

# Synergistic actions of insulin and troglitazone on contractility in endothelium-denuded rat aortic rings

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**Goud, Chetan, Bertram Pitt, R. Clinton Webb, and Joyce M. Richey.** Synergistic actions of insulin and troglitazone on contractility in endothelium-denuded rat aortic rings. *Am. J. Physiol.* 275 (*Endocrinol. Metab.* 38): E882–E887, 1998.—Insulin attenuates vascular contraction via inhibition of voltage-operated  $\text{Ca}^{2+}$  channels and by enhancement of endothelium-dependent vasodilation. Thus it has been suggested that hypertension-associated insulin resistance results from an insensitivity to the hormone's effects on vascular reactivity. This hypothesis has been strengthened by reports that thiazolidinediones, a class of insulin-sensitizing agents, lower blood pressure and improve insulin responsiveness in hypertensive, insulin-resistant animal models. We tested the hypothesis that troglitazone enhances the vasodilating effect of insulin via inhibition of voltage-operated  $\text{Ca}^{2+}$  channels in vascular smooth muscle cells. Rat thoracic aortic rings (no endothelium) were suspended in tissue baths for isometric force measurement. Rings were incubated with 0.1 DMSO vehicle (control), troglitazone ( $10^{-5}$  M), insulin ( $10^{-7}$  U/l), or both troglitazone and insulin (1 h) and then contracted with phenylephrine (PE), KCl, or BAY K 8644. Troglitazone increased the  $\text{EC}_{50}$  values for PE and KCl. Contractions to BAY K 8644 in troglitazone-treated rings were virtually abolished. Insulin alone had no effect on contraction. However, when insulin was combined with troglitazone, the  $\text{EC}_{50}$  values for PE and KCl were further increased. Additionally, the maximum contractions to both PE ( $14 \pm 4\%$  of control) and KCl ( $12 \pm 2\%$  of control) were reduced. Measurement of  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]$ ) with fura 2-AM in dispersed vascular smooth muscle cells indicated that neither insulin nor troglitazone alone altered PE-induced increases in intracellular  $[\text{Ca}^{2+}]$ . However, troglitazone and insulin together caused a significant reduction in PE-induced increases in intracellular  $[\text{Ca}^{2+}]$  (expressed as percentage of preincubation stimulation to PE:  $47 \pm 10\%$ , treated;  $102 \pm 13\%$ , vehicle). These results demonstrate that troglitazone inhibits  $\text{Ca}^{2+}$  influx and that it acts synergistically with insulin to attenuate further vascular contraction via inhibition of voltage-operated  $\text{Ca}^{2+}$  channels.

vascular smooth muscle; calcium channels

CLINICAL AND EPIDEMIOLOGICAL studies have provided evidence that a correlation exists between insulin resistance and essential hypertension (2, 3, 14). This association has prompted investigations into the role of insulin in the modulation of blood pressure. Investigators have demonstrated that insulin can directly alter activity of isolated cells by acting on transmembrane cation transporters, such as the  $\text{Ca}^{2+}$ -ATPase,  $\text{Na}^+$ - $\text{K}^+$ -

ATPase,  $\text{Na}^+$ / $\text{H}^+$  antiport, and  $\text{Ca}^{2+}$ / $\text{Na}^+$  exchange systems (9, 10, 13, 22, 29). Several studies have also shown that insulin can directly attenuate vasoconstrictor responses of vascular smooth muscle via inhibition of voltage-operated  $\text{Ca}^{2+}$  channels (22, 25), as well as by enhancing endothelium-dependent vasodilation through the nitric oxide (NO)-guanosine 3',5'-cyclic monophosphate pathway (20, 23, 27). Recently, studies have suggested that insulin resistance is involved in the development and maintenance of hypertension in such animal models as the obese Zucker rat (3, 30), fructose-fed rat (17), and spontaneously hypertensive rat (SHR; 7, 12). Thus many investigators believe that a resistance to the vasodilating effects of insulin may lead to the development of hypertension (18, 21). This hypothesis has been strengthened by reports that thiazolidinediones, a class of insulin-sensitizing agents, lower blood pressure in addition to improving insulin responsiveness in hypertension (1, 5). More recently, pioglitazone and troglitazone, two related insulin-sensitizing agents, were demonstrated to inhibit vascular smooth muscle contraction, as well as reduce L-type voltage-gated  $\text{Ca}^{2+}$  current in A7r5 vascular smooth muscle cells (11, 21, 28). The current study was performed to test the hypothesis that the insulin-sensitizing agent troglitazone can enhance the vasodilating effects of insulin via inhibition of voltage-gated  $\text{Ca}^{2+}$  channels in vascular smooth muscle cells. To eliminate the potential involvement of endothelium-derived factors (18, 20, 23), we conducted these studies in endothelium-denuded segments of rat aorta.

## METHODS

The methods and procedures described in the present report were reviewed by the animal protocol review committee of the University of Michigan Medical School and are in accordance with institutional guidelines.

**Isolated tissue bath protocol.** Adult male Sprague-Dawley rats (weight, 275 g; Harlan Industries and Charles Rivers) were anesthetized with pentobarbital sodium (50 mg/kg ip) and exsanguinated. Thoracic aortas were removed and placed into cold physiological salt solution (PSS; in mmol/l: 130 NaCl, 4.7 KCl, 1.6  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ , 1.18  $\text{KH}_2\text{PO}_4$ , 1.17  $\text{MgSO}_4$ , 5.5 dextrose, 14.9  $\text{NaHCO}_3$ , and 0.03  $\text{CaNa}_2\text{EDTA}$ ). The vessels were cleaned of connective tissue and cut into 4-mm cylindrical segments under a dissecting microscope. The endothelium was removed from the arterial ring preparations by cannulating the lumen with microforceps and gently rolling the vessel between the forceps and palm. Finta et al. (4) have previously reported that this rubbing procedure removes at least 95% of the endothelium. The absence of endothelium was confirmed by lack of a response to the endothelium-dependent vasodilator acetylcholine ( $10^{-6}$  mol/l) in rings contracted with phenylephrine (PE;  $5 \times 10^{-8}$  mol/l).

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**Muscle bath experiments.** Vessel segments were mounted in 50-ml jacketed organ baths containing PSS after the lumen was cannulated with two wire hooks; one hook was fastened to a stationary stainless steel rod, the other to an isometric force transducer. PSS was maintained at 37°C and aerated with 95% O<sub>2</sub>-5% CO<sub>2</sub> throughout the experiment. Rings were placed under optimal resting tension (passive tension placed on tissue that results in maximal isometric performance, determined as 4 g for aortic rings) and equilibrated for ~60 min with washing. Tissues were incubated with indomethacin ( $5 \times 10^{-6}$  mol/l) during the incubation period in order to block cyclooxygenase activity. After the equilibration period, drugs were added directly to the muscle bath. Tissues were incubated with insulin and/or troglitazone for 1 h before evaluation of contractile activity. These agents remained in the bath while the aortic rings were contracted in a cumulative fashion with PE, the depolarizing agent KCl, or the voltage-gated Ca channel agonist BAY K 8644. Isometric tension was measured as grams of force and then normalized to maximal contraction to either PE or KCl.

**Isolation of vascular smooth muscle cells and fura 2-AM loading.** Single vascular smooth muscle cells were isolated via enzymatic dispersion from arteries removed from rats according to the methods of Wilde et al. (26) as modified by Tostes et al. (24). Briefly, thoracic aortas were quickly excised from pentobarbital-anesthetized rats (50 mg/kg, ip) and placed into 0.1 mmol/l Ca<sup>2+</sup> Hanks' balanced salt solution containing (in mmol/l) 140 NaCl, 5.4 KCl, 0.44 KH<sub>2</sub>PO<sub>4</sub>, 0.42 NaH<sub>2</sub>PO<sub>4</sub>, 4.17 NaHCO<sub>3</sub>, 0.026 CaNa<sub>2</sub>EDTA, 0.10 CaCl<sub>2</sub>·H<sub>2</sub>O, 5.0 HEPES, and 5.5 dextrose (pH 7.35). Vessels were placed into 5 ml of 0.1 mmol/l Ca<sup>2+</sup> Hanks' PSS containing 0.05 g bovine serum albumin (type I), 5 mg soybean trypsin inhibitor, 5 mmol/l taurine, 2 mg dithiothreitol, 3 mg type I collagenase, and 3 mg papain (all from Sigma, St. Louis, MO). Vessels were incubated in a shaking water bath at 37°C for 45 min. After incubation, vessel fragments were removed by pipette and placed in 15 ml plastic capped tubes and resuspended in 0.1 mmol/l Ca<sup>2+</sup> Hanks' solution containing albumin, trypsin inhibitor, and taurine as described above. The aortic fragment was then loaded with the intracellular, fluorescent Ca<sup>2+</sup> indicator fura 2 by incubation with the membrane-permeant, acetoxymethyl ester form, fura 2-AM, at a concentration of 5 μmol/l for 60 min. Excess fura 2 was removed from the cell suspension by washing the tissue with 0.1 mmol/l Ca<sup>2+</sup> Hanks' balanced salt solution.

Vascular smooth muscle cells were released by gentle pipette agitation. Loaded cells were allowed 30 min for complete cytosolic deesterification of the fura 2-AM. Small aliquots of fura 2-loaded cells were placed on coverslips in a controlled-atmosphere (95% O<sub>2</sub>-5% CO<sub>2</sub>), controlled-temperature (37°C) superfusion chamber. This bath was positioned on the stage of a Leitz Diavert inverted microscope. The superfusion system delivered the PSS containing Ca<sup>2+</sup> (1.6 mmol/l) at a rate of 3 ml/min.

**Measurement of intracellular Ca.** Intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]) was monitored by alternately exciting fura 2-AM at 340 and 380 nm. The fluorescence emission, taken as the ratio of emissions at 340 and 380 nm excitation wavelength (340/380 ratio), was recorded. Previous studies have demonstrated the increase in fura 2-AM 340/380 ratio has a linear relationship with the increase in the intracellular free [Ca<sup>2+</sup>] (16, 24, 26). Isolated vascular smooth muscle cells did not exhibit autofluorescence. Maximum and minimum levels for intracellular [Ca<sup>2+</sup>] were determined at the end of each experimental protocol by treating the cells with buffer containing 1.6 mmol/l Ca<sup>2+</sup> with 1 μmol/l ionomycin and 15 mmol/l EGTA (0 mmol/l Ca<sup>2+</sup>). The 340/380 ratios were used to

calculate intracellular free [Ca<sup>2+</sup>] as described previously by Tostes et al. (24). Vascular smooth muscle cells were initially stimulated with PE ( $3 \times 10^{-6}$  mol/l) and after the fura 2-AM 340/380 ratio reached plateau, PE was washed out with fresh PSS. Cells were then incubated with vehicle (0.1% DMSO), troglitazone, insulin, or a combination of troglitazone and insulin. After the incubation period, cells were again stimulated with PE ( $3 \times 10^{-6}$  mol/l). The effects of the various incubations on the fura 2-AM 340/380 ratio in response to PE are expressed as percentage of response to PE before the specific incubation.

**Drugs.** Troglitazone was obtained as a gift from Warner-Lambert Pharmaceuticals (Ann Arbor, MI). BAY K 8644 was purchased from Calbiochem. Bovine pancreatic insulin, indomethacin, PE, acetylcholine, sodium nitroprusside, and all other chemicals were purchased from Sigma. Troglitazone was prepared in DMSO, and BAY K 8644 and indomethacin were prepared in 95% ethanol. All other drugs were prepared in distilled water. Each drug was prepared on the day of the experiment from its powder form or was diluted from a frozen stock solution.

**Statistics.** Data are means ± SE. Statistical analyses were performed by Student's *t*-test. The criterion for statistical significance was  $P \leq 0.05$ . The Bonferroni correction was applied in multiple-testing procedures.

## RESULTS

A number of studies have suggested that insulin can lower [Ca<sup>2+</sup>] and inhibit the contraction of isolated vascular smooth muscle cells (9, 12, 29, 30). Incubation of endothelium-denuded rings of rat thoracic aorta with insulin ( $10^{-7}$  U/l) for 1 h had no effect on the cumulative concentration-response curve to PE ( $10^{-9}$  to  $3 \times 10^{-6}$  mol/l; Fig. 1). However, incubation with troglitazone ( $10^{-5}$  mol/l) significantly shifted the concentration response to PE downward and to the right at concentrations ranging from  $3 \times 10^{-9}$  to  $3 \times 10^{-8}$  mol/l compared with control (incubated with 0.1% DMSO vehicle) and insulin-incubated tissues. These results suggest that troglitazone attenuates contractile activity mediated by receptor activation with the α-adrenergic agonist (Fig. 1). The addition of insulin combined with troglitazone further inhibited PE-induced contractions at a much broader range of concentrations ( $3 \times 10^{-9}$  to  $3 \times 10^{-6}$  mol/l). In a separate set of experiments, a concentration-dependent relationship between troglitazone and inhibition of PE-mediated contraction was established by varying troglitazone ( $10^{-6}$  to  $10^{-5}$  mol/l) while maintaining insulin at  $10^{-7}$  U/l. The EC<sub>50</sub> values for PE (concentration required to reach half-maximal response;  $-\log$  mol/l) were increased for tissues incubated with troglitazone compared with the control and insulin-incubated aortic rings. At  $10^{-6}$  mol/l,  $3 \times 10^{-6}$  mol/l, and  $10^{-5}$  mol/l troglitazone, the EC<sub>50</sub> ( $-\log$  mol/l) values were  $7.45 \pm 0.12$ ,  $7.07 \pm 0.06$ , and  $6.29 \pm 0.10$ , respectively. Control and insulin-incubated tissues had similar EC<sub>50</sub> values of  $7.56 \pm 0.11$  and  $7.26 \pm 0.19$  mol/l, respectively (Fig. 2).

To investigate the changes in [Ca<sup>2+</sup>] paralleling the alterations in vascular reactivity induced by troglitazone and insulin, we used fura 2 epifluorescence microscopy to measure changes in the 340/380 ratio of isolated rat aortic smooth muscle cells. Vascular segments

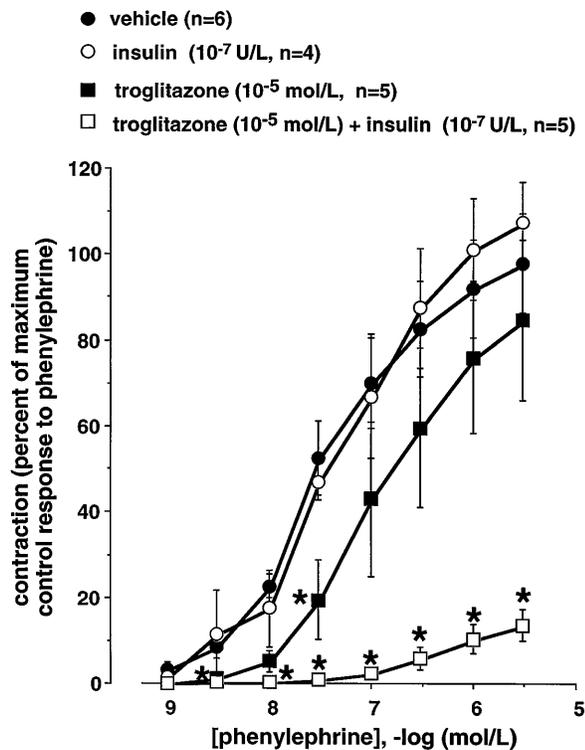


Fig. 1. Troglitazone combined with insulin inhibits contractile responses to phenylephrine (PE) in rat aortic rings. Aortic rings (no endothelium) were made to contract to PE under the following experimental conditions: 1) 0.1% DMSO vehicle (control); 2) incubation with insulin; 3) incubation with troglitazone; and 4) incubation with troglitazone + insulin. PE-induced contractions were reduced only when aortic segments were incubated in combination with insulin and troglitazone;  $n$  = no. of rats. \*Significant change from control,  $P < 0.05$ .

stimulated with PE ( $3 \times 10^{-6}$  mol/l) were shown to result in maximum contractile force production in the isolated tissue bath experiments (Fig. 1), and this concentration of the agonist was used to evaluate changes in the 340/380 ratio. Basal  $[Ca^{2+}]$  levels were  $38.6 \pm 2.2$  nmol/l ( $n = 22$  experiments) and the initial exposure to PE ( $3 \times 10^{-6}$  mol/l) increased  $[Ca^{2+}]$  levels to  $86.5 \pm 6.2$  nmol/l ( $n = 22$ ;  $P < 0.05$ ). After this initial exposure to PE, the isolated smooth muscle cells were washed and then incubated with either vehicle (0.1% DMSO), insulin ( $10^{-7}$  U/l), troglitazone ( $10^{-5}$  mol/l), or a combination of insulin ( $10^{-7}$  U/l) and troglitazone ( $10^{-5}$  mol/l). After these incubations PE ( $3 \times 10^{-6}$  mol/l) was added again, and the magnitude of the change in the 340/380 ratio was measured and expressed as a percentage of the initial exposure to PE (Fig. 3). Exposure to vehicle (0.1% DMSO) did not alter the magnitude of the PE-stimulated increase in intracellular  $[Ca^{2+}]$  ( $101.67 \pm 12.47\%$ ; Fig. 3). Although there was a tendency for insulin and troglitazone alone to reduce the increased level of the 340/380 ratio produced by PE stimulation, these differences were not statistically significant (expressed as a percentage of response to PE before incubation: insulin =  $85.14 \pm 13.77\%$ ; troglitazone =  $83.67 \pm 17.74\%$ ; Fig. 3). However, the increases in the 340/380 ratio for cells stimulated with PE and incubated with both troglitazone and insulin

( $46.75 \pm 10.31\%$ ) were significantly reduced compared with control ( $101.67 \pm 12.4\%$ ;  $P < 0.05$ ; Fig. 3).

To provide a more precise understanding of how troglitazone and insulin may act to modulate vascular reactivity, cumulative concentration-response curves were then performed with KCl. Elevating  $K^+$  provides a means for assessing vasoconstriction mediated by voltage-gated  $Ca^{2+}$  channels. As with PE, insulin had no effect on contractile responses to KCl (Fig. 4). However, troglitazone appeared to be much more effective in shifting the cumulative concentration-response curve for KCl (15 to 100 mmol/l) to the right compared with control than was observed for PE-induced contraction (Fig. 4). Troglitazone in combination with insulin significantly shifted the concentration-response curve to the right compared with control at all concentrations of KCl tested (Fig. 4). Thus troglitazone is more efficacious in reducing contraction induced by voltage-gated  $Ca^{2+}$  channels than those regulated by ligand-receptor complexes. As observed for PE, insulin and troglitazone synergistically inhibited KCl-induced contractile responses.

In order to address the possibility that troglitazone acts specifically on voltage-gated  $Ca^{2+}$  channels, cumulative concentration-response curves were performed to BAY K 8644, a specific agonist for voltage-gated  $Ca^{2+}$  channels. Again, insulin had no effect on contractile activity. Troglitazone almost completely abolished the response to BAY K 8644 ( $3 \times 10^{-8}$  to  $3 \times 10^{-7}$  mol/l) compared with control, providing further evidence that troglitazone depresses contractile activity by acting as an antagonist of voltage-gated  $Ca^{2+}$  channels (Fig. 5).

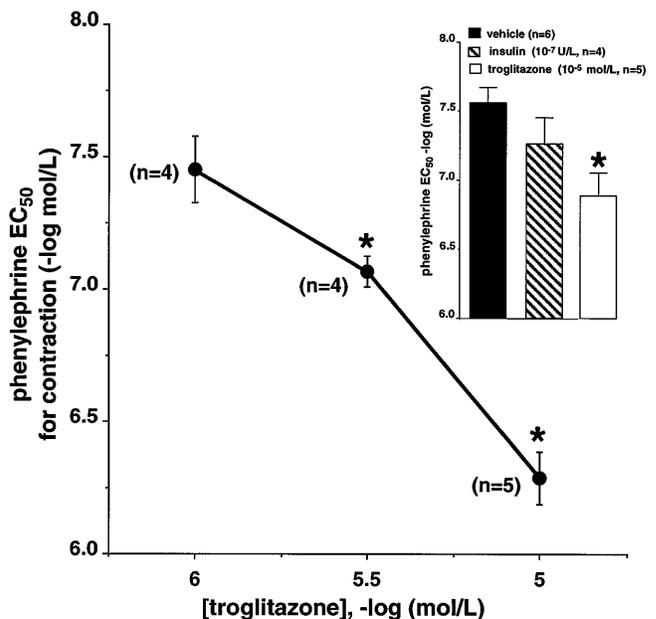


Fig. 2.  $EC_{50}$  values for PE are reduced by troglitazone + insulin. Aortic rings (no endothelium) were made to contract to PE in presence of insulin ( $10^{-7}$  U/l) and 3 different concentrations of troglitazone ( $10^{-6}$  to  $10^{-5}$  mol/l). Troglitazone increased  $-\log EC_{50}$  values for PE in presence of insulin, indicating that insulin-sensitizing drug reduced contractile sensitivity to PE. Inset:  $-\log EC_{50}$  values for PE in aortic strips treated with vehicle alone, insulin alone, and troglitazone alone;  $n$  = no. of rats. \*Significant change from control (vehicle),  $P < 0.05$ .

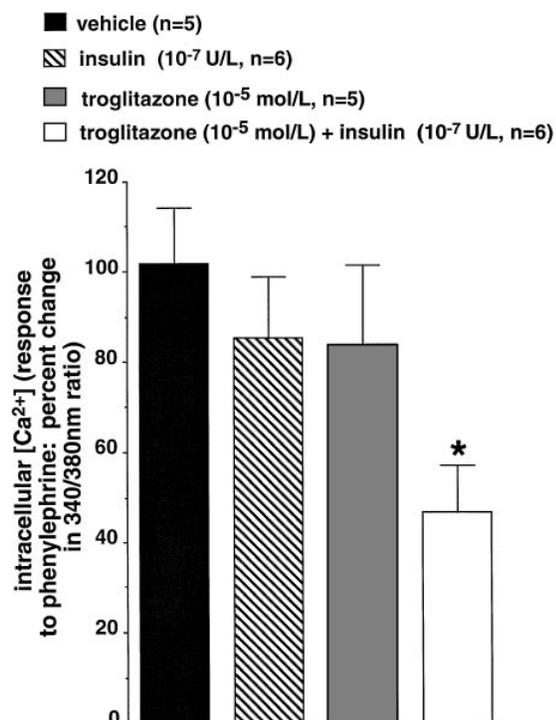


Fig. 3. Troglitazone + insulin inhibits PE-induced increases in intracellular [Ca<sup>2+</sup>]. Isolated smooth muscle cells were stimulated with PE (3 × 10<sup>-6</sup> mol/l) under the following conditions, and intracellular [Ca<sup>2+</sup>] was measured using fura 2 epifluorescence microscopy: 1) 0.1% DMSO vehicle (control); 2) incubation with insulin; 3) incubation with troglitazone; and 4) incubation with troglitazone + insulin. PE-induced increases in intracellular [Ca<sup>2+</sup>] were reduced only when smooth muscle cells were incubated with troglitazone + insulin; *n* = no. of rats. \*Significant change from control, *P* < 0.05.

## DISCUSSION

Whereas a correlation between insulin resistance and hypertension has been observed, the exact nature of this relationship is unclear (2, 14). Insulin has been shown in previous studies to modulate the activity of transmembrane cation transporters such as Ca<sup>2+</sup>-ATPase, Na<sup>+</sup>-K<sup>+</sup>-ATPase, Na<sup>+</sup>/H<sup>+</sup> antiport, and Ca<sup>2+</sup>/Na<sup>+</sup> exchange systems (9, 10, 13, 22, 29). Recent studies have suggested that insulin resistance is involved in the development and maintenance of hypertension in such animal models as the obese Zucker rat (3, 30), fructose-fed rat (17), and SHR (7, 12). This has prompted investigators to hypothesize that hypertension in insulin-resistant individuals is the result of an inability of insulin to directly attenuate vasoconstriction (18, 20, 30). This hypothesis has been strengthened by reports that thiazolidinediones, a class of insulin-sensitizing agents, inhibit contraction of vascular tissue and lower blood pressure in the SHR (1). The current study supports previous reports that the insulin-sensitizing agent troglitazone inhibits contraction of rat vascular smooth muscle via reduction of intracellular Ca<sup>2+</sup>, independent of NO release from the endothelium (21). We also show for the first time that insulin and troglitazone synergistically inhibit contractility in an endothelium-denuded aortic ring preparation.

Previous studies have shown that insulin attenuates the contractility of isolated smooth muscle cells and

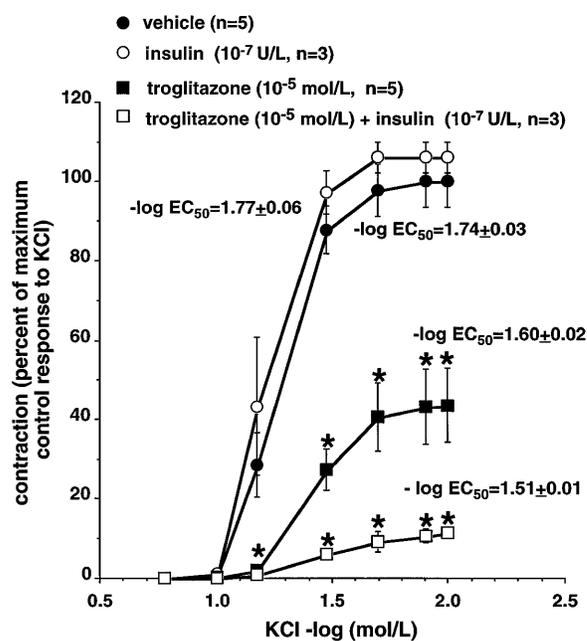


Fig. 4. Troglitazone alone and in combination with insulin inhibits contractile responses to KCl. Aortic rings (no endothelium) were made to contract to KCl under the following experimental conditions: 1) 0.1% DMSO vehicle (control); 2) incubation with insulin; 3) incubation with troglitazone (10<sup>-5</sup> mol/l); and 4) incubation with troglitazone + insulin. KCl-induced contractions were reduced when aortic segments were incubated with troglitazone alone and when incubated with insulin + troglitazone. EC<sub>50</sub> values for KCl (−log mol/l) are indicated; *n* = no. of rats. \*Significant change from control, *P* < 0.05.

intact vessels (9, 22, 27). The vasodilating effects of insulin have been attributed to stimulation of NO production by the endothelium (20, 23, 27) and inhibition of Ca<sup>2+</sup> entry into vascular smooth muscle cells (9). Insulin alone did not have an effect on PE- or KCl-induced contractions in our experiments. The lack of an

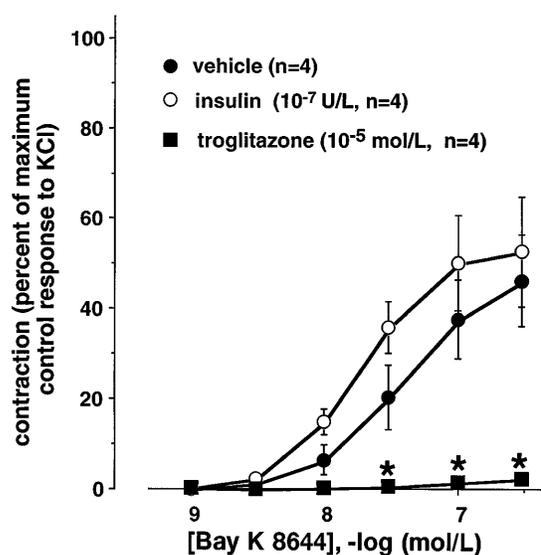


Fig. 5. Troglitazone inhibits contractile responses to BAY K 8644. Aortic rings (no endothelium) were made to contract to BAY K 8644 in presence and absence of either insulin or troglitazone. Insulin did not inhibit contractions to calcium-channel agonist, whereas troglitazone completely inhibited responses to BAY K 8644; *n* = no. of rats. \*Significant change from control (vehicle), *P* < 0.05.

insulin effect on vascular reactivity may be attributed to denudation of the vascular rings. By removing the endothelium, we can assume that production of the endothelium-derived relaxing factor NO or a hyperpolarizing factor is negligible. Thus any attenuation of vascular reactivity mediated by the effects of insulin on synthesis and release of these substances was eliminated. In addition, the lack of an insulin effect on vascular reactivity suggests that the inhibition of  $\text{Ca}^{2+}$  entry into smooth muscle cells by insulin in our aortic ring preparation is not sufficient to reduce contraction. However, our results do support the view that insulin can modulate contractile responses of rat aortic smooth muscle when used in combination with troglitazone.

Previous studies by Zhang et al. (31) demonstrated that pioglitazone, also an insulin-sensitizing agent, may lower blood pressure by its inhibitory effects on current mediated by what is considered a distinct voltage-gated  $\text{Ca}^{2+}$  channel. In our study, troglitazone, which is of the same chemical class as pioglitazone, decreased both the sensitivity to KCl as well as the maximum response. Whereas troglitazone also reduced sensitivity to PE, it did not cause a reduction in maximal force production. Studies by Godfraind et al. (6) suggest that  $\text{Ca}^{2+}$  antagonists are less potent against  $\text{Ca}^{2+}$  entry stimulated by receptor than by high  $\text{K}^+$ -induced depolarization. Because PE-induced activation of  $\alpha$ -adrenoreceptors causes both release of  $\text{Ca}^{2+}$  from intracellular stores as well as influx from the extracellular medium via the phosphoinositide pathway, we would expect this type of contraction to be less sensitive to troglitazone. However, agonist-induced contraction is believed to rely partially on depolarization and activation of potential-operated  $\text{Ca}^{2+}$  channels in the plasma membrane, thus providing a possible explanation for the decrease in sensitivity to PE in the presence of troglitazone (Fig. 1). Although fura 2 measurements did not demonstrate a significant reduction by troglitazone in the increased level of intracellular  $[\text{Ca}^{2+}]$  stimulated by a high concentration of PE ( $3 \times 10^{-6}$  mol/l, a concentration producing maximal force in isolated aortic rings, see Fig. 1), it is possible that the rightward shift in the concentration-response effect for contraction reflects a decrease in intracellular  $[\text{Ca}^{2+}]$  at lower concentrations of PE (Fig. 1). Intracellular  $[\text{Ca}^{2+}]$  is significantly reduced in the presence of both troglitazone and insulin.

To elucidate further the mechanism by which troglitazone and insulin modulate vascular reactivity, we contracted aortic rings in the muscle bath by the cumulative addition of increasing concentrations of KCl. Elevating  $\text{K}^+$  provides a means for assessing contraction mediated by voltage-induced influx of extracellular  $\text{Ca}^{2+}$  through channels in the plasma membrane. To gain a more accurate assessment of the effects of troglitazone on vascular reactivity, we characterized responses to BAY K 8644, a specific agonist of L-type  $\text{Ca}^{2+}$  channels. Responses to BAY K 8644 were almost completely abolished by troglitazone, suggesting that troglitazone acts specifically on voltage-gated  $\text{Ca}^{2+}$  channels. These results are consistent with find-

ings by Song et al. (21) demonstrating that troglitazone inhibits L-type  $\text{Ca}^{2+}$  currents in freshly dissociated rat-tail artery and in aortic and cultured vascular smooth muscle cells.

The fact that insulin alone had no effect on vascular reactivity but in combination with troglitazone produced a profound inhibition of vascular smooth muscle contraction led us to speculate about the possibility of one  $\text{Ca}^{2+}$  channel possessing two different gating mechanisms. We propose that insulin has a small inhibitory effect on gating of the  $\text{Ca}^{2+}$  channel by both agonists and membrane potential, whereas troglitazone potentially inhibits activation of the  $\text{Ca}^{2+}$  channel by voltage. When combined, there is an enhancement of the inhibitory effects of insulin on the  $\text{Ca}^{2+}$  channel, in addition to troglitazone's own antagonizing effects, resulting in attenuated contractile responses to both PE and KCl. Indeed, investigators have been able to modulate the degree of inhibition of norepinephrine-induced contraction by nisoldipine, a voltage-gated  $\text{Ca}^{2+}$  channel antagonist that can distinguish between receptor-mediated and voltage-dependent responses (6).

Another tenable explanation for greater attenuation of aortic contraction in the presence of insulin and troglitazone may be attributed to stimulation of  $\text{Ca}^{2+}$ -ATPase by insulin. Previous studies by Zemel et al. (29) provide evidence that insulin increases  $\text{Ca}^{2+}$  efflux as the rate of spontaneous relaxation after PE washout. Whereas the increase in  $\text{Ca}^{2+}$ -ATPase activity caused by insulin alone may not be sufficient to shift the response curves to KCl or PE, troglitazone may facilitate the effects of insulin, thus providing an explanation for the greater inhibition of contractile activity observed in the presence of troglitazone and insulin.

Stimulation of the action of vasodilating factors by troglitazone may provide an alternative mechanism for the synergism with insulin. Itoh et al. (8) demonstrated the presence of  $\gamma$ -gene transcripts for peroxisome proliferator-activated receptors (the purported target receptors for thiazolidinediones) in rat cultured vascular smooth muscle cells. Troglitazone was found to cause a significant increase of basal secretion of an endothelium-derived relaxing factor (19). Because the current study was conducted in endothelium-denuded aortic rings, it is unlikely that troglitazone increased an endothelium-derived relaxing factor. However, it is conceivable that troglitazone resulted in the production of a smooth muscle relaxing factor. Consistent with this observation, Muniyappa et al. (15) have provided evidence that insulin-like growth factor 1 can increase arterial smooth muscle NO production. The notion that insulin and troglitazone activated transcription factors to induce NO production at the level of the smooth muscle cell is doubtful considering the relatively short exposure time (1 h).

In summary, we have demonstrated that the insulin-sensitizing agent troglitazone decreases contractile responses of endothelium-denuded rat aortic rings to both agonist-mediated and depolarization-induced contraction via reduction of intracellular  $[\text{Ca}^{2+}]$ . Furthermore, insulin and troglitazone have synergistic effects

on attenuating vascular reactivity. These data support the notion that resistance to the direct effects of insulin on the vasculature may be an important contributor to the development of hypertension in insulin resistant states.

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