mountain View, CA) before the animals were killed. This study was approved by the Institutional Laboratory Animal Care and Use Committee of Samsung Biomedical Research Institute of Sungkyunkwan University School of Medicine.

Histologic Analysis and Morphometry

Two or three individual sections from the middle of the common carotid arteries were analyzed. Cross-sections of carotid arteries were embedded in paraffin, and transverse histologic sections (0.4 μm) were taken from each segment and stained with hematoxylin-eosin (H&E). The slides were photographed through a microscope at a magnification of $\times$40, and morphometry was performed by using a digitizer (Artz II; Wacom, Vancouver, BC, Canada) linked to a computer graphics program (Graphics Tablet Software V2.45, Wacom), which allowed the manual selection and delineation of areas of interest. The intimal area (defined as the area between the lumen and the internal elastic laminae), the medial area (between the internal and the external elastic laminae), and the intimal to medial area were calculated. Plasma glucose was determined by the glucose oxidase method with YSI 2300 (YSI, Yellow Springs, OH). Plasma insulin level was measured by an insulin radioimmunoassay kit (Linco, St Charles, MO). Plasma triglyceride and total cholesterol levels were measured with commercial kits (Yeong-Dong Diagnostics, Kyunggido, Korea).

Cell Culture

VSMCs were prepared from SD rats by enzymatic dissociation according to the method previously described.[21] VSMCs of passage 4 to 10 were used in the experiments. VSMCs were cultured in Dulbecco's modified Eagle's medium (DMEM) F12 (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL), penicillin (100 U/mL), streptomycin (10 mg/mL), and 25 mmol/L HEPES (pH 7.4) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were subcultured after trypsinization on a weekly basis, and each plate was replenished with fresh medium twice a week. The purity and identity of the VSMC cultures were verified by staining with a monoclonal antibody against smooth muscle α-actin (Dako, Carpinteria, CA). For all experiments, VSMCs were grown to subconfluence and then starved in serum-free DMEM containing 0.1% bovine serum albumin (Sigma, St Louis, MO) for 48 hours.

[^{3}H]-Thymidine Incorporation Assay

Relative rates of DNA synthesis in VSMCs were assessed by measurement of the incorporation of [^{3}H]-thymidine. VSMCs were seeded at a density of 3×10^4 cells/well and grown to confluence. After serum starvation for 48 hours, VSMCs were stimulated for 24 hours in the presence of insulin and/or TGZ. During the final 4 hours of assay, 0.5 μCi of [^{3}H]-thymidine (NEN, Boston, MA) was added to each well. Cells were washed twice with cold calcium- and magnesium-free phosphate-buffered saline (PBS) and lysed in the presence of 5% trichloroacetic acid (Sigma). Cellular residues were rinsed in 95% ethanol and solubilized in 0.4 N NaOH at 4°C. Radioactivity was measured by a liquid scintillation counter (LS6500 TD; Beckman Instruments, Irvine, CA). Counts of thymidine were normalized to those of control and expressed as percentage value of controls. Experiments were performed in triplicate.

Statistical Analyses

Values of the experimental studies are expressed as means $\pm$ SEM. Statistical significance was determined with unpaired Student's *t* test or Tukey's multiple comparison procedure applied after a 1-way analysis of variance (ANOVA) conducted on transformed data. For all comparisons, a *P* value less than .05 was considered significant.

RESULTS

Metabolic Characteristics of OLETF Rats

Certain metabolic characteristics of the OLETF rats at 2 weeks after balloon injury are presented in Table 1. There were no significant differences in body weight between OLETF rats treated with TGZ and untreated controls, indicating that the 0.15% TGZ food admixture was not toxic to the rats (Table 1). Plasma glucose concentrations did not differ significantly between TGZ-treated and untreated groups. Drug administration led to significant decreases in plasma insulin, triglyceride, and total cholesterol levels. However, free fatty acid and high-density lipoprotein-cholesterol (HDL-C) levels were similar between the 2 groups.

Morphometric Analysis

To investigate the effect of TGZ on intimal proliferation, balloon injury was performed in the left common carotid arteries of the OLETF rats. In TGZ-treated groups, neointimal formation was significantly reduced compared with the untreated groups (0.197 ± 0.013 mm^2 *v* 0.157 ± 0.011 mm^2, $P < .05$). The neointimal area/medial area (I/M) ratio also significantly decreased in the TGZ-treated group compared with the untreated group (1.292 ± 0.083 *v* 1.651 ± 0.148, $P < .05$).

Table 1. Metabolic Characteristics of OLETF Rats

	Control	TGZ-Treated
Body weight (g)	416.6 ± 7.8	409.9 ± 11.4
Plasma glucose (mmol/L)	9.43 ± 1.29	9.14 ± 0.94
Plasma insulin (pmol/L)	12.8 ± 1.4	8.9 ± 0.7*
Triglyceride (mmol/L)	2.33 ± 0.16	0.94 ± 0.06†
Total cholesterol (mmol/L)	2.46 ± 0.08	2.18 ± 0.09*
HDL cholesterol (mmol/L)	1.18 ± 0.04	1.16 ± 0.04
Free fatty acid (μEq/L)	1,548.3 ± 82.4	1,414.5 ± 34.7

NOTE. Data are means ± SEM.
*$P < .05$.
†$P < .0001$ *v* control.

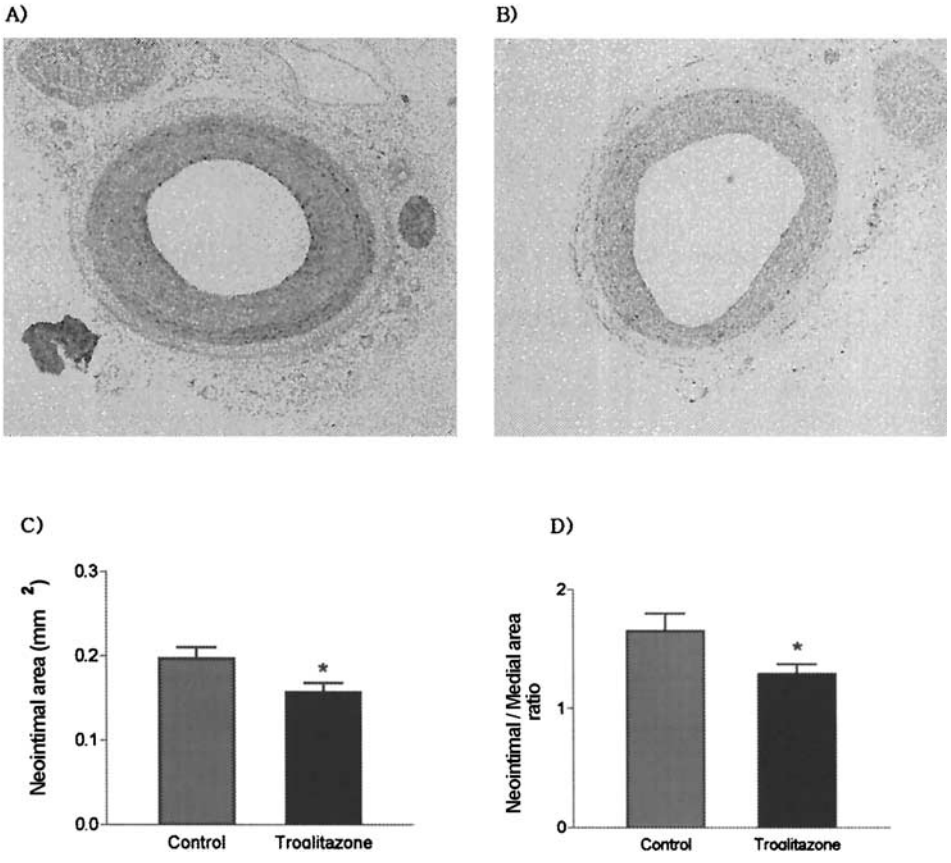

Fig 1. Effects of TGZ on neointima formation 2 weeks after balloon injury in OLETF rats. H&E-stained cross sections of representative injured arteries from (A) control group and (B) TGZ-treated group are presented (original magnification ×40). Both (C) neointimal area and (D) neointimal/medial area ratio were significantly decreased by TGZ treatment (n = 8/group). Results are expressed as mean ± SEM. *$P < .001$ *v* insulin.

There was no significant change in the medial area after TGZ treatment (0.123 ± 0.004 *v* 0.123 ± 0.004 mm^2, Fig 1).

Effects of TGZ on Insulin-Induced VSMC Proliferation

To determine whether the change of insulin level in OLETF rats treated with TGZ affected the neointimal formation, we examined the effects of insulin on VSMC proliferation in the culture condition. All of the experiments were performed in a serum-free environment to eliminate the effects of other potential growth factors on VSMCs. Insulin stimulated VSMC proliferation in a dose-dependent manner, as measured by [^{3}H]-thymidine incorporation assay (Fig 2). Insulin (17.3 nmol/L) stimulated VSMC DNA synthesis by 237% compared with untreated cells, but insulin at a lower concentration did not significantly stimulate DNA synthesis. Insulin-induced VSMC proliferation was significantly inhibited by TGZ at a concentration of 45 μmol/L (106.43 ± 4.23%, Fig 3). Growth inhibition was observed 24 hours after TGZ treatment.

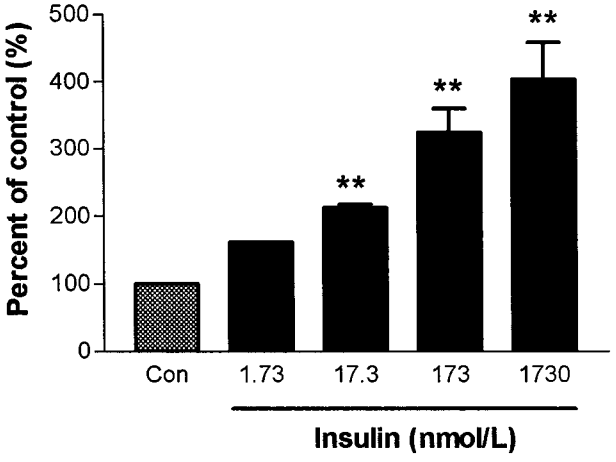

Fig 2. Dose-dependent stimulation of DNA synthesis by insulin in VSMCs. Growing VSMCs were seeded in 24-well plates at a density of 3 × 10^4 cells/well and incubated in DMEM/F12 media containing 10% FBS. The cells were starved in serum-free DMEM and then treated with insulin (0 to 1,730 nmol/L) for 24 hours. During the final 4 hours of incubation, VSMCs were labeled with 0.5 μCi [^{3}H]-thymidine. Radioactivity of [^{3}H]-thymidine incorporated into DNA was measured as described in Materials and Methods. Results are expressed as mean percent of control ± SEM. **$P < .001$ *v* control.

Blood Flow Velocity

To determine whether TGZ can affect blood flow, the blood flow velocities of injured and uninjured carotid arteries were measured by Doppler ultrasonography in OLETF rats 2 weeks after balloon injury before they were killed. In the untreated control group of OLETF rats, systolic blood flow velocity significantly decreased in the injured carotid arteries compared with the uninjured ones (0.633 ± 0.049 *v* 1.030 ± 0.023 meters

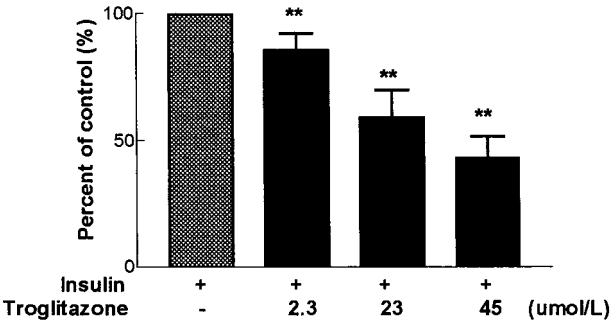

Fig 3. Effects of TGZ on insulin-induced DNA synthesis by VSMCs. Quiescent VSMCs were stimulated with insulin (17.3 nmol/L) in the presence of TGZ (2.3 to 45 μmol/L) for 24 hours. Insulin-induced DNA synthesis was significantly decreased by TGZ in a dose-dependent manner. Experiments were performed in triplicate on 3 different occasions. Results are expressed as mean percent of control ± SEM. **$P < .001$ *v* control.

per second [m/s], $P < .05$). In the treated group, however, there was a significant increase in systolic blood flow velocity in the injured carotid arteries compared with that of the untreated group (0.906 ± 0.043 m/s, $P < .01$, Fig 4).

DISCUSSION

Although it has been shown that TGZ markedly inhibits VSMC proliferation and neointimal formation in normal SD and obese Zucker rats,[4,5] there has been no report on the effects of TGZ on intimal hyperplasia and blood flow in OLETF rats, the recently established insulin-resistant type 2 diabetic rat model. This is the first report in which both postmortem histomorphometry of the vascular structure and blood flow velocity measurement in living animals treated with TGZ was undertaken. Migration and proliferation of VSMCs are critical events in the development of restensis after balloon injury, as well as in the progression of atherosclerosis.[6] Although smooth muscle cell proliferation can persist near the luminal surface as late as 12 weeks after balloon injury, the number of arterial smooth muscle cells does not increase after 2 weeks.[22] Therefore, in this study, we showed that the administration of TGZ in OLETF rats prevented the neointimal formation 2 weeks after balloon injury to carotid arteries. In contrast to the effects on neointimal formation and I/M ratio, TGZ failed to affect vascular remodelling, indicated by there being no change in the medial area. The antiproliferative effect of TGZ was also observed in cultured VSMCs. Moreover, the blood flow velocity of balloon-injured carotid arteries was increased by TGZ treatment.

TGZ has been shown to decrease plasma glucose and insulin levels in both insulin-resistant diabetic animals and in humans with type 2 diabetes.[1-3] In this study, the plasma insulin level was decreased by TGZ without a change in blood glucose concentration, which implies an improvement in insulin resistance. The OLETF rats usually show hyperglycemia after 24 weeks of age. Similarly, TGZ did not affect the body weight in OLETF rats, indicating that 0.15% TGZ was not toxic to the rats. In addition to its antihyperglycemic effect, TGZ also reduces circulating levels of triglycerides and total cholesterol.[23] In our study, serum triglyceride and cholesterol levels significantly decreased after TGZ treatment. However, there was no significant change in HDL-C levels. Lefebvre et al[24] have reported that rosiglitazone, another member of the TZD family, lowers serum triglyceride-rich lipoprotein without affecting HDL concentrations. FFA level was not changed by TGZ. In this study, experimental animals were fasted for 12 hours and blood sampling was performed before the animals were killed. Oakes et al[25] have reported that in obese Zucker rats after a 7-hour fast, FFA levels tended to increase by treatment with TZD (rosiglitazone and darglitazone), which lowered insulin and triglyceride level. There has also been a rapid change in FFA levels by TZD treatment. These data suggest that TZD may lose its effect on FFA level depending on the state of fasting. The observed decrease in triglyceride levels in animals treated with TGZ shows an added advantage of TGZ therapy in correcting the dyslipidemia frequently observed in people with type 2 diabetes. The protective effect of TGZ on neointimal formation and/or blood flow velocity may be related to a decline in hyperinsulinemia and hypertriglyceridemia. However, the vascular protective effect of TGZ was also reproduced in insulin-sensitive SD rats (data not shown). Moreover, although insulin could stimulate VSMC proliferation in vitro, the serum insulin concentration of the OLETF rats (12.8 ± 1.4 pmol/L) was too low to have a significant effect in vivo. Therefore, it is likely that the effects of TGZ may be direct on the vascular system.[6] However, whether the vascular protective effects of TGZ on neointimal formation were mediated first, perhaps by an improvement in blood flow, remains to be determined. It has been shown that there is an association between insulin resistance and essential hypertension.[7-9] Insulin resistance is associated with the increase in peripheral vascular resistance.[10,11] TGZ has also been shown to lower blood pressure,[1,3,12] induce vasorelaxation,[13,14] and increase

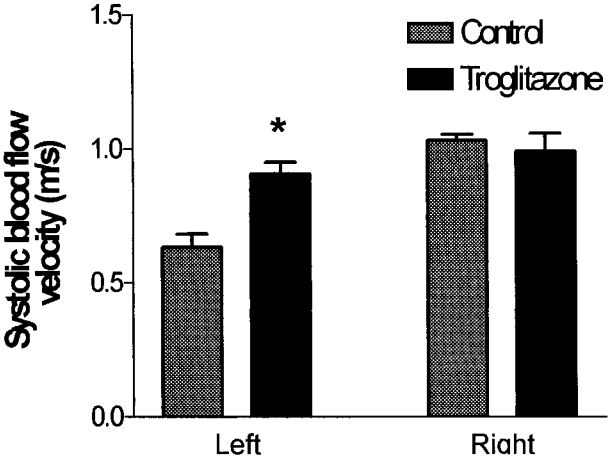

Fig 4. Effects of TGZ on systolic blood flow velocity 2 weeks after balloon injury in OLETF rats. Systolic blood flow velocity of left and right carotid arteries (control) was measured by ultrasonography before animals were killed (n = 8/group) and compared with TGZ-treated and nontreated groups. TGZ treatment significantly increased blood flow velocity in balloon-injured left common carotid arteries. Results are expressed as means ± SEM. **$P < .05$ *v* control left carotid arteries.

skin blood flow.[15,16] However, TZDs, including TGZ, have recently been demonstrated to bind with high affinity to the peroxisome proliferator-activated receptor-γ (PPAR-γ), which acts as a transcription factor and belongs to the nuclear receptor subfamily of PPARs.[26] Iijima et al[27] demonstrated previously that PPAR-γ is expressed in VSMCs, and PPAR-γ expression in VSMCs may be related to the VSMC proliferation. In fact, PPAR-γ activation by TGZ reduces the expression of matrix metalloproteinase (MMP)-9 in VSMCs,[28] which implies it has antiatherosclerotic effects.[29-32]

In summary, we have demonstrated that the insulin-sensitizing agent, TGZ, inhibited VSMC proliferation in vitro, attenuated the intimal hyperplasia after balloon injury to carotid arteries, and increased the blood flow velocity in the carotid arteries of OLETF rats. TZD may, therefore, be a useful adjunct to prevent restenosis after balloon angioplasty in patients with insulin resistance and type 2 diabetes mellitus.

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