Full Paper

Mechanisms Underlying Pioglitazone-Mediated Relaxation in Isolated Blood Vessel

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Abstract. Pioglitazone is a widely used anti-type 2 diabetic drug. Beside its insulin-sensitizing effects, pioglitazone exerts preventive roles against ischemic heart disease. Since one possible explanation is anti-hypertensive action, we examined effects of pioglitazone on contractility of isolated blood vessel. Endothelium-intact [End (+)] or -removed [End (−)] rat aorta is isolated and isometric tension is recorded. In both End (+) and End (−) aorta, pretreatment with pioglitazone ($3 – 10\,\mu$M, 30 min) inhibited noradrenaline (NA) ($1\,$nM $– 1\,\mu$M)-induced contraction. In NA ($100\,$nM)-pre-contracted aorta, pioglitazone ($1 – 10\,\mu$M) directly induced a relaxation. The relaxant effect is higher in End (−) aorta than in End (+) aorta. In End (+) aorta, N^G-nitro-L-arginine methyl ester ($100\,\mu$M) significantly inhibited the relaxation. In End (−) aorta, neither indomethacin nor cimetidine affected the relaxation, but tetraethylammonium ($10\,$mM) inhibited it. Furthermore, the relaxation was significantly inhibited by a voltage-dependent K^+ (K_V)-channel blocker, 4-aminopyridine ($1\,$mM), or an inward rectifying K^+ (K_{IR})-channel blocker, $BaCl_2$ ($1\,$mM). GW9662 ($2\,\mu$M), a blocker of peroxisome proliferator-activated receptor (PPAR)-γ was ineffective against the relaxation. The present study demonstrated that pioglitazone causes PPAR-γ–independent relaxation. While endothelium-dependent relaxation is mediated via nitric oxide, the endothelium-independent one is responsible for smooth muscle K^+ (K_V, K_{IR})-channel opening.

Keywords: diabetes, endothelium, smooth muscle, nitric oxide, potassium channel

Introduction

Thiazolidinedione derivatives (TZDs) such as pioglitazone, troglitazone, and rosiglitazone are anti-type 2 diabetic drugs. Pioglitazone is an insulin sensitizer that promotes glucose metabolism without increasing insulin secretion (1). It is also known that TZDs act as agonists for peroxisome proliferator-activated receptor (PPAR)-γ (2).

Ischemic heart disease is one of the leading causes for increased mortality and morbidity in the developed world. Diabetes, hypertension, and atherosclerosis are identified as primary risk factors for ischemic heart disease. Besides its insulin-sensitizing effects, accumulating evidence indicate that pioglitazone exerts preventive roles against ischemic heart disease (3). One possible explanation might be anti-hypertensive action, since pioglitazone has been reported to lower blood pressure in both humans (4, 5) and experimental animals (6 – 8). However effects of pioglitazone on contractile responses in isolated blood vessel are not fully understood.

In the present study, we examined effects of pioglitazone on contractility of rat aorta and found that pioglitazone induced PPARγ-independent vasodilation, which is caused by endothelium-derived nitric oxide (NO) and opening smooth muscle K_V (voltage-dependent K^+) and/or K_{IR} (inward rectifying K^+) channels.

Materials and Methods

Tissue preparation

Male Wistar rats (175 – 385 g; 5 – 10-week-old) were anesthetized with urethane (1.5 g/kg, i.p.) and euthanized by exsanguinations. The thoracic aorta was isolated.

After removal of fat and adventitia, each aorta was cut into strips approximately 4-mm-wide, 8-mm-long for the measurement of isometric contraction. The endothelium was removed by rubbing the intimal surface with the flat face of a pair of forceps. Removal of the endothelium was confirmed by the lack of relaxation induced by acetylcholine (ACh) (1 μM). Animal care and treatment were conducted in conformity with institutional guidelines of Kitasato University.

Measurement of isometric contraction

The arterial strips were placed in normal physiological salt solution, which contained 136.9 mM NaCl, 5.4 mM KCl, 1.5 mM CaCl$_2$, 1.0 mM MgCl$_2$, 23.8 mM NaHCO$_3$, 5.5 mM glucose, and 1 μM EDTA. The high-K$^+$ (72.7 mM) solution was prepared by replacing NaCl with equimolar KCl. These solutions ware saturated with a 95% O$_2$ – 5% CO$_2$ mixture at 37°C and pH 7.4. Smooth muscle contraction was recorded isometrically with a force-displacement transducer (Nihon Kohden, Tokyo) as described previously (9). Each muscle strip was mounted in a 10-ml organ bath under a resting tension of 1.0 g. After 30-min equilibration, each strip was repeatedly exposed to high-K$^+$ solution until the responses became stable (45 – 60 min). Concentration-response curves were obtained by the cumulative application of pioglitazone to the arteries pre-contracted with noradrenaline (NA) (100 nM).

Statistical analysis

Results are each expressed as the mean ± S.E.M. Statistical evaluation of the data was performed by Student's *t*-test. Comparison in the identical arterial strip was performed by the paired *t*-test, whereas that in the different strips was done by the unpaired *t*-test. Results were considered significant when *P* was less than 0.05. All PD$_2$ values were calculated as the $-\log_{10}$EC$_{50}$ (i.e., that concentration at which the half maximal effect occurred) by sigmoidal curve fitting.

Chemicals

The chemicals used were as follows: EDTA, NA, indomethacin, cimetidine, tetraethylammonium (TEA), glibenclamide, apamin, 4-aminopyridine (4-AP), BaCl$_2$ (Sigma-Aldrich, St. Louis, MO, USA), pioglitazone (Alexis, San Diego, CA, USA), N^G-nitro-L-arginine methyl ester (L-NAME; Dojindo, Kumamoto), charybdotoxin (Peptide Institute, Osaka), GW9662 (Cayman, Ann Arbor, MI, USA), and ACh (Daiichi Pharmaceutical, Tokyo).

Results

Effects of pretreatment with pioglitazone on NA-induced contraction of rat aorta

We first examined effects of pretreatment of rat aorta with pioglitazone (1 – 10 μM) on concentration-force relationship for NA (1 nM – 1 μM). In the endothelium-denuded [End (–)] rat aorta, NA caused concentration-dependent contraction (Fig. 1a, open circle, n = 4). Pretreatment with pioglitazone (1 μM, 30 min) had no effect on the NA-induced contraction (Fig. 1a, closed circle, n = 4). Pretreatment of End (–) aorta with pioglitazone (3 – 10 μM) shifted the concentration-force relationship for NA to the right (PD$_2$ for NA is 8.38 ± 0.10 without pioglitazone and 7.97 ± 0.05 with 3 μM pioglitazone, n = 4, *P*<0.01, Fig. 1b; PD$_2$ for NA is 8.14 ± 0.02 without pioglitazone and 7.82 ± 0.04 with 10 μM pioglitazone, n = 4, *P*<0.01, Fig. 1c). In the endothelium-intact [End (+)] rat aorta, NA caused concentration-dependent contraction (Fig. 1d, open circle, n = 5). Pretreatment with pioglitazone (1 μM, 30 min) had no effect on the NA-induced contraction (Fig. 1d, closed circle, n = 5). Pretreatment of End (+) aorta with pioglitazone (3 – 10 μM) shifted the concentration-force relationship for NA to the right (PD$_2$ for NA is 8.16 ± 0.09 without pioglitazone and 7.89 ± 0.10 with 3 μM pioglitazone, n = 4, *P*<0.05, Fig. 1e; PD$_2$ for NA is 8.06 ± 0.03 without pioglitazone and 7.81 ± 0.04 with 10 μM pioglitazone, n = 4, *P*<0.01, Fig. 1f).

Relaxant effects of pioglitazone on NA- or high-K$^+$– induced precontraction

We next examined whether pioglitazone directly induces relaxation of rat aorta pre-contracted with NA or high-K$^+$. As shown in Fig. 2a, pioglitazone caused concentration-dependent (1 – 10 μM) relaxation in End (–) or End (+) rat aorta pre-contracted with NA (100 nM). Maximal relaxation caused by 10 μM pioglitazone was significantly higher in End (–) (Fig. 2b, open circle, 29.4 ± 0.9%, n = 4) than End (+) aorta (closed circle, 18.5 ± 2.9%, *P*<0.05, n = 4). In contrast, pioglitazone was ineffective to the aorta pre-contracted with high-K$^+$ (72.7 mM) in the absence or presence of endothelium (Fig. 2: c and d, n = 4).

Mechanisms of pioglitazone-induced relaxation in End (+) rat aorta

To explore mechanisms of pioglitazone-induced vasodilation in End (+) rat aorta, we examined effects of pretreatment with NO synthase (NOS) inhibitor, L-NAME (100 μM, 30 min). The pioglitazone (1 – 10 μM)-induced concentration-dependent relaxation was significantly inhibited by the treatment with L-NAME [maxi-
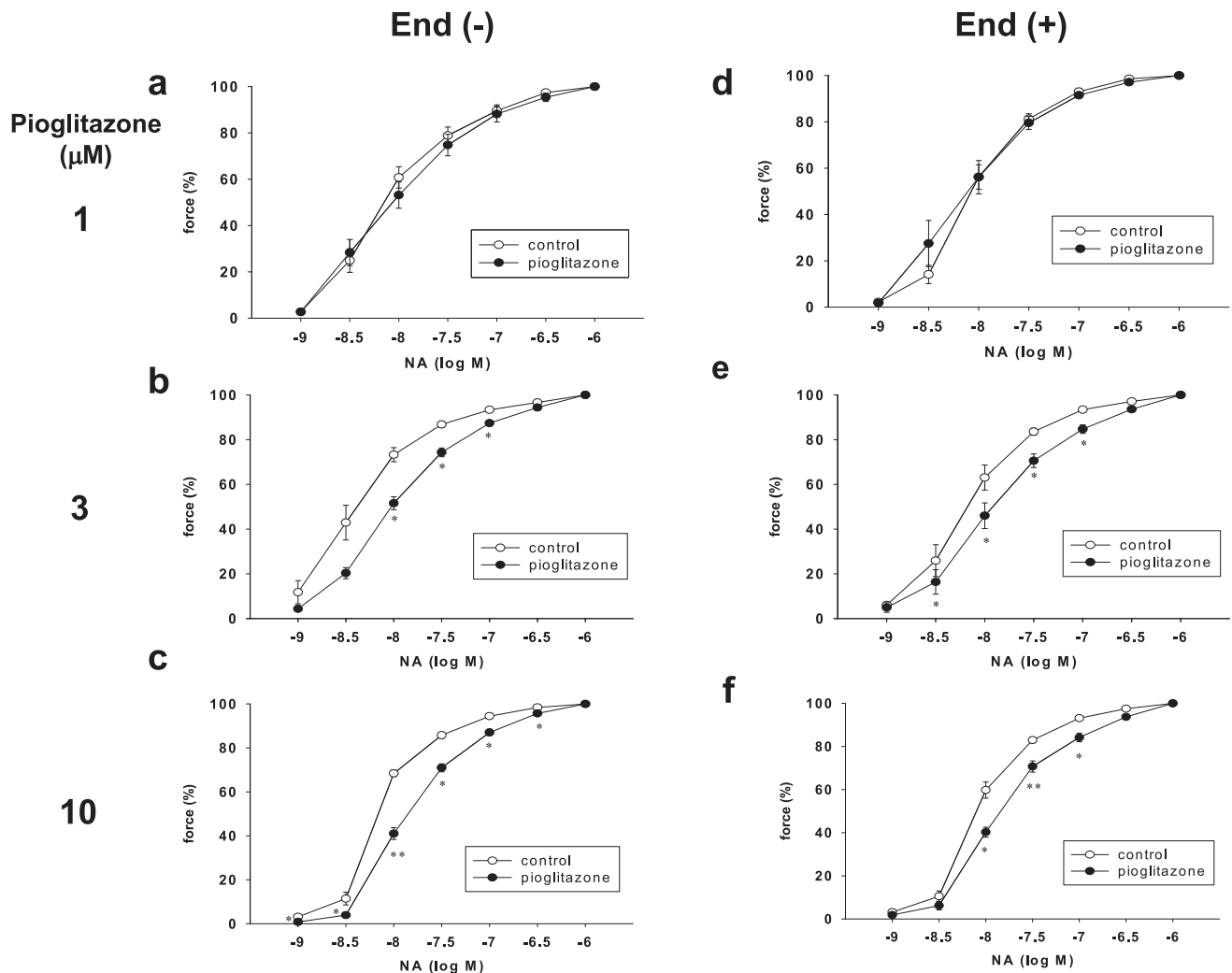
End (−)

End (+)

Fig. 1. Effect of pioglitazone pretreatment on concentration-force relationships for noradrenaline (NA) in an endothelium-denuded [End (−); a, b, c] or -intact [End (+); d, e, f] rat aorta. NA (1 nM – 1 μM) was cumulatively added to the artery. Pioglitazone (1 μM: a, d; 3 μM: b, e; 10 μM: c, f) was added 30 min before the addition of NA. 100% represents NA (1 μM)-induced maximal contraction. Results are each expressed as the mean ± S.E.M. of 4 – 5 experiments. *, **: Significantly different from the control at $P<0.05$ and $P<0.01$, respectively.

mal relaxation caused by 10 μM pioglitazone was 10.1 ± 2.2% in the presence of L-NAME, n = 4 *vs* 37.2 ± 8.8% in the absence of L-NAME (control), n = 4, $P<0.05$, Fig. 3].

Mechanisms of pioglitazone-induced relaxation in End (−) rat aorta

To explore mechanisms of pioglitazone-induced vasodilation in End (−) rat aorta, we examined effects of pretreatment with an inhibitor of cyclooxygenase, indomethacin; a histamine H_2–receptor antagonist, cimetidine; or a non-selective K^+-channel blocker TEA. The pioglitazone (1 – 10 μM)-induced concentration-dependent relaxation was not inhibited by the treatment with either indomethacin (10 μM, 30 min, n = 4, Fig. 4a)

or cimetidine (30 μM, 30 min, n = 4 *vs* control, n = 6, Fig. 4b). In contrast, the relaxation was significantly inhibited by the treatment with TEA (10 mM, 30 min) [maximal relaxation caused by 10 μM pioglitazone was 6.1 ± 3.8% in the presence of TEA, n = 4 *vs* 33.1 ± 3.2% in the absence of TEA (control), n = 4, $P<0.01$, Fig. 4c].

To determine which K^+ channel is contributing to the pioglitazone-induced relaxations in End (−) aorta, we examined effects of the following K^+ channel blockers: glibenclamide [1 μM, a selective ATP-sensitive K^+ (K_{ATP})-channel blocker], charybdotoxin [300 nM, a large conductance Ca^{2+}-activated K^+ (BK_{Ca})-channel blocker], apamin [1 μM, a small conductance Ca^{2+}-activated K^+ (SK_{Ca})-channel blocker], 4-AP [1 mM, a K_V channel blocker] or $BaCl_2$ [1 mM, a K_{IR} channel blocker].
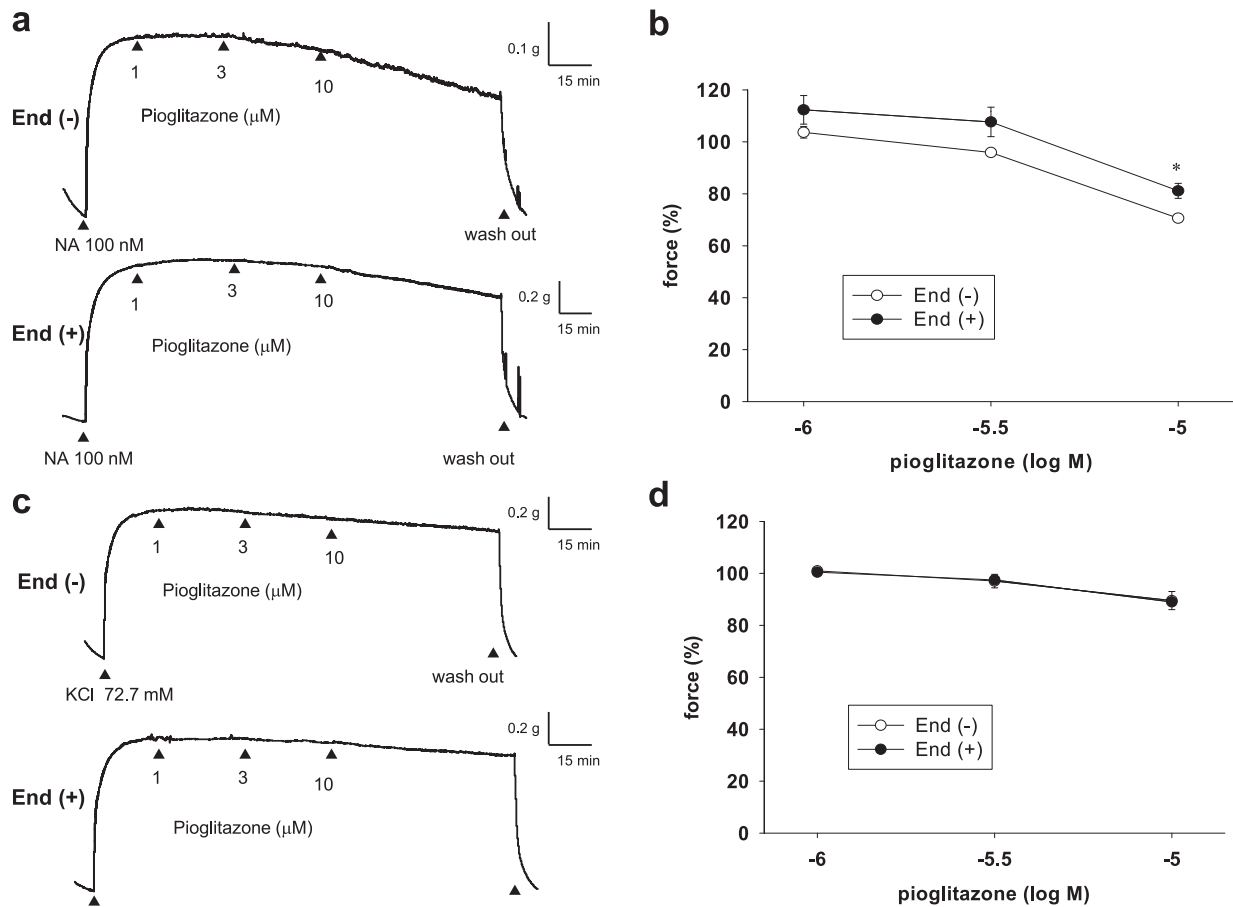
Fig. 2. Representative recordings of the relaxant effect of pioglitazone (1 – 10 μM) in an endothelium-denuded [End (−)] or -intact [End (+)] artery pre-contracted with NA (100 nM) (a) or KCl (72.7 mM) (c). Pioglitazone (1 – 10 μM) was cumulatively added after the contraction induced by NA or KCl had reached a steady state. b and d: Concentration-force relationships for pioglitazone in End (−) or End (+) arteries pre-contracted with NA (b) or KC l (d). 100% represents NA- or KCl-induced pre-contraction. Results are each expressed as the mean ± S.E.M. of 4 experiments. *: Significantly different from End (−) at *P*<0.05.

The pioglitazone (1 – 10 μM)-induced concentration-dependent relaxation was not inhibited by the treatment with either glibenclamide (Fig. 5a, n = 4), charybdotoxin (Fig. 5b, n = 5), or apamin (Fig. 5c, n = 4). In contrast, the relaxation was significantly inhibited by the treatment with 4-AP [relaxation caused by 3 μM pioglitazone was 1.9 ± 0.6% in the presence of 4-AP, n = 5 *vs* 8.5 ± 0.1% in the absence of 4-AP (control), n = 4, *P*<0.01, Fig. 5d] as well as by the treatment with BaCl$_2$ [maximal relaxation caused by 10 μM pioglitazone was 11.9 ± 0.8% in the presence of BaCl$_2$, n = 4 *vs* 28.9 ± 2.7% in the absence of BaCl$_2$ (control), n = 8, *P*<0.01, Fig. 5e].

Effects of PPAR-γ antagonist GW9662 on pioglitazone-induced relaxation

Finally, we examined whether PPAR-γ contributes to the pioglitazone-induced relaxation in rat aorta.

The pioglitazone (1 – 10 μM)-induced concentration-dependent relaxation in either End (−) or End (+) aorta was not inhibited by the treatment with a PPAR-γ antagonist, GW9662 (2 μM, 30 min) [maximal relaxation caused by 10 μM pioglitazone in End (−) was 44.0 ± 12.4% in the presence of GW9662, n = 4 *vs* 29.8 ± 2.1% in the absence of GW9662 (control), n = 4, Fig. 6a; maximal relaxation caused by 10 μM pioglitazone in End (+) was 50.2 ± 5.6% in the presence of GW9662, n = 5 *vs* 44.1 ± 4.3% in the absence of GW9662 (control), n = 4, Fig. 6b].

Discussion

The major findings of the present study are that pretreatment of rat aorta with pioglitazone (3 – 10 μM) decreased the sensitivity of contractile responses to NA. Further, pioglitazone directly induced a relaxation of rat
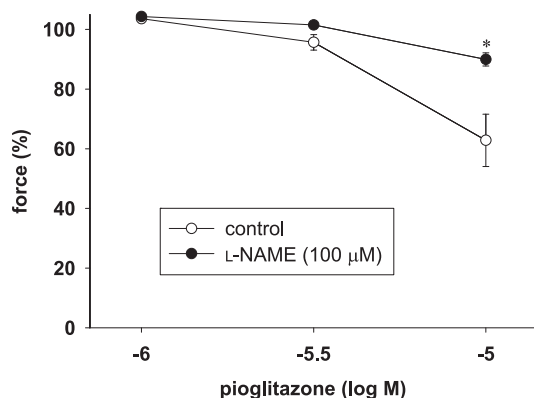
Fig. 3. Concentration-force relationships for pioglitazone in the absence (control) or presence of L-NAME (100 μM). Pioglitazone (1 – 10 μM) was cumulatively added after the contraction induced by NA had reached a steady state. L-NAME (100 μM) was added 30 min before the addition of NA. 100% represents NA (100 nM)-induced pre-contraction. Results are each expressed as the mean ± S.E.M. of 4 experiments. *: Significantly different from the control at $P<0.05$.

aorta pre-contracted with NA but not high-K⁺. The relaxant effect of pioglitazone was independent of PPAR-γ. We determined that pioglitazone-induced

relaxation in End (+) aorta was mediated by NO. In End (−) aorta, the relaxation was not mediated by producing prostacyclin or histamine, but caused by opening smooth muscle K⁺ channels. Specifically, K_V and/or K_{IR} channels are responsible for the pioglitazone-induced relaxation (Fig. 7). Our preliminary data also showed that the inhibitory effects of pioglitazone on smooth muscle contractility were observed in carotid and mesenteric arteries of rats (data not shown), suggesting that the effects of pioglitazone are not specific to the muscular type vessels. To the best of our knowledge, the results are the first demonstration and we clarified the novel effects of pioglitazone on contractility of isolated blood vessels.

Several results regarding the effects of pioglitazone on contractility of isolated blood vessel were previously reported. It was demonstrated in End (+) aorta that pioglitazone (0.1 μM – 10 mM) directly induced a relaxation of rat aorta pre-contracted with phenylephrine, which was inhibited by L-NAME (10). It was also reported that chronic treatment with pioglitazone restored the impaired NO-mediated, endothelium-dependent relaxation in the diabetic rat aorta (11). These
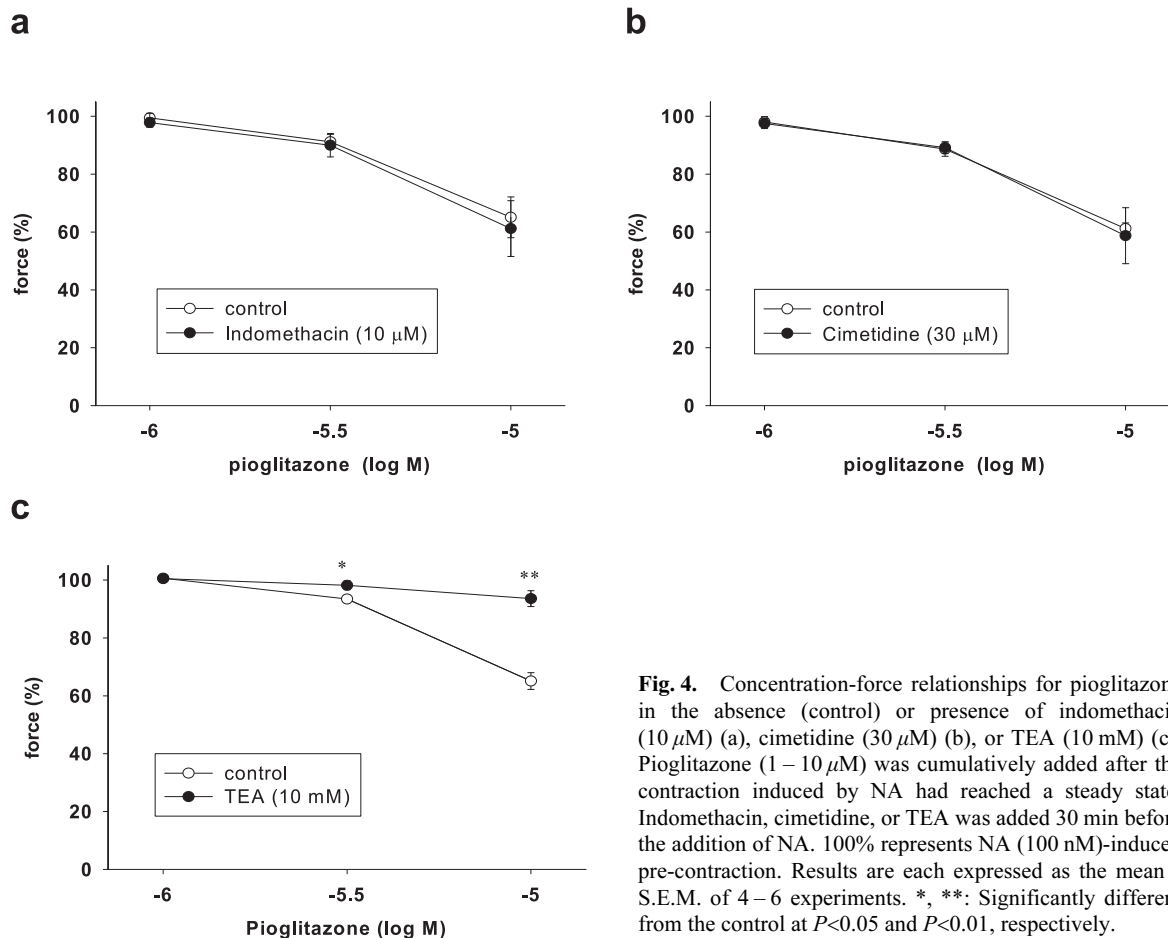
a

b

c

Fig. 4. Concentration-force relationships for pioglitazone in the absence (control) or presence of indomethacin (10 μM) (a), cimetidine (30 μM) (b), or TEA (10 mM) (c). Pioglitazone (1 – 10 μM) was cumulatively added after the contraction induced by NA had reached a steady state. Indomethacin, cimetidine, or TEA was added 30 min before the addition of NA. 100% represents NA (100 nM)-induced pre-contraction. Results are each expressed as the mean ± S.E.M. of 4 – 6 experiments. *, **: Significantly different from the control at $P<0.05$ and $P<0.01$, respectively.
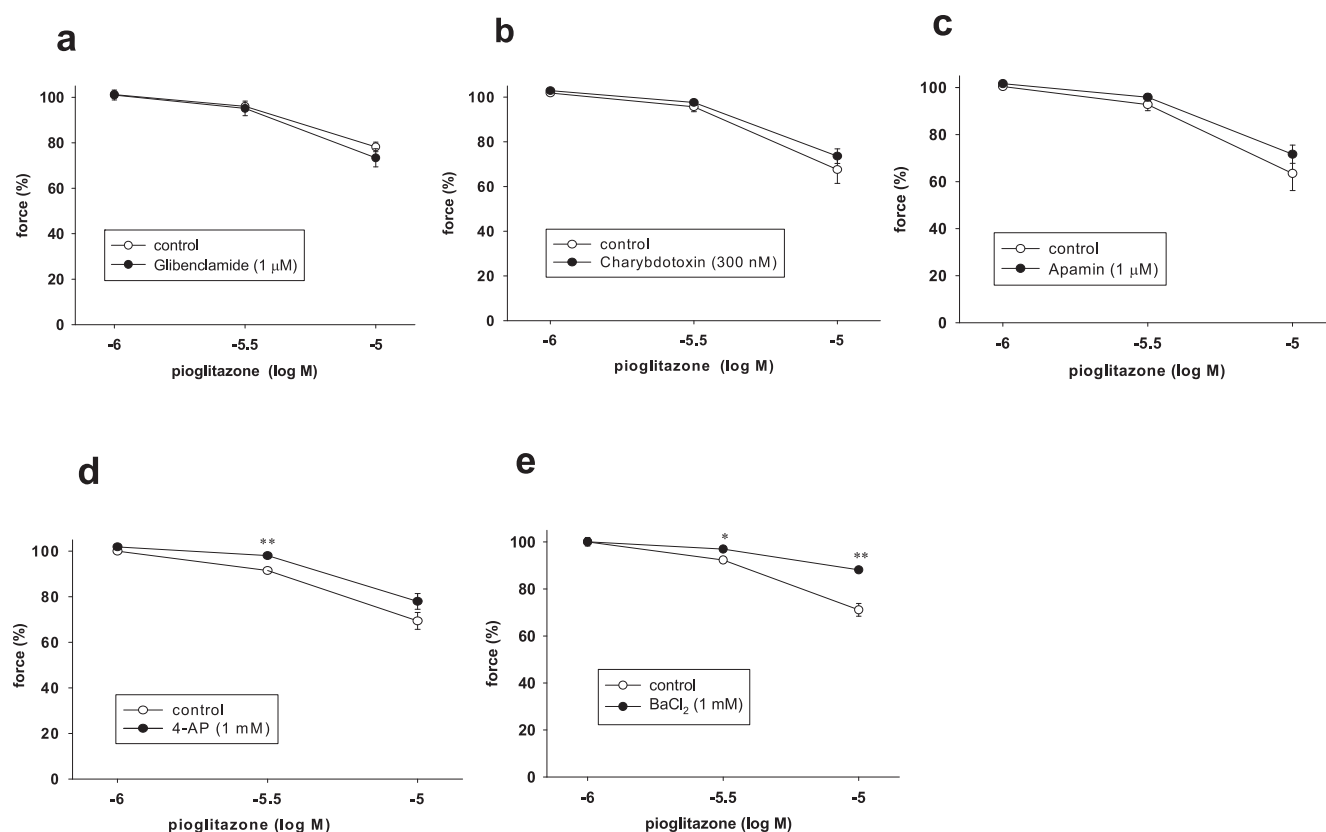
Fig. 5. Concentration-force relationships for pioglitazone in an endothelium-denuded artery pre-contracted with NA in the absence (control) or presence of glibenclamide (1 μM) (a), charybdotoxin (300 nM) (b), apamin (1 μM) (c), 4-AP (1 mM) (d), or BaCl$_2$ (1 mM) (e). Pioglitazone (1 – 10 μM) was cumulatively added after the contraction induced by NA had reached a steady state. Each inhibitor was added 30 min before the addition of NA. 100% represents NA (100 nM)-induced pre-contraction. Results are each expressed as the mean ± S.E.M. of 4 – 8 experiments. *, **: Significantly different from the control at $P<0.05$ and $P<0.01$, respectively.
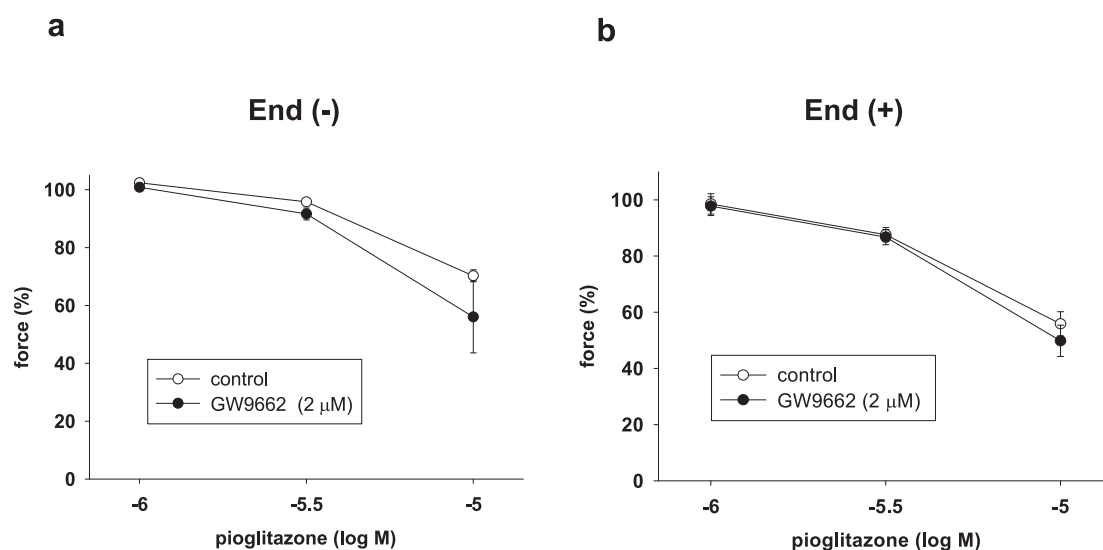

Fig. 6. Concentration-force relationships for pioglitazone in an endothelium-denuded [End ($-$)] (a) or -intact [End ($+$)] (b) artery pre-contracted with NA (100 nM) in the absence (control) or presence of GW9662 (2 μM). Pioglitazone (1 – 10 μM) was cumulatively added after the contraction induced by NA had reached a steady state. GW9662 was added 30 min before the addition of NA. 100% represents NA (100 nM)-induced pre-contraction. Results are each expressed as the mean ± S.E.M. of 4 – 5 experiments.

**Mechanisms of pioglitazone-induced vasodilation
in rat aorta   (PPAR-γ–independent mechanism)**

Pioglitazone

Endothelium   Smooth muscle

↑ NO   Opening of K$^+$ channels
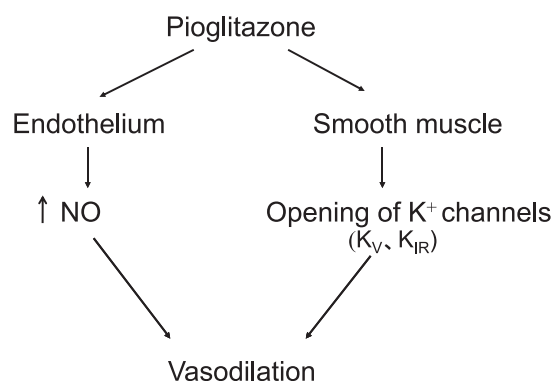(K_V、K_{IR})

Vasodilation

Fig. 7. Mechanisms of pioglitazone-induced vasodilation in rat aorta. NO: nitric oxide, K_V: voltage-dependent K$^+$, K_{IR}: inward rectifier K$^+$.

results support our results. In contrast, it was shown that pioglitazone ($0.01 – 10\,\mu$M) was ineffective on the phenylephrine-induced contraction in End (−) aorta, although high concentration of pioglitazone ($100\,\mu$M – 10 mM) was effective (10). The effects of pioglitazone on high-K$^+$–induced contraction seem different depending on the vessel types since it was reported that pretreatment with pioglitazone ($0.1 – 100\,\mu$M) inhibited the high-K$^+$–induced contraction in mesenteric arteries of guinea pigs (12) in contrast to our results in rat aorta.

To advance the previous findings, we demonstrated that pioglitazone activates smooth muscle K$^+$ channels in rat aorta. There are several reports exploring the effects of pioglitazone on activity of ion channels using isolated vascular smooth muscle cells (VSMCs). It was shown that pioglitazone inhibited voltage-operated Ca^{2+} currents (I_{Ca}) in VSMCs from mesenteric arteries of guinea pigs (12, 13) and rats (14) or rat aorta (15). On the other hand, pioglitazone was shown to have no effect on voltage-dependent K$^+$ currents ($I_{K(V)}$) and Ca^{2+}-activated K$^+$ currents ($I_{K(Ca)}$) in mesenteric arterial VSMCs (13). In the present study, we found that pioglitazone activated K_V and/or K_{IR} but not K_{ATP}, BK$_{Ca}$, and SK$_{Ca}$ channels. Some discrepancies seem exist and they might be derived from the differences between the isolated cells and blood vessels, since it is known that isolated VSMCs change their phenotypes from contractile to synthetic ones (16). It was also shown that roglitazone, one of the other TZDs, inhibited I_{Ca}, $I_{K(Ca)}$, and $I_{K(V)}$ in VSMCs from rat aorta (17) and guinea-pig mesenteric artery (13). On the other hand, it was reported that troglitazone caused vasorelaxation via activation of K_V channel in rat tail artery (18). Therefore it seems likely that in addition to cell and tissue

difference, another difference also exists between the TZDs in the effects on ion channel activities.

In the present study, mechanisms of pioglitazone-induced NO-dependent relaxation remain unclear. Although there is no report so far that phosphorylation of endothelial NOS (eNOS) is induced by pioglitazone but not troglitazone (19) or rosiglitazone (20), it might be possible that eNOS is post-translationally regulated (e.g., via phosphorylation or protein-protein interaction) by pioglitazone. It also remains unclear how pioglitazone activates K$^+$ channels in smooth muscle. There are two possible explanations. Pioglitazone might directly (physically) interact with K$^+$ channels to regulate its opening. Alternatively, pioglitazone might indirectly open K$^+$ channels by regulating intracellular signal transduction (e.g., second messengers). Further investigation is needed to clarify the underlying mechanisms.

In the previous clinical reports, treatment with pioglitazone ($4 – 12$ weeks) improved endothelium-dependent vasodilation (flow-induced one) in patients with type 2 diabetes (21) and in non-diabetic patients with coronary artery diseases (22), while endothelium-independent vasodilation (nitroglycerin-induced one) was not affected. In the present study, we showed in the isolated rat aorta that pioglitazone directly caused both endothelium-dependent and -independent vasodilation. We speculate that the discrepancy might be derived from the differences in the experimental settings (in vivo long-term effects of pioglitazone as measured by nitroglycerin-induced vosodilation *vs* ex vivo direct vasodilating effects of pioglitazone). Nevertheless, since both endothelium-dependent and -independent arterial relaxations are important to regulate blood pressure in vivo, we postulate that the present ex vivo results would explain at least in part the beneficial effects of pioglitazone against ischemic heart diseases beyond its insulin sensitizing action.

In summary, the present results demonstrated that the anti-type 2 diabetic drug pioglitazone induces PPAR-γ–independent relaxation in isolated blood vessel. The relaxation is caused by endothelium-derived NO and opening smooth muscle K_V and/or K_{IR} channels. The present findings might contribute to gain mechanistic insights into the preventive roles of pioglitazone against diabetes-associated ischemic heart diseases.

Acknowledgments

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