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The Inhibitory Effect of Pioglitazone on Agonist-dependent Vascular Contractility

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Accepted 16 January 2008

Abstract

The present study was undertaken to determine whether pioglitazone treatment influences on the agonist-induced vascular smooth muscle contraction and, if so, to investigate the related mechanism. The measurement of isometric contractions using a computerized data acquisition system was combined with molecular experiments. Pioglitazone decreased Rho-kinase activating agonist-induced contraction but not phorbol ester-induced contraction suggesting the least involvement of Ca^{2+}-independent thin filament regulation of contractility. Furthermore, pioglitazone decreased thromboxane A_2 mimetic-induced phosphorylation of MYPT1 at Thr855, the newly-highlighted site, instead of Thr696. In conclusion, this study provides the evidence and possible related mechanism concerning the vasorelaxing effect of pioglitazone as an antihypertensive on the agonist-induced contraction in rat aortic rings regardless of endothelial function.

Keywords: MYPT1, Phorbol ester, Pioglitazone, Rho-kinase, Thromboxane A_2 mimetic

Cardiovascular disease is one of the leading causes of death in the western world and diabetes mellitus has been identified as a primary risk factor[1], due to the alteration in vascular responsiveness to several vasoconstrictors and vasodilators[2]. Most of the complications in diabetes are due to increased serum glucose and increased generation of oxygen-derived free radicals, which lead to endothelium dysfunction.

Pioglitazone, a peroxisome proliferators activated receptors (PPAR) gamma agonist, improves insulin-mediated glucose uptake into skeletal muscle without increasing endogenous insulin secretion and has been demonstrated to be effective in the treatment of non-insulin dependent diabetes mellitus with insulin resistance[3-5]. It activates PPARγ, leading to improved insulin sensitivity and reduced blood pressure in both humans and animals through a mechanism that has not been completely elucidated[6]. This finding, coupled with the observation that PPARγ is expressed in both vascular muscle[7] and endothelium[8], suggest that it may play an important role in the regulation of blood pressure and vascular tone.

Vascular smooth muscle contraction in response to both agonists and increased intravascular pressure has been shown to be largely dependent on the classic smooth muscle biochemical pathway involving Ca^{2+}-calmodulin-induced myosin regulatory light chain phosphorylation. The requirement for these pathways has been directly confirmed in studies of isolated vascular smooth muscles in combination with fluorescence measurements of intracellular Ca^{2+} and electrophoretic estimations including Ca^{2+} sensitization and time-dependent phenomena such as cytoskeletal and cellular reorganization may contribute to contractile responsiveness. These mechanisms may support the Ca^{2+}-myosin light chain phosphorylation pathway and, further, provide a mechanism for maintained tension development at comparatively low energy cost.

It is generally accepted that the initiation of smooth muscle contractility is predominantly controlled by a Ca^{2+}-dependent increase in myosin light chain 20 kDa (MLC_{20}) phosphorylation[9]. However, other pathways have now been described that may regulate the contractility of smooth muscle by regulating the phosphorylation of MLC_{20} independently of a rise in intracellular Ca^{2+} [10-12]. The phosphorylation of MLC_{20} promotes the interaction of actin and myosin II and the contraction of smooth muscle. The degree of MLC_{20} phosphorylation or contraction does not always paral-

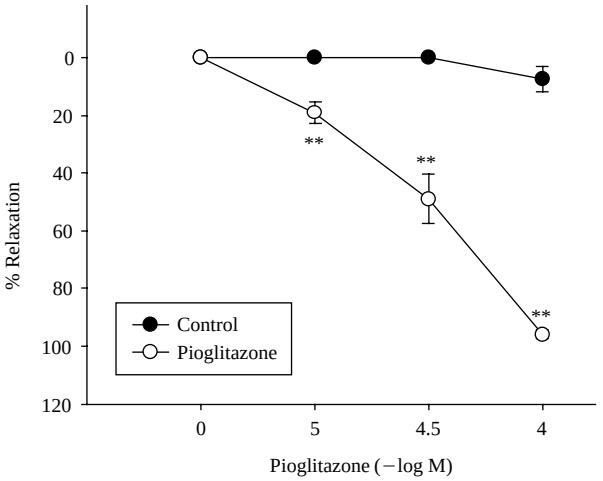

Figure 1. Direct effect of pioglitazone on 0.1 μM thromboxane A_2 mimetic U46619-induced contraction in the aortic rings with endothelium denuded. Developed relaxation is expressed as a percentage of the maximum contraction to 0.1 μM thromboxane A_2 mimetic U46619. Data are expressed as means of 3-5 experiments with vertical bars showing SEM. $^{**}P < 0.01$, presence versus absence of pioglitazone.

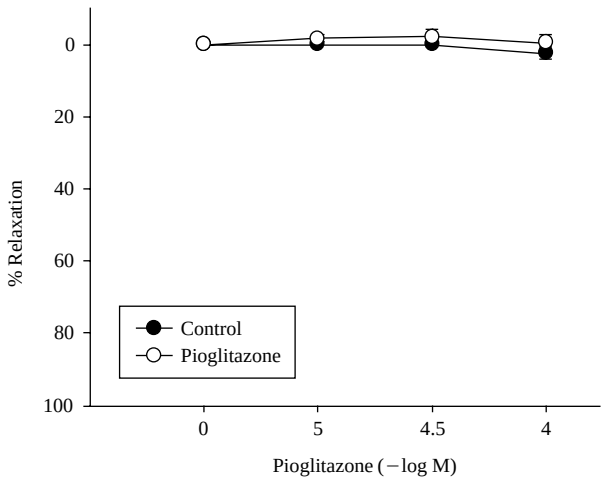

Figure 2. Direct effect of pioglitazone on 1 μM phorbol 12,13-dibutyrate (PDBu)-induced contraction in the aortic rings with endothelium denuded. Developed relaxation is expressed as a percentage of the maximum contraction to 1 μM PDBu. Data are expressed as means of 3-5 experiments with vertical bars showing SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, presence versus absence of pioglitazone.

lel the Ca^{2+} concentration. The extent of MLC_{20} phosphorylation or force of contraction induced by agonist stimulation is usually higher than that caused by an increase in the Ca^{2+} concentration, a finding explained by so-called Ca^{2+} sensitization[9]. Thus, an additional mechanism of regulation that modulates the levels of MLC_{20} phosphorylation and degree of contraction has been proposed. Subsequent studies have revealed that inhibition of MLC phosphatase is a major pathway in Ca^{2+} sensitization[13]. These pathways are generally stimulated by contractile agonists that activate heterotrimeric G protein-coupled receptors, probably *via* $G_{12/13}$ stimulation of Rho GEFs (guanine nucleotide-exchange factors)[14]. Activation of Rho A leads to subsequent activation of a recently isolated downstream target of Rho, a p160 Rho-associated coiled-coil-containing protein kinase (Rho-kinase)[15,16].

There are few reports of the effect and the related mechanism of pioglitazone on agonist-induced vascular constriction. Hence the purpose of the present study was to investigate the vasorelaxation effect and possible related mechanism of an antidiabetic pioglitazone treatment on aortic contractibility induced by a specific agonist.

Effect of Pioglitazone on Agonist-induced Regulation of Contractility of Denuded Aorta

The addition of 0.1 μM thromboxane A_2 mimetic U-46619 produced the contraction in rat aorta with endothelium denuded (Figure 1). The absence of the endothelium was verified by the lack of relaxation after the addition of acetylcholine (1 μM) to precontracted ring segments. Pioglitazone significantly inhibited thromboxane A_2 mimetic-induced contraction regardless of endothelial function (Figure 1).

Thromboxane A_2 mimetic is believed to increase Rho-kinase activity[17]. Thus we thought that Rho-kinase activity might be more inhibited by pioglitazone compared to control.

Effect of Pioglitazone on Phorbol Ester-induced Rregulation of Contractility of Denuded Aorta

There was no significant difference in phorbol ester -induced contractility between control and pioglitazone treated rat aorta with endothelium denuded (Figure 2). Thus we postulated that protein kinase C activity or other downstream kinases such as mitogen-activated protein kinase kinase (MEK) and extracellular signal regulated kinase (ERK) 1/2 might not be inhibited by pioglitazone treatment.

Effect of Pioglitazone on Depolarization-induced Regulation of Contractility of Denuded Aorta

There was no significant difference in depolarization-induced contractility between control and pioglitazone treated rat aorta with endothelium denuded (Figure 3). Thus we postulated that thick or myosin

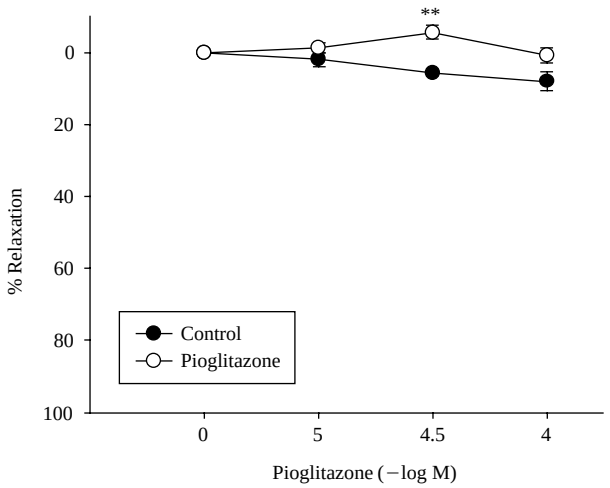

Figure 3. Direct effect of pioglitazone on depolarization-induced contraction in the aortic rings with endothelium denuded. Developed relaxation is expressed as a percentage of the maximum contraction to 50 mM KCl. Data are expressed as means of 3-5 experiments with vertical bars showing SEM.

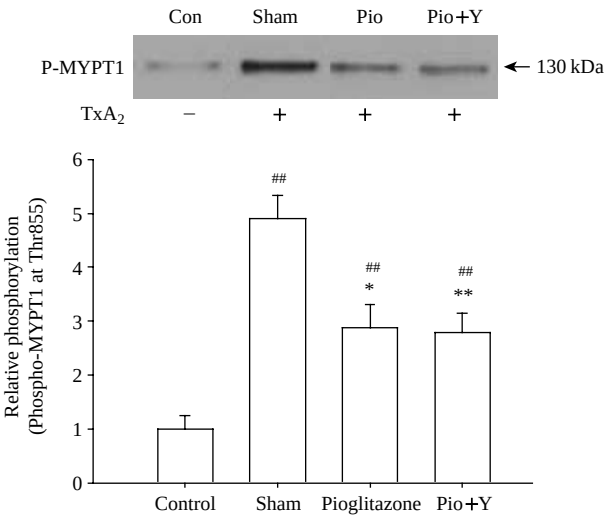

Figure 4. Decreased phospho-MYPT1$_{Thr855}$ protein levels in quick frozen pioglitazone-added rat aorta in the absence of endothelium compared to the vehicle-added rat aorta precontracted with thromboxane A_2 mimetic U46619. Upper panel shows a typical blot and lower panel shows average densitometry results on the relative level of phospho-MYPT1. Data are expressed as means of 3-5 experiments with vertical bars showing SEM. $*P<0.05$, $**P<0.01$, $^{\#\#}P<0.01$, versus sham or control respectively. Pio: pioglitazone; TxA_2: thromboxane A_2; Y: 0.1 μM Y-27632.

filament regulation including myosin light chain kinase inactivation might not be inhibited by pioglitazone.

Effect of Pioglitazone on Tthe Level of Phospho-MYPT1 at Thr-855

To confirm the role of pioglitazone in thick filament regulation of smooth muscle contractility, we measured the levels of MYPT1 (myosin-targeting subunit of myosin light chain phosphatase) and phospho-MYPT1 in the muscles quick frozen after 30 min exposure to 0.1 μM thromboxane A_2 mimetic U-46619. Interestingly, there was a significant decrease in the 0.1 μM thromboxane A_2 mimetic U-46619-induced MYPT1 phosphorylation at the newly-highlighted site of Thr855 instead of Thr696[17,19] in quick frozen pioglitazone treated rat aorta in the absence of endothelium compared to the vehicle treated rat aorta (Figure 4). Thus thick or myosin filament regulation including myosin phosphatase activation through RhoA/Rho-kinase inactivation might be involved in decreased contractility in pioglitazone treated rat aorta.

Discussion

It is generally accepted that the initiation of vascular smooth muscle contractility is predominantly controlled by a Ca^{2+}-dependent increase in myosin light chain 20 kDa (MLC_{20}) phosphorylation[9]. However, other pathways have now been described that may regulate the contractility of smooth muscle by regulating the phosphorylation of MLC_{20} independently of a rise in intracellular Ca^{2+} [10-12]. The phosphorylation of MLC_{20} promotes the interaction of actin and myosin II and the contraction of smooth muscle. The degree of MLC_{20} phosphorylation or contraction is not always linearly related to the Ca^{2+} concentration. The extent of MLC_{20} phosphorylation or force of contraction induced by agonist stimulation is usually higher than that caused by an increase in the Ca^{2+} concentration, a finding explained by so-called Ca^{2+} sensitization[9] involving the inhibition of myosin phosphatase[13] or thin filament regulation[20].

The signaling pathways involved in vascular smooth muscle contractility may be categorized broadly as leading to either thick or thin filament regulation. As described above, thick or myosin filament regulation encompasses both Ca^{2+} activation and Ca^{2+} sensitization as it involves both activation of myosin light chain kinase by Ca^{2+}-calmodulin and regulation of myosin light chain phosphatase activity. On the other hand, thin or actin filament regulation includes the possible disinhibition of actin-myosin interactions by phosphorylation of caldesmon, possibly by protein kinase C, mitogen-activated protein kinase kinases or extracellular signal regulated kinases that are translo-

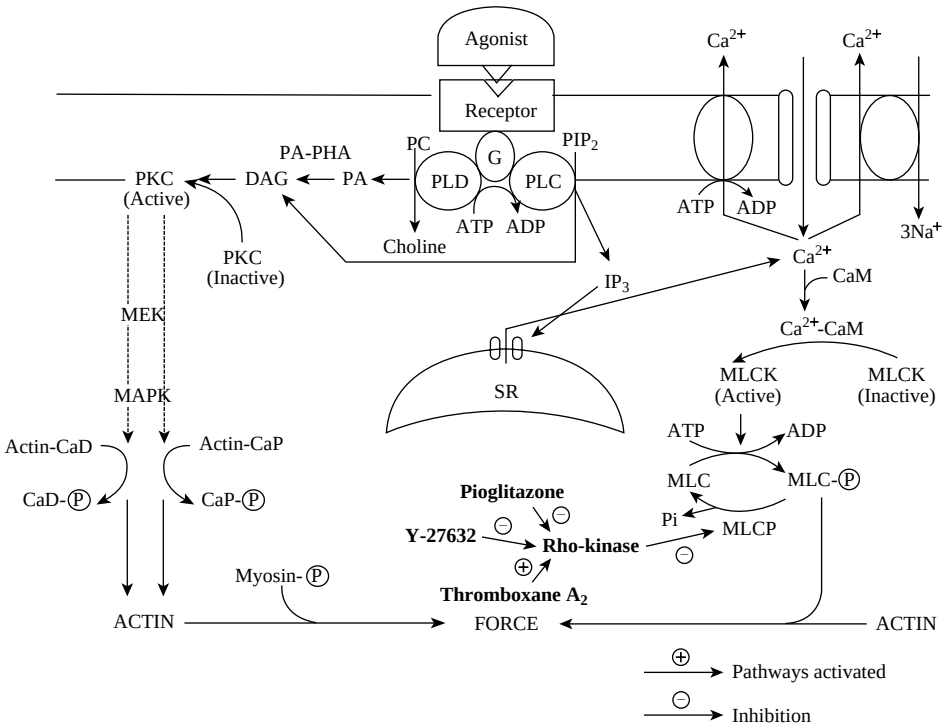

Figure 5. Putative vascular smooth muscle relaxation pathway by pioglitazone in rat aorta. Pioglitazone-induced vasorelaxation is mediated via Rho-kinase, which subsequently decreases the activity of myosin phosphatase. On the contrary, thromboxane A_2 increases contractility inducing Rho-kinase activation.

cated during their activation[21].

The main finding of the present study is that pioglitazone decreased thromboxane A_2 mimetic U-46619 induced vasoconstriction regardless of endothelial function but not phorbol ester- or depolarization-induced contraction suggesting that thin or actin filament regulation and myosin light chain kinase activity might not be involved but Rho-kinase activity and subsequent myosin phosphatase activity might be inhibited (Figure 5). Molecular result also supports the possible mechanism (Figure 4). Furthermore, another experiment for vascular contraction, where various concentration of pioglitazone and Rho-kinase inhibitor Y-27632 were directly coadministrated to rat aorta precontracted with 0.1 μM thromboxane A_2 mimetic U-46619, showed that there was no additional relaxation effect of Y-27632 compared to administration of pioglitazone alone (Je H. D., unpublished data) indicating that the relaxation mechanism of pioglitazone is similar to that of Y-27632. Recently, it was reported that pioglitazione decreased blood pressure with the endothelial function[22]. However, pioglitazone inhibited the special agonist-induced vasoconstriction regardless of endothelial function (Figure 1). On the other hand, pioglitazone did not affect the contractile response to phorbol ester or depolarization in the aortic rings with endothelium denuded (Figure 2) suggesting that thin filament regulation or myosin light chain kinase activity might not be important in pioglitazone-induced vasorelaxation.

In summary, our results indicate that pioglitazone relaxed thromboxane A_2-induced contraction in rat aorta inhibiting Rho-kinase activity regardless of endothelial function. Subsequent interactions between myosin phosphatase and inactivated Rho-kinase may lead to attenuated Ca^{2+} sensitivity of the myofilament and vascular relaxation. Therefore, this study provides the evidence concerning the vasorelaxing effect and the related mechanism of an antidiabetic pioglitazone as an antihypertensive on the Rho-kinase activating agonist-specific vascular contraction in rat aortic rings regardless of endothelial function.

Methods

Tissue Preparation

Male Sprague-Dawley rats, weighing 320-350 g, were anesthetized by sodium pentobarbital (50 mg/kg i.p.) followed by cervical dislocation, in agreement with procedures approved by the Institutional Animal Care and Use Committee. The thoracic aorta was quickly removed and immersed in oxygenated (95% O_2/5% CO_2) physiological saline solution composed of (mM): 115.0 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25.0 $NaHCO_3$, 1.2 KH_2PO_4, and 10.0 dextrose (pH 7.4). The aorta was cleaned of all adherent connective tissue, and the endothelium was removed by gentle

abrasion with a cell scraper.

Contraction Measurements

Circular strips (4 mm wide) were prepared and attached to a force transducer. The strips were allowed to equilibrate at 37°C for at least 1 h and challenged with a depolarizing solution containing 50 mM KCl. Muscle strips were then washed and allowed to equilibrate for 1 h before beginning the experiment. The absence of the endothelium was verified by the lack of relaxation after the addition of acetylcholine (1 μM) to precontracted ring segments.

Western Blot Analysis

Muscle strips were quick frozen by immersion in a dry ice/acetone slurry containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT). Muscles were stored at −80°C until used. Tissues were brought to room temperature in a dry ice/acetone/TCA/DTT mixture. Then samples were homogenized in a buffer containing 20 mM mops, 4% SDS, 10% glycerol, 10 mM DTT, 20 mM β-glycerophosphate, 5.5 μM leupeptin, 5.5 μM pepstatin, 20 KIU aprotinin, 2 mM Na_3VO_4, 1 mM NaF, 100 μM $ZnCl_2$, 20 μM 4-(2-aminoethyl) benzenesulphonyl fluoride (AEBSF) and 5 mM EGTA. Protein-matched samples (modified Lowry protein assay, DC Protein Assay Kit, Bio-Rad) were electrophoresed on SDS-PAGE (Protogel, National Diagnostics), transferred to PVDF membranes and subjected to immunostaining and densitometry, as above, using the appropriate antibody. The success of protein matching was confirmed by Naphthol Blue Black staining of the membrane and densitometry of the actin band. Any mismatch of lane loading was corrected by normalization to actin staining. Each set of samples from an individual experiment was run on the same gel and densitometry was performed on the same film.

Chemicals and Antibodies

Sodium fluoride, U46619, potassium chloride, sodium bicarbonate, phenylephrine hydrochloride and D-(+)-glucose were obtained from Sigma (St Louis, MO, USA). Pioglitazone hydrochloride was obtained from Dong-A pharmaceutical (Youngin-si, Gyeonggi-do, Korea), dithiothreitol, trichloroacetic acid and acetone from Fisher Scientific (Hampton, NH, USA), and enhanced chemiluminescence (ECL) from Pierce (Rockford, IL, USA). The phospho-MYPT1Thr855 antibody (1 : 2,000) was purchased from Upstate Biotechnology (Lake Placid, NY, USA) to check the level of RhoA/Rho-kinase activity[17,18]. Anti-mouse IgM (goat) or anti-rabbit IgG (goat), conjugated with horseradish peroxidase, were used as secondary antibodies (1 : 2,000, 1 : 2,000, respectively, Upstate, Lake Placid, NY). General laboratory reagents were of analytic grade or better and were purchased from Sigma or Fisher Scientific.

Statistics

The data were expressed as mean ± standard error of the mean (SEM). Student's unpaired *t* test was used to determine the statistical significance of the means between two groups using SPSS 12.0 (SPSS Inc., Chicago, Illinois, U.S.A.). P values < 0.05 were regarded as statistically significant.

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