Mechanisms of Vasorelaxation Induced by Troglitazone, a Novel Antidiabetic Drug, in the Porcine Coronary Artery

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Background—Troglitazone (TRO), a novel antidiabetic drug, has been reported to decrease blood pressure and relax vascular strips. The mechanism of relaxation induced by TRO was determined in terms of 

Ca\(^{2+}\) signaling in smooth muscle cells.

Methods and Results—Front-surface fluorometry and fura 2–loaded medial strips of porcine coronary artery were used to examine the effects of TRO on cytosolic 

Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)\(_i\)]) and contractions. The sustained contraction induced by 100 nmol/L U46619 was similar to that induced by 60 mmol/L K\(^{+}\) depolarization (60K\(^{+}\)). TRO concentration dependently decreased [Ca\(^{2+}\)\(_i\)], and the force of these contractions. The concentration of TRO required to induce 50% inhibition of U46619-induced force (2.9 μmol/L) was significantly lower than that required in the case of 60K\(^{+}\)-induced force (7.3 μmol/L). Replacing extracellular Ca\(^{2+}\) with Mn\(^{2+}\) gradually quenched fluorescence at 360 nm excitation. This decline was accelerated by 100 nmol/L U46619 and 30K\(^{+}\) to a similar extent, indicating a similar activation of Ca\(^{2+}\) influx. TRO completely inhibited U46619-activated influx but partly inhibited depolarization-activated influx. Cumulative applications of extracellular Ca\(^{2+}\) during stimulations with U46619 or 118K\(^{+}\) induced stepwise increases in [Ca\(^{2+}\)\(_i\)], and force. TRO shifted the [Ca\(^{2+}\)\(_i\)]-force relation to the right during both stimulations.

Conclusions—TRO relaxes coronary artery by decreasing [Ca\(^{2+}\)\(_i\)] and Ca\(^{2+}\) sensitivity of contractile apparatus. Inhibition of Ca\(^{2+}\) influx was important in decreasing [Ca\(^{2+}\)\(_i\)]. TRO more effectively inhibits receptor-operated Ca\(^{2+}\) influx than voltage-operated Ca\(^{2+}\) channels. (Circulation. 1998;98:2446-2452.)

Key Words: vasodilation ■ calcium channels ■ diabetes mellitus ■ fura 2

Troglitazone (TRO), (±)-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-ylmethoxy) benzyl]-2,4-thiazolidinedione, one of the thiazolidinediones, is a newly developed antidiabetic drug.1 Kellerer et al2 reported that TRO improves the response of fibroblasts to insulin impaired by high glucose and restores autophosphorylation of insulin receptors. Besides its antidiabetic effects, TRO was found to decrease blood pressure.3,4 It was reported that TRO inhibited L-type Ca\(^{2+}\) channels of smooth muscle and induced relaxation of arterial strips without endothelium.5 Pioglitazone, another member of the thiazolidinediones, was shown to have similar vasorelaxing effects.6 However, the mechanism of vasorelaxation induced by the thiazolidinediones has yet to be determined, especially in terms of Ca\(^{2+}\) signal transduction in smooth muscle.

In the present study, the mechanisms of vasorelaxation induced by TRO was elucidated by measuring the effects of TRO on cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) and force in fura 2–loaded porcine coronary arterial medial strips. A Mn\(^{2+}\) quenching technique was used to directly evaluate the inhibitory effects of TRO on Ca\(^{2+}\) influx.

Methods

Preparation of Strips of Porcine Coronary Artery and Fura 2 Loading

Left circumflex coronary arteries (20 to 30 mm from the origin) were isolated from fresh porcine hearts at a local slaughterhouse. The adventitia was trimmed away, and the endothelium was removed by rubbing it with a cotton swab. Then, the segments were cut into strips (1×5×0.1 mm). The functional loss of the endothelium was confirmed by the lack of relaxation by 1 μmol/L bradykinin.

The strips were incubated with 25 μmol/L fura 2 acetoxymethyl ester (fura 2-AM) in Dulbecco’s modified Eagle medium containing 5% fetal bovine serum for 4 hours at 37°C. After loading with fura 2, the strips were equilibrated in normal physiological salt solution (PSS) for at least 1 hour at 37°C. Loading the strips with fura 2, per se, did not affect contractility.2,3

Simultaneous Measurement of [Ca\(^{2+}\)\(_i\)] and Force

The fura 2–loaded strips were mounted vertically in a quartz organ bath, and the isometric tension was measured as described.3 The resting load was adjusted to 300 mg. The responsiveness of each strip to 118 mmol/L K\(^{+}\) PSS was recorded as reference. The values of developed force in 5.9 mmol/L K\(^{+}\) PSS (at rest) and at steady state of contraction induced by 118 mmol/L K\(^{+}\) were assigned to be 0% and 100%, respectively.
Changes in the fluorescence intensity of the fura 2–loaded strips were monitored with a front-surface fluorometer (CAM-OF3, Japan Spectroscopic Co), as previously described. The fluorescence ratio was monitored as an indicator of [Ca\(^{2+}\)](i). The absolute values of [Ca\(^{2+}\)](i) for 0% and 100% levels were estimated in separate measurements to be 108±27 and 715±103 nmol/L (n=10), respectively. These [Ca\(^{2+}\)](i) values are considered to be an approximation of the true [Ca\(^{2+}\)](i) value. Therefore, a statistical analysis of the [Ca\(^{2+}\)](i) signal was performed with the percent values.

**Mn\(^{2+}\) Quenching Protocol**

To examine the effect of TRO on the Ca\(^{2+}\) influx, Mn\(^{2+}\) quenching protocol was used. In the protocol, the fluorescence intensity at 360 nm excitation (F360) was monitored. After exposing strips to Ca\(^{2+}\)-free PSS, 0.3 mmol/L Mn\(^{2+}\) was added, which induced a gradual decline of F360 (Mn\(^{2+}\) quenching). The declines of F360 during stimulation with U46619 or K\(^{+}\) depolarization were examined in the absence and presence of 10 μmol/L TRO. After 20 minutes' recording of the Mn\(^{2+}\) quenching, strips were exposed to 1 μmol/L ionomycin in the presence of Mn\(^{2+}\) for 10 minutes to obtain a minimal level of F360, which was determined to be comparable to autofluorescence level. The fluorescence intensity was expressed as a percentage, assigning the values obtained in 5.9 mmol/L K\(^{+}\) and in 118 mmol/L K\(^{+}\) PSS to be 0% and 100%, respectively. In all experiments, strips were pretreated with 1 μmol/L thapsigargin for 10 minutes before the addition of Mn\(^{2+}\) to avoid any possible interference by Ca\(^{2+}\) store sites. The pretreatment with thapsigargin had no effect on the basal Mn\(^{2+}\) influx. The F360 decline during the initial 5 minute period was fitted to the exponential decline curve with the following formula:

\[
F = F_0 e^{-t/\tau}
\]

where \(F_0\) is the F360 value at the beginning of Mn\(^{2+}\) quenching, \(t\) is time in minutes, and \(\tau\) is a time constant indicating the time required for F360 to decline by 1/e.

**Drugs and Solutions**

The composition of normal PSS was as follows (mmol/L): NaCl 123, KCl 4.7, NaHCO\(_3\) 15.5, KH\(_2\)PO\(_4\) 1.2, MgCl\(_2\) 1.2, CaCl\(_2\) 1.25, and d-glucose 11.5. High-K\(^{+}\) PSS was prepared by replacing equimolar NaCl with KCl. PSS was bubbled with 95% O\(_2\) and 5% CO\(_2\). Fura 2-AM was purchased from Dojindo. Bovine serum albumin and thapsigargin were purchased from Sigma. TRO (C\(_{24}\)H\(_{27}\)NO\(_5\)S, MW 441.55, dissolved in DMSO) was donated by Sankyo Co (Tokyo, Japan). U46619 (9,11-dideoxy-9a,11e-methanooxy prostaglandin F\(_{2a}\)) was purchased from Funakoshi.

**Data Analysis**

The values were expressed as mean±SEM. Student’s t test was used to determine statistical significance, and ANOVA was used to determine the concentration-dependent effect of TRO. The significant differences in curves such as concentration-response curves and the [Ca\(^{2+}\)](i)-force relation curves were determined by ANCOVA. P<0.05 were considered to have statistical significance. All data were collected by the use of a computerized data acquisition system (MacLab, Analog Digital Instruments).

**Results**

**Effects of TRO on [Ca\(^{2+}\)](i) and Force of Coronary Arterial Strips During U46619- and K\(^{+}\)-Induced Contraction**

Figure 1a and 1b shows the representative recordings of 10 μmol/L TRO-induced decreases in the [Ca\(^{2+}\)](i), and force during contractions induced by 100 mmol/L U46619 and 60 mmol/L K\(^{+}\), respectively. The application of U46619 induced a rapid increase in [Ca\(^{2+}\)](i), which reached a peak in 5 minutes (74.0±4.4%, n=5), then slightly decreased to a steady level within 15 minutes (64.4±2.5%, n=5), and was subsequently sustained at similar level (60.6±1.7%, n=5 at 75 minutes). Similarly, force rapidly developed and reached a plateau level within 15 minutes (84.7±3.9%, n=5) and was sustained at this level (81.6±3.4%, n=5 at 75 minutes). The sustained elevation of [Ca\(^{2+}\)](i) was not observed in the absence of extracellular Ca\(^{2+}\) (Figure 5a). TRO was applied 15 minutes after the application of U46619, which rapidly decreased the level of both [Ca\(^{2+}\)](i) and force (Figure 1a). By 30 minutes, 10 μmol/L TRO decreased both [Ca\(^{2+}\)](i) and force to 0%. The responses to 118 mmol/L K\(^{+}\) and U46619 were completely restored after TRO was washed out (data not shown).

The stimulation with 60 mmol/L K\(^{+}\) also induced rapid and subsequent sustained increases in [Ca\(^{2+}\)](i) and force, which were maintained for more than 70 minutes. The levels of
[Ca\textsuperscript{2+}], and force were 98.4±2.6% and 93.7±2.1% at 10 minutes, and 91.7±3.3% and 96.7±2.7% (n=3) at 70 minutes, respectively (Figure 1b, 1c, and 1d). Thus, although the [Ca\textsuperscript{2+}], level at the sustained phase obtained with 60 mmol/L K\textsuperscript{+} was significantly higher than that obtained with U46619, the extent of force development was similar. When 10 μmol/L TRO was applied at 10 minutes after stimulation with 60 mmol/L K\textsuperscript{+} (sustained phase), both [Ca\textsuperscript{2+}], and force decreased. Contrary to the case of U46619-induced contraction, 10 μmol/L TRO only partially inhibited the [Ca\textsuperscript{2+}], (40.9±3.7%, n=4) and force (23.4±2.3%, n=4) at 60 minutes after application (Figure 1b). Figure 1c and 1d show the concentration-dependent inhibition of [Ca\textsuperscript{2+}], and force, respectively, by TRO obtained at 60 minutes after application during contractions induced by 100 mmol/L U46619 and 60 mmol/L K\textsuperscript{+}. The concentration-response (force) curve obtained with 60 mmol/L K\textsuperscript{+} (EC\textsubscript{50}, 7.26 μmol/L) is located to the right (P<0.005) of that obtained with U46619 (EC\textsubscript{50}, 2.89 μmol/L).

Thirty mmol/L K\textsuperscript{+} induced an increase in [Ca\textsuperscript{2+}], (67.9±2.2%, n=4) that was similar to that induced by 100 mmol/L U46619 (62.1±2.1%, n=5). The [Ca\textsuperscript{2+}], levels at 60 minutes after the application of 10 μmol/L TRO during contractions induced by 30 mmol/L/K\textsuperscript{+} and 100 mmol/L U46619 were 30.0±4.5% (n=4) and −3.2±1.3% (n=5), respectively (P<0.01). Again, TRO only partially inhibited [Ca\textsuperscript{2+}], elevation induced by 30 mmol/L K\textsuperscript{+}.

**Effect of TRO on Ca\textsuperscript{2+} Influx in Porcine Coronary Artery**

Mn\textsuperscript{2+} quenching protocol was used to directly assess the effects of TRO on Ca\textsuperscript{2+} influx.\textsuperscript{12} In the absence of extracellular Ca\textsuperscript{2+}, application of 0.3 mmol/L Mn\textsuperscript{2+} caused a gradual decline of fura 2 fluorescence at 360 nm excitation (F360) without any stimulation, which fell to <50% at ~20 minutes (n=4; Figure 2a, C and 2b, F). This decline indicates a basal passive influx of Mn\textsuperscript{2+} during the resting state. TRO had no effect on this basal decline (data not shown). To examine the effects of TRO on the Mn\textsuperscript{2+} influx induced by U46619 or high K\textsuperscript{+}, strips were stimulated with 100 nmol/L U46619 or 30 mmol/L K\textsuperscript{+} in Ca\textsuperscript{2+}-free PSS, and 0.3 mmol/L Mn\textsuperscript{2+} was subsequently applied. Both 100 mmol/L U46619 (Figure 2a, A) and 30 mmol/L K\textsuperscript{+} (Figure 2b, D) accelerated the F360 decline to a similar extent. During stimulation with U46619 and K\textsuperscript{+}, it took about 7 minutes to obtain a 50% decrease. Ten μmol/L TRO completely inhibited the U46619-induced acceleration of F360 decline (Figure 2a, B), although it only partially inhibited K\textsuperscript{+}-induced acceleration (Figure 2b, E).

The decline of F360 was fitted to an exponential curve and a time constant was determined; 19.0±1.7 (basal decline, n=4), 11.0±1.3 (U46619-accelerated decline, n=4), 22.0±0.85 (U46619-accelerated decline in the presence of TRO, n=4), 9.4±0.62 (K\textsuperscript{+}-accelerated decline, n=4), and 12.7±1.4 (K\textsuperscript{+}-accelerated decline in the presence of TRO n=4). There was no difference in time constants between U46619- and K\textsuperscript{+}-accelerated declines in the absence of TRO. The time constants obtained with U46619 and 30 mmol/L K\textsuperscript{+} in the absence of TRO were both significantly (P<0.05) smaller than those obtained in the presence of 10 μmol/L TRO. The time constant obtained with U46619 in the presence of TRO did not significantly differ from that of the basal decline, whereas the time constant obtained with 30 mmol/L K\textsuperscript{+} in the presence of TRO was significantly smaller (P<0.05).

**Effects of TRO on Increases in [Ca\textsuperscript{2+}], and Force Induced by Histamine**

Histamine (HIS) induced a rapid increase in [Ca\textsuperscript{2+}], with a sharp peak at 30 seconds followed by a sustained elevation that gradually declined and developed force rapidly to a peak at 3 to 5 minutes, followed by a gradual decline, as described\textsuperscript{3, 8} (Figure 3a). When strips were treated with 10 μmol/L TRO 10 minutes before the stimulation with HIS, both [Ca\textsuperscript{2+}], elevation and force were inhibited (Figure 3b). It should be noted that the early rapid phase caused by Ca\textsuperscript{2+}
release (Figure 3c and 3d, peak) and the declining phase (Figure 3c and 3d, 30 minutes and 60 minutes) of [Ca^{2+}]_{i}, elevation and force were inhibited by TRO.

Effects of TRO on Ca^{2+} release from the intracellular store sites were further examined by the use of HIS and caffeine as stimuli to induce two different mechanisms of Ca^{2+} release. In Figure 4, representative recordings show 10 μmol/L HIS- and 20 mmol/L caffeine-induced increases in [Ca^{2+}]_{i}, and force in the absence (Figure 4a and 4e) and presence (Figure 4b and 4f) of 10 μmol/L TRO. When the strip was exposed to Ca^{2+}-free PSS containing 2 mmol/L EGTA, the [Ca^{2+}]_{i} level gradually decreased to reach a steady state after 10 minutes. Subsequent application of HIS or caffeine induced transient increases in both [Ca^{2+}]_{i} and force. The application of TRO at the time of exposure to Ca^{2+}-free PSS had no effect on the decreases in [Ca^{2+}]_{i}, observed in Ca^{2+}-free PSS. However, the transient increases in [Ca^{2+}]_{i} and force induced by HIS were significantly inhibited by TRO in a dose-dependent manner (Figure 4b, 4c, and 4d). TRO, even at 10 μmol/L, had no effect on caffeine-induced increases in [Ca^{2+}]_{i} and force. (Figures 4f, 4g, and 4h).

Effects of TRO on the [Ca^{2+}]_{i}-Force Relationships

In Figure 5, representative recordings show the effect of TRO on the changes in [Ca^{2+}]_{i}, and force induced by cumulative applications of extracellular Ca^{2+} during stimulation with 100 nmol/L U46619 (Figure 5a and 5b) and 118 mmol/L K^{+} (Figure 5d and 5e). When U46619 was applied to the Ca^{2+}-free PSS, there was only a small, if any, transient rise in [Ca^{2+}]_{i}, although there was a rapid and small sustained
development of force (Figure 5a). Subsequent cumulative applications of the extracellular Ca$_2$$^+$ (0 to 1.25 mmol/L) induced a stepwise increase in [Ca$_2$$^+$]$_i$ and force. As shown in Figure 5b, when TRO was added 10 minutes before stimulation with U46619, the small sustained force developed by U46619 in Ca$_2$$^+$$-$free medium was inhibited. The levels of force induced by 100 nmol/L U46619 without and with 3, 6, and 10 µmol/L TRO were 17.7±5.1%, 13.0±1.5%, 7.69±1.7%, and 0.29±0.7%, respectively. The subsequent elevation of [Ca$_2$$^+$], and force induced by the cumulative applications of extracellular Ca$_2$$^+$ was inhibited by TRO. Figure 5c shows [Ca$_2$$^+$]$_i$-force relation curves during the stimulation with U46619 in the absence and presence (3, 6, and 10 µmol/L) of TRO. TRO concentration-dependently shifted the [Ca$_2$$^+$]$_i$-force relation curve to the right. TRO also inhibited the elevation of [Ca$_2$$^+$], and force induced by the cumulative application of extracellular Ca$_2$$^+$ during 118 mmol/L K$^+$ depolarization and shifted the [Ca$_2$$^+$]$_i$-force relation to the right (Figure 5d, 5e, and 5f).

**Discussion**

We found that a novel antidiabetic drug, TRO, relaxed the medial strips of the porcine coronary artery by decreasing [Ca$_2$$^+$], and by shifting the [Ca$_2$$^+$]$_i$-force relation to the right in smooth muscle. The rightward shift of [Ca$_2$$^+$]$_i$-force relation indicated the decrease in Ca$_2$$^+$ sensitivity of the contractile apparatus as a whole. The decrease in [Ca$_2$$^+$] was mainly caused by an inhibition of Ca$_2$$^+$ influx and partly caused by an inhibition of agonist/receptor-mediated Ca$_2$$^+$ release. TRO completely inhibited [Ca$_2$$^+$]$_i$ increase and Mn$_2$$^+$ influx induced by 100 nmol/L U46619, but only partially inhibited those induced by 30 mmol/L K$^+$, although these two stimulations induced similar [Ca$_2$$^+$]$_i$ elevation and Mn$_2$$^+$ influx. This finding suggested that there was a difference in efficacy of TRO in inhibiting Ca$_2$$^+$ influx between stimulation with U46619 and K$^+$ depolarization. On the other hand, although 60 mmol/L K$^+$ induced similar force development to that obtained with 100 nmol/L U46619, TRO inhibited U46619-activated force development more potently than that induced by K$^+$ depolarization. This observation suggests that TRO preferentially inhibits agonist-activated Ca$_2$$^+$ influx and force development.

A voltage-operated L-type Ca$_2$$^+$ channel (VOC) is one of the well-characterized Ca$_2$$^+$ influx pathways in smooth muscle. High K$^+$ solution is considered to activate VOC and induce increases in [Ca$_2$$^+$]$_i$. In the present study, TRO...
inhibited an extracellular Ca\(^{2+}\)-dependent increase in [Ca\(^{2+}\)], induced by high K\(^+\) depolarization, indicating an inhibition of VOC. This finding is consistent with the reports that VOC in smooth muscle was inhibited by TRO.\(^3\) In the present study, however, we found that TRO inhibits Ca\(^{2+}\) influx pathways activated by agonists in preference to VOC.

There are at least 4 mechanisms to be considered for Ca\(^{2+}\) influx induced by agonist in vascular smooth muscle cells. First, some agonists depolarize membrane potential and activate VOC (U46619 depolarized membrane potential of porcine coronary artery\(^15\)). Second, VOC can be activated by intracellular second messengers or by trimeric G proteins activated by agonist.\(^14\) The third mechanism is the so-called receptor-operated Ca\(^{2+}\) channel.\(^15\) The fourth mechanism is the capacitative Ca\(^{2+}\) influx pathway.\(^16\) The observed preferential inhibition of U46619-induced Ca\(^{2+}\) influx suggests that different mechanisms of Ca\(^{2+}\) influx other than VOC are involved in the U46619-induced Ca\(^{2+}\) influx in porcine coronary artery, and that TRO inhibits agonist-activated Ca\(^{2+}\) channels other than VOC. These effects of TRO on Ca\(^{2+}\) influx pathways in smooth muscle are similar to those observed with SKF 96365, which was reported to be an inhibitor of receptor-operated Ca\(^{2+}\) channels, capacitative Ca\(^{2+}\) entry and VOC.\(^17\) There is no similarity between the chemical structure of TRO and that of SKF 96365. The effect of TRO on the agonist-activated Ca\(^{2+}\) influx has to be examined in nonexcitable cells such as endothelial cells that lack VOC, before concluding that TRO inhibits the Ca\(^{2+}\) entry pathways other than VOC.

The present study suggests that TRO inhibits inositol trisphosphate (IP\(_3\))-induced Ca\(^{2+}\) release but not the caffeine-induced one (Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism). The mechanism of this inhibition of IP\(_3\)-induced Ca\(^{2+}\) release remains unidentified. However, it is suggested that the inhibition of IP\(_3\)-induced Ca\(^{2+}\) release may partly be linked to inhibition of agonist-activated Ca\(^{2+}\) influx through the capacitative Ca\(^{2+}\) entry.\(^16\) It is also possible that TRO inhibits either receptor–G protein interaction or phospholipase C, which, then, may inhibit IP\(_3\) production. This possibility remains to be elucidated.

During the relaxations induced by TRO, the [Ca\(^{2+}\)]-force relationships of the contractions were shifted to the right. Furthermore, TRO inhibited the sustained force development accompanied by only a transient [Ca\(^{2+}\)], increase induced by U46619 in the Ca\(^{2+}\)-free media (Figure 5). These findings suggest that TRO decreases Ca\(^{2+}\) sensitivity of the contractile apparatus in the porcine coronary artery. Increases in cAMP or cGMP were shown to be linked to the decrease in Ca\(^{2+}\) sensitivity.\(^7,10,18\) Several kinases, such as protein kinase C, tyrosine kinase, and rhoA-associated kinase, were suggested to increase Ca\(^{2+}\) sensitivity.\(^19 –21\) TRO was shown to improve autophosphorylation of insulin receptor and phosphorylation of insulin receptor substrate 1 in rat fibroblasts impaired by high glucose.\(^8\) It is not known whether TRO has any effect on these phosphorylation-dependent regulations of Ca\(^{2+}\) sensitivity.

The plasma concentration of TRO in healthy volunteers and NIDDM patients reached 2.56 µmol/L and 3.14 µmol/L, respectively.\(^22\) Since IC\(_{50}\) values of TRO to inhibit contrac-

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