Troglitazone Reduces Contraction by Inhibition of Vascular Smooth Muscle Cell Ca^{2+} Currents and Not Endothelial Nitric Oxide Production

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The insulin-sensitizing compound troglitazone has evolved into a promising therapeutic agent for type II diabetes. It improves insulin sensitivity and lipoprotein metabolic profiles and lowers blood pressure in humans and rodents. Because troglitazone has insulin-like effects on a number of tissues, we hypothesized that it may reduce vascular tone through stimulation of endothelial-derived nitric oxide (NO) production or by diminution of vascular smooth muscle cell (VSMC) intracellular calcium ($[Ca^{2+}]_i$). Our results show that troglitazone decreases norepinephrine-induced contractile responses in the rat tail artery, an effect not reversed by the NO inhibitor L-nitroarginine methyl ester (L-NAME). In contrast, troglitazone significantly inhibited L-type Ca^{2+} currents in freshly dissociated rat tail artery and aortic VSMCs and in cultured VSMCs. The data suggest that troglitazone attenuates vascular contractility via a mechanism involving VSMC $[Ca^{2+}]_i$ but independent from endothelial generation of NO. Because insulin has been shown to affect vascular tone by both of these mechanisms, troglitazone only partially mimics insulin action in this tissue. *Diabetes* 46:659–664, 1997

Hypertension is considerably more common in diabetic individuals than in those without clinical carbohydrate intolerance. The prevalence of coexistent hypertension and type II diabetes is rising, in part, because both conditions increase with age and obesity (1,2). Insulin resistance is commonly observed in essential hypertension (1,2) and in type II diabetes (3,4). Enhanced vascular responsiveness to vasoconstrictor agents and blunted vasodilatation are among the proposed mechanisms leading to hypertension in association with insulin resistance (5–12). Recent reports that insulin-sensitizing agents can reduce vascular contractility (13–16) and lower blood pressure (17–23) lend credence to the notion that insulin resistance contributes to the development of hypertension.

Thiazolidinediones, the latest of a class of compounds developed for the oral treatment of type II diabetes, increase the insulin sensitivity in resistant states and improve the concomitant hyperinsulinemia and hyperglycemia (24–28). These effects have been ascribed to a number of mechanisms, including enhancement of adipogenesis (29), GLUT4 expression (30,31), and insulin-mediated suppression of hepatic glucose output (32). However, different members of this class of compounds exhibit different actions on glucose transport and metabolism in different models of insulin resistance (33,27). A member of this class of antidiabetic drugs, troglitazone, will likely be released for treatment of type II diabetes in the near future (21,23). Troglitazone appears to enhance insulin action in skeletal muscle, liver, and adipose tissue (26,28) and to lower blood pressure (21,23). The hypotensive effects of troglitazone may not be solely mechanistically linked to improvement of insulin resistance and may be more related to its insulin-like actions on vascular tissue. Insulin has been reported to dilate vessels through endothelium-dependent nitric oxide (NO) generation (30,34,35) and by decreasing vascular smooth muscle cell (VSMC) intracellular calcium ($[Ca^{2+}]_i$) (10–12). The literature remains divided as to which of these two mechanisms is involved in the vascular relaxation produced by pioglitazone, another thiazolidinedione (17,36). Therefore, we investigated the role of both of these potential mechanisms in attenuation of vascular contractility by troglitazone.

RESEARCH DESIGN AND METHODS

Animals. Male Wistar rats, 200–250 g, were purchased from Harlan (Indianapolis, IN), housed two per cage, and given food and water ad libitum. On the day of the experiment, the animals were anesthetized with sodium pentobarbital (30 mg/kg i.p.) and the tail artery removed for contractility studies. Other animals were used for preparation of aortic and tail vascular smooth muscle cells for patch-clamp experiments. The entire procedure was approved by the Wayne State University Animal Investigation Committee.

Vascular contractility. The tail artery was cleaned of fat in an ice-chilled buffer containing the following: 130 mol/l NaCl, 15 mol/l $NaHCO_3$, 4.7 mol/l KCl, 1.2 mol/l KH_2PO_4, 1.6 mol/l $CaCl_2$, 1.2 mol/l $MgSO_4$, 0.03 mol/l EDTA, and 5.6 mol/l glucose. Here, 3-mm rings were suspended from isometric force transducers (Gould Instruments, Cleveland, OH) in muscle baths containing the same buffer at 37°C, aerated with 95% O_2 and 5% CO_2 to maintain pH at 7.4. All rings were stretched to 1 g of tension and allowed to stabilize for 30 min before addition of agonists (37). In experiments where rings were preincubated in vitro, troglitazone (10 μmol/l; Parke-Davis, Ann Arbor, MI) and/or an equivalent volume of the DMSO vehicle were added 90 min before stepwise addition of norepinephrine (NE) (1 nmol/l to 100 μmol/l). A full dose response was

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ANOVA, analysis of variance; DMEM, Dulbecco's modified Eagle's medium; iNOS, inducible nitric oxide synthase; L-NAME, L-nitroarginine methyl ester; NE, norepinephrine; PI, phosphatidylinositol; PPARα, peroxisome proliferation-activated receptor α; TEA, tetraethylammonium; VSMC, vascular smooth muscle cell.

performed; the next higher dose was added after contraction to the previous dose had reached a plateau. L-nitroarginine methyl ester (L-NAME, 100 μmol/l) was added 5 min before troglitazone to evaluate the role of NO. To test the direct effects of troglitazone, rings were precontracted with a half-maximal dose (5 μmol/l) of NE, and troglitazone (dissolved in DMSO) was then added at the following concentrations: 1, 10, and 30 μmol/l. Control rings received an equal volume of DMSO. Acetylcholine (10 μmol/l) was used to assess absence of endothelium in rings that had been rolled against blunt forceps tips to ensure denudation. Denudation was considered complete when acetylcholine evoked no response.

Calcium channel currents. The effect of troglitazone on currents through voltage-dependent Ca^{2+} channels was studied using whole-cell patch-clamp analysis of A7r5, VSMCs derived from embryonic rat thoracic aorta (ATCC, Rockville, MD), and freshly dissociated rat aortic and rat tail artery VSMCs. A7r5 cells were used for the initial experiments because they are easy to culture, have large currents, and behave in a manner similar to freshly dissociated cells (15,38–40). As previously described (12), cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 9% fetal bovine serum (Gibco, Grand Island, NY), 0.2% Tylosin, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma, St. Louis, MO) in 10-cm petri dishes at 37°C in a water-jacketed incubator under 95% air, 5% CO_2, and 100% humidity. Before recording, confluent cells were released by enzyme treatment (0.2% trypsin, 1 mmol/l EGTA in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution), centrifuged for 5 min at 1,000 rpm, resuspended, and plated onto pieces of 10- × 4-mm-glass coverslips on the bottom of a 35-mm petri dish. One hour later, the coverslips were transferred to the experimental chamber (volume = 0.5 ml) for patch-clamp studies.

Freshly dissociated VSMCs were isolated from rat aortas and tail arteries using techniques previously described (15,35). Dispersed freshly dissociated VSMCs were placed directly onto 10 × 4 mm^2 glass coverslips in 0.5 ml of bath solution in the recording chamber and allowed 5–10 min to attach before continuous superfusion with medium at room temperature (3 ml/min). Extracellular solutions are described below.

Cells were recorded in whole-cell patch-clamp configuration using an Axopatch 200 patch-clamp amplifier (Axon Instruments) controlled by a Labmaster TL-1 DMA A/D, D/A interface and pClamp software (Axon Instruments), as described previously (38–40). Whole-cell configuration was attained after acquisition of giga seals by gentle suction while holding the electrode at –80 mV. Cell capacitance and series resistance were corrected to 90%. To study L-type Ca^{2+} channels, Ba^{2+} was substituted for Ca^{2+} in the extracellular solution, and potassium current was blocked with tetraethylammonium (TEA) in the extracellular solution and with Cs^+ in the electrode solution. Because these cells do not have a voltage-dependent Na^+ current (38), no Na^+ channel blocker was needed. The extracellular medium contained 83 mol/l NaCl, 30 mol/l TEA, 2 mol/l $MgCl_2$, 20 mol/l $BaCl_2$, and 10 mol/l HEPES and had a pH of 7.4, plus vehicle (0.1% DMSO) or test drug (troglitazone 1–23 μmol/l) in vehicle. The electrode solution contained 120 mol/l CsCl, 10 mol/l CsEGTA, 1.4 mol/l $MgCl_2$, 3.6 mol/l Mg-ATP, 1 mol/l Na-GTP, and 10 mol/l HEPES and had a pH of 7.2.

Voltage-dependent current was elicited at 20-s intervals by a ramp command potential, going from –100 to 50 mV in 300 ms, with a digital resolution of 1 mV. Between ramps, cells were held at –80 mV (14). Previous studies by this laboratory (38,40) have demonstrated that the major current peak elicited by this ramp protocol is L-type current: it is dihydropyridine-sensitive, dependent on the presence of Ca^{2+} or Ba^{2+}, not contaminated by either T-type current, which inactivates before peak current is reached, or sodium current (not present at all). The ramp rate is such that L-type currents activate almost completely but hardly inactivate at all during the ramp, and the voltage dependence and drug effects on the current elicited by the ramp protocol are nearly identical to L-type current voltage dependence and drug effects measured with pulse-type voltage protocols. In addition, the ramp protocol has the advantage that its entire I-V curve can be measured in less than 1 s and as frequently as once every 20 s.

Statistical analysis. Population statistics and determinations of significance with analysis of variance (ANOVA), Dunnett's procedure (for vascular reactivity), or Bonferroni's correction for multiple pairwise comparisons (for the patch-clamp experiments) was calculated using GraphPADInstat (GraphPAD Software, San Diego, CA) and Pharm-PC (Springer-Verlag, New York, NY). Population statistics are given as means ± SE. P values < 0.05 were considered significant.

RESULTS

Previous experiments in our laboratory demonstrated that insulin, IGF-I, and the antidiabetic agent metformin required preincubation of at least 90 min to diminish vascular contractility (15,37,41). In this study, preincubation of endothelium intact rings with 10 μmol/l troglitazone for 90 min reduced contractility (P = 0.026 via two-way ANOVA) in response to NE (400 ± 127 vs. 632 ± 95 mg in controls at the highest dose [100 μmol/l]), an effect not prevented by coincubation with 100 μmol/l L-NAME, suggesting that endothelium may not be required. We then examined whether troglitazone could directly relax precontracted tail artery rings lacking intact endothelium (unresponsive to acetylcholine). A representative experiment is shown in Fig. 1*A*. Troglitazone elicited relaxation that began almost immediately on addition and could be reversed with the removal of the drug and the addition of fresh NE (data not shown). A summary of the data is presented in Fig. 1*B*. The drug produced a downward trend in tension at 10 μmol/l, and a statistically significant reduction of tension (P = 0.02) was obtained at the highest dose (30 μmol/l).

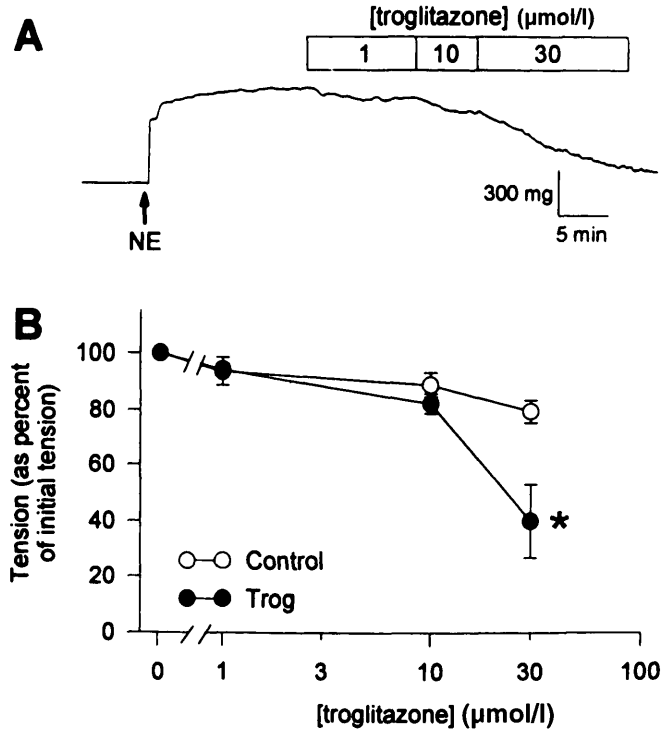

FIG. 1. ***A*: representative tracing of the result of direct addition of troglitazone to precontracted (5 μmol/l NE) rat tail artery rings unresponsive to acetyl choline (100 μmol/l). *B*: vasorelaxation to troglitazone as percent of initial contraction (n = 5; P = 0.02 at 30 μmol/l).**

To determine whether troglitazone attenuated contraction by direct inhibition of Ca^{2+} currents in VSMCs, we studied both cultured and freshly dissociated cells. Initial experiments on A7r5 cells demonstrated that concentrations of troglitazone as low as 1 μmol/l were able to decrease Ba^{2+} current through L-type Ca^{2+} channels. In Fig. 2, currents were recorded for at least 2 min in medium containing 0.1% DMSO before troglitazone application. A nonsignificant decrease of 2.0 ± 1.5% (n = 13 cells) in peak current was seen during DMSO application. Two minutes after application of 1 μmol/l troglitazone, the current decreased significantly to 87.7 ± 3.7% of its magnitude (Fig. 3*B*; n = 12 cells, P < 0.05 compared with 2 min in vehicle alone), without change in the voltage dependence of the current (Fig. 2*A*). In four of the cells,

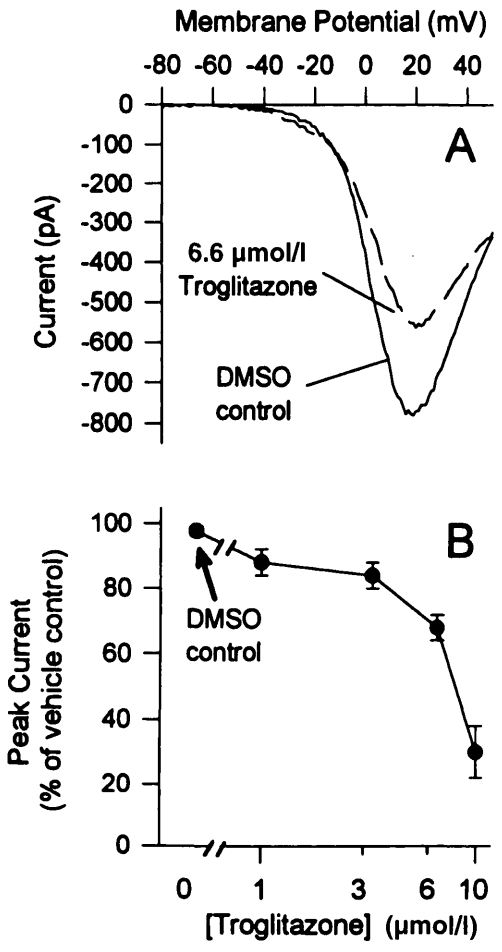

FIG. 2. Effect of troglitazone on Ba^{2+} current through L-type Ca^{2+} channels. A7r5 VSMCs were held at –80 mV, and currents were elicited by a voltage ramp going from –100 to 50 mV in 300 ms. Cells were sequentially exposed to the vehicle (0.1% DMSO control, n = 13 cells) for >2 min, and then troglitazone in DMSO (0.1%) at concentrations of 1 µmol/l (n = 12 cells), 3.3 µmol/l (n = 4 cells), 6.6 µmol/l (n = 4 cells), and 10 µmol/l (n = 4 cells). *A*: example of currents observed in DMSO control and 6.6 µmol/l troglitazone in a representative cell. *B*: summary of changes in peak current at the 2-min points in the DMSO control and each troglitazone concentration, as a percentage of the current elicited in DMSO before troglitazone application. ANOVA, $P < 0.001$. $P < 0.001$ for 6.6 µmol/l and 10 µmol/l troglitazone compared with DMSO control (Bonferroni-corrected t test). $P < 0.05$ for 1 µmol/l and 3.3 µmol/l troglitazone compared with DMSO control (Dunnett's multiple comparison procedure).

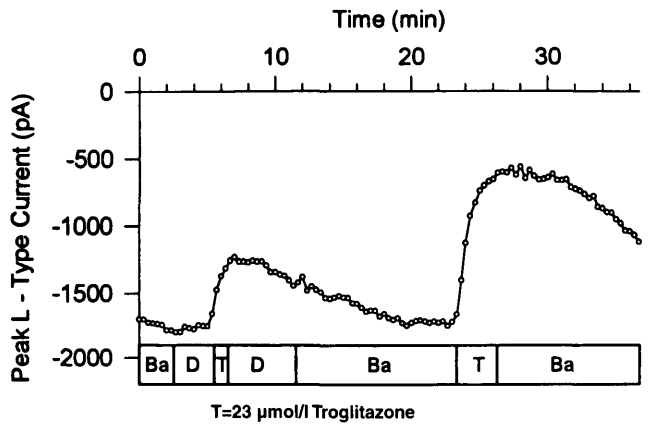

FIG. 3. Reversibility of the inhibitory effect of troglitazone on current through Ca^{2+} channels in a representative A7r5 cell. Patch-clamp methods were as described in Fig. 2. Various solutions were present in the recording chamber, according to the following abbreviations: Ba, Ba^{2+}-containing external medium described in METHODS; D, 0.1% DMSO in Ba medium; T, 23 µmol/l troglitazone in D medium.

cumulative additions of troglitazone (2-min duration for each concentration) to higher concentrations decreased the peak current still further (Fig. 2B, ANOVA, $P < 0.0001$), with an apparent ED_{50} between 6.6 and 10 µmol/l.

To ascertain that decreases in L-type current were not due simply to current rundown, we examined the reversibility of the troglitazone effects. Figure 3 shows the time course of the change in peak current in a typical experiment in which 23 µmol/l troglitazone was applied briefly and then washed off first with DMSO-containing medium (vehicle) for >5 min. As before, the current was steady in DMSO-containing solution (0.6 ± 0.5% change during 2–5 min) before application of the drug. Here, 1 min of 23 µmol/l troglitazone immediately initiated a reduction of current that was partially reversed in DMSO-containing medium within 5 min and completely reversed in medium without DMSO. In four cells treated with 23 µmol/l troglitazone for 3 min, the current was reduced to 22.5 ± 5.3% of vehicle control and returned to 65.8 ± 4.1% of the pretroglitazone level when washed 5 min in vehicle. The current during troglitazone application was significantly lower than both the vehicle control and current after 5 min washout (ANOVA and all Bonferroni-corrected paired comparisons, $P < 0.01$). Previous experiments in A7r5 showed current rundown to be minimal (<20%) within 1 h of vehicle application (data not shown). These observations suggest that current rundown did not have a significant impact on our results although some contribution cannot be totally excluded.

In VSMCs from tail artery (Fig. 4), change in vehicle did not alter current (n = 9 cells), but separate applications of 1 µmol/l (n = 8) and 10 µmol/l troglitazone (n = 5) significantly decreased it (ANOVA, $P < 0.0001$; Bonferroni-corrected comparisons with control, $P < 0.001$). Vehicle washes for 5 min were partially effective at reversing these effects. Current recordings in Fig. 4*A* show that as with A7r5 cells, troglitazone caused no change in the voltage dependence of the elicited current. Similar results were seen in aortic VSMCs, where 1 µmol/l troglitazone decreased current to 73.7 ± 3.2% of control (n = 6 cells; ANOVA, $P < 0.001$; Bonferroni-corrected comparisons of 1 µmol/l troglitazone to vehicle, $P < 0.01$; wash, $P < 0.05$), suggesting the effect is common to several VSMC types.

DISCUSSION

Unlike previous oral antidiabetic agents, thiazolidinediones exert antihyperglycemic action primarily by enhancing peripheral insulin action and do not stimulate insulin secretion (21–28). Vasodilatory effects of insulin (5–12) have been attributed to a number of mechanisms, including stimulation of NO production by the endothelium (34,35) and inhibition of Ca^{2+} entry into VSMCs (10–12,40). In human volunteers, short-term infusion of L-NAME prevents insulin-induced vasodilation in skeletal muscle beds (34,35). L-NAME also negates both insulin-mediated and IGF-I–mediated (3,42–44) vasodilation. Previous reports on the actions

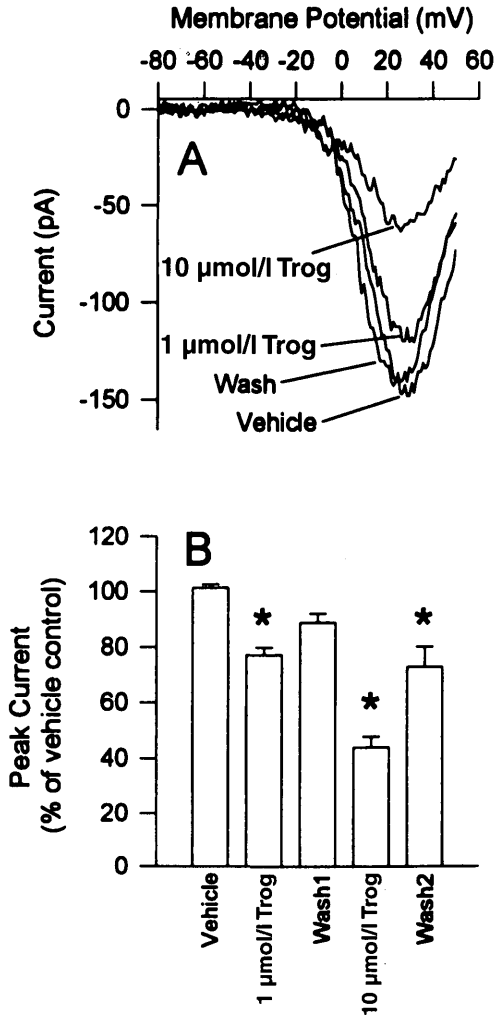

FIG. 4. Effect of troglitazone on current through L-type Ca^{2+} channels before, during, and after application of 1 and 10 µmol/l troglitazone to freshly dissociated rat tail artery VSMCs. Patch-clamp methods were as described in Fig. 2. *A*: results obtained with a representative cell exposed to vehicle (0.1% DMSO), 1 µmol/l troglitazone (1 µmol/l trog) for 2 min, washed in vehicle for 5 min (Wash), and 10 µmol/l trog for 2 min. *B*: summary of changes in several cells in response to troglitazone. Taking the magnitude of the current in vehicle (0.1% DMSO) just before 1 µmol/l troglitazone application as the reference point, bars show the change in current during 2-min application of vehicle (n = 9 cells), after 2 min in 1 µmol/l troglitazone (n = 8 cells), after 5-min washout with vehicle (n = 5 cells), after 2 min in 10 µmol/l troglitazone (n = 5 cells), and again after 5-min washout with vehicle (n = 3 cells): ANOVA, $P < 0.0001$. *$P < 0.001$ compared with vehicle (Bonferroni-corrected t test).

of pioglitazone on aortic contractility have demonstrated endothelium-dependent (17) and endothelium-independent effects (36), leaving the exact mechanism of thiazolidinedione action on the vasculature in question. In this study, we observed that long-term preincubation with troglitazone, like insulin and IGF-I (37,41,43,44), significantly decreased NE-induced contractile responses in rat tail arteries. However, unlike insulin and IGF-I (41,43,44), attenuation by troglitazone was not affected by L-NAME, a blocker of NO synthesis, suggesting that troglitazone does not inhibit adrenergically mediated contraction through stimulation of endothelium-derived NO production. On the other hand, troglitazone directly relaxed acetylcholine unresponsive tail artery rings precontracted with NE. Thus, although this agent has been shown to enhance insulin sensitivity in other tissues, it does not appear to effect vasodilation by mimicking insulin action on endothelial cells. Direct actions on VSMCs are more consistent with the drug's relaxation of NE-elicited contractions of endothelium-denuded blood vessels and are further supported by the inhibition of current through VSMC Ca^{2+} channels observed in this study. Indeed, troglitazone effectively reduced current through L-type channels in VSMCs from three different sources: a cell culture line derived from rat embryonic aorta and freshly dissociated VSMCs from rat aorta and tail artery.

The rat tail artery is abundantly innervated by sympathetic nerves. Although some changes in adrenergic responses of tail arteries have been attributed to modulation of release and uptake of NE by presynaptic endings in this tissue (45), our results suggest an action on the postreceptor adrenergic coupling process. Norepinephrine causes contraction through increases in VSMC Ca^{2+}, in part, as a result of influx through voltage-dependent Ca^{2+} channels (46). Therefore, the inhibition of Ca^{2+} channels observed in our experiments provides a plausible mechanism by which troglitazone attenuates the contractile response to norepinephrine.

Because of the critical role of $[Ca^{2+}]_i$ in the development of vascular tone, inhibition of Ca^{2+} influx through voltage-dependent channels is likely to contribute to the hypotensive effects of troglitazone in insulin-resistant humans (21,23) and rats (20). Specific Ca^{2+} channel blockers lower vascular tone through decreases in VSMC $[Ca^{2+}]_i$ (46). Furthermore, the concentrations of troglitazone shown to have an impact on Ca^{2+} currents in this study, as low as 1 µmol/l, are within the therapeutically effective dose range of troglitazone (21). Our results with troglitazone are reminiscent of the direct inhibition of Ca^{2+} channels by insulin (10–12,40) and other thiazolidinedione derivatives (14,19,27) and may be relevant to other VSMC functions, including proliferation and DNA synthesis. Indeed, since the increased peripheral vascular resistance associated with hypertension appears to be partially caused by accelerated growth (hyperplastic or hypertrophic) of VSMCs in small arteries and arterioles (47), the antihypertensive effects of troglitazone may be further related to these actions of reduced VSMC Ca^{2+} influx.

Although troglitazone itself was not tested, experiments with three other thiazolidinediones in insulin-receptor–transfected Chinese hamster ovary cells showed these drugs not to modify insulin binding, tyrosine kinase activity, or phosphorylation of the receptor or its substrate (IRS-1). Activity was confined to specific modulation of phosphatidylinositol (PI) 3-kinase (48). Similar effects on PI 3-kinase were reported in rat adipose tissue (49) in a time course compatible with activation of the peroxisome proliferation-activated receptor α (PPARα) (50). Results of these in vitro studies provide support for the facilitated translocation and expanded intracellular pool of GLUT4 seen in adipocytes of insulin-resistant mice after thiazolidinedione treatment (51). However, enhanced glucose transport was ruled out as a mechanism for increased relaxation to pioglitazone in the rat aorta (17). This does not negate possible transcriptional activation of an unknown protein (or proteins) and PPARα that may be responsible, in part, for the longer-term (90 min) troglitazone effects seen in this study. Some thiazolidinediones have been shown to inhibit tyrosine kinase activity, particularly epi-

dermal growth factor autophosphorylation (52). This fact, and others stated above, indicates that the thiazolidinediones may mimic insulin action in some tissues and not in others because their mechanisms of action are totally distinct. Thus, like activation of PI-3 kinase, inhibition of Ca^{2+} may be another specific action of this class of compounds and need not be related to other insulin-like actions, such as activation of the Na^+-K^+-ATPase.

The possibility exists that troglitazone may enhance the actions of NO-producing agents. In preliminary experiments, troglitazone had a greater rather than additive effect on nitroprusside-induced relaxation of denuded blood vessels (data not shown). Similarly, NO-mediated vascular relaxation by insulin and IGF-I (34-43) may be increased by troglitazone, not by direct stimulation of NO production by endothelial cells but by enhancement of endothelial-derived NO action on VSMCs. The thiazolidinediones may also regulate inducible nitric oxide synthase (iNOS) in either endothelial cells or VSMC. In this regard, we have recently reported that IGF-I stimulates both iNOS expression and NO production by VSMC (53). This possibility needs to be addressed in future studies because it will likely play a role in the long-term effects of these agents on vascular tone and growth.

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