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NO-1886, a lipoprotein lipase activator, attenuates vascular smooth muscle contraction in rat aorta

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Abstract

The chemical compound [4-(4-bromo-2-cyano-phenylcarbamoyl)-benzyl]-phosphonic acid diethyl ester (NO-1886) is a lipoprotein lipase activator having beneficial effects on both diabetes control and the cardiovascular system. Preventing accumulation of lipids in the cell wall, in addition to improving insulin actions on vasculature, may indirectly contribute to the reducing effect of NO-1886 on vascular resistance. However, the direct effect of NO-1886 on vascular resistance, i.e., whether NO-1886 directly modulates the function of vascular endothelium and/or smooth muscle cells has not been investigated. In this study we therefore investigated the direct effect of NO-1886 on vascular contractility using rat aortic rings and cultured smooth muscle cell-line A10. The results show that administration of NO-1886 attenuated aortic contraction induced by phenylephrine and/or a high K^+ environment, in both the presence and absence of aortic endothelium. 1-(5-Chloronaphthalene-1-sulfonyl) homopiperazine hydrochloride (ML-9), a myosin light chain kinase (MLCK) inhibitor, blocked this inhibitory effect of NO-1886, whereas inhibitors of other signaling molecules such as calmodulin, protein kinase C and Rho-kinase had no effect. The vasorelaxant effect of NO-1886 was blocked in the absence of extracellular Ca^{2+}, or in the presence of the Ca^{2+} channel inhibitor, verapamil. NO-1886 attenuated smooth muscle contraction induced by the cumulative addition of $CaCl_2$. In A10 cells, NO-1886 inhibited the membrane depolarization-induced initial peak of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. This inhibition did not occur in the absence of extracellular Ca^{2+}. Taken together these results demonstrate that NO-1886 attenuates smooth muscle contraction and causes vasorelaxation by an extracellular Ca^{2+}- and MLCK-dependent mechanism.

Keywords: NO-1886; Vascular smooth muscle; Extracellular Ca^{2+}; Myosin light chain kinase

1. Introduction

Impaired vascular function increases vascular resistance and leads to the development of hypertension and arteriosclerosis (Vogel, 1997). It is also known that vascular dysfunction is associated with insulin resistance (Baron, 1994; de Jongh et al., 2004). In fact, insulin resistance causes impaired endothelium-dependent vasodilation and increases vascular smooth muscle contraction in both humans (de Jongh et al., 2004; Fleischhacker et al., 1999) and animals (Khalil and Granger, 2002; Lagaud et al., 2001; Naik et al., 2006). Thus, the reduction of vascular resistance is associated closely with the improvement of metabolic disturbances *in vivo*.

Several anti-diabetic drugs such as the ligands of the proliferator-activated receptor (PPAR)-α (bezafibrate) and -γ (pioglitazone) have been reported to have beneficial effects on the cardiovascular system (Julius et al., 2001; Kurtz, 2006). On the other hand, some antihypertensive drugs such as long-acting dihydropiridine calcium channel antagonists, α blockers and angiotensin converting enzyme inhibitors have been reported to improve insulin resistance in diabetic humans and animals (Harada et al., 1999; Harano et al., 1995; Iimura et al., 1995; Lithell, 1996). Therefore it is now necessary to choose anti-diabetic drugs that have beneficial effects on both diabetes control and the cardiovascular system.

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The chemical compound [4-(4-bromo-2-cyano-phenylcarbamoyl)-benzyl]-phosphonic acid diethyl ester (NO-1886) is a lipoprotein lipase activator discovered by the Otsuka Pharmaceutical Factory, Inc. (Tsutsumi et al., 1993). NO-1886 activates tissue lipoprotein lipase and has been shown in experimental animals to cause a reduction in the levels of plasma triacylglycerol and increase the levels of high density lipoprotein cholesterol (Tsutsumi et al., 1993, 1995). The effect of NO-1886 to improve insulin resistance status has also been reported in insulin-resistant animal models in which a reduction in plasma lipids would be expected to contribute to such beneficial effects (Kusunoki et al., 2000). Chronic administration of NO-1886 has also been reported to attenuate high-cholesterol feeding-induced atherosclerosis in the coronary artery of rats (Tsutsumi et al., 1993) and the aorta of rabbits (Chiba et al., 1997). In addition, Hara et al. (Hara et al., 1998) showed that treatment of aged rats with NO-1886 resulted in an improvement in endothelium-dependent relaxation in the thoracic aorta. Therefore, NO-1886 may be a beneficial drug for both diabetic and hypertensive conditions. It is possible the protective effect of NO-1886 on the cardiovascular system is attributable to many different *in vivo* biological factors. For example, preventing accumulation of lipids in the cell wall, in addition to improving insulin actions on vasculature, may indirectly reduce vascular resistance. However, the direct effect of NO-1886 on vascular resistance, i.e., whether NO-1886 directly modulates the function of vascular endothelium and/or smooth muscle cells has not been investigated. Therefore, we carried out a study to examine whether NO-1886 has a direct effect on vascular contractility in rat aortic rings and a cultured cell-line of aortic smooth muscle cells.

2. Materials and methods

2.1. Agents

Agent NO-1886 was synthesized in the New Drug Research Laboratory of Otsuka Pharmaceutical Factory Inc., Tokushima, Japan. N_{ϖ}-Nitro-L-arginine (L-NNA) and verapamil hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA), (5-isoquinolinesulfonyl)homopiperazine dihydrochloride (HA-1077), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), and (S)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]homopiperazine dihydrochloride (H-1152) were from Calbiochem (San Diego, CA, USA), staurosporine, nicotinamide and ryanodine from Wako Pure Chemical Industries (Osaka, Japan) and 1-(5-Chloronaphthalene-1-sulfonyl)homopiperazine hydrochloride (ML-9) from Biomol International, L.P. (Exeter, UK). NO-1886, staurosporine and ML-9 were dissolved in dimethyl sulfoxide (DMSO), while verapamil, HA-1077, W-7, H-1152, ryanodine and nicotinamide were dissolved in distilled water.

2.2. Preparation of aortic rings and tension measurements

All animal procedures were in accordance with the institutional guidelines for the care and use of laboratory animals of Tokushima University. Male Wistar rats (Japan SLC, Shizuoka, Japan) weighing 250–350 g were anesthetized by an intraperitoneal injection of pentobarbital. The thoracic aortas were dissected free of connective tissue and cut into ring segments, 3- to 4-mm in length. In some experiments, the aortic endothelium was removed by inserting a cotton thread into the lumen, followed by gentle rubbing. Each ring was then placed in a 3 ml organ bath (Micro Easy Magnus, Kishimoto Medical; Kyoto, Japan) and mounted on two stainless steel wires, one of which was fastened to the bath and the other connected to a force transducer for the measurement of isometric tension. The bath was filled with Krebs-Ringer bicarbonate buffer (KRB) solution at 37 °C and bubbled with a mixture of 95% O_2–5% CO_2. The KRB contained (in mmol/l) 118 NaCl, 4.6 KCl, 2.5 $CaCl_2$, 24.8 $NaHCO_3$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, and 5.6 glucose. The rings were equilibrated for 60 min under a resting tension of 1.5 g and the solution changed at 30 min intervals. The presence or absence of endothelium was confirmed by the addition of the endothelium-dependent vasodilator, acetylcholine (1 μM), after contractions had been induced by phenylephrine (1 μM). Following washout of phenylephrine and acetylcholine with KRB, the aortic rings were contracted by a high K^+ concentration solution (50 mM KCl in bath solution). After recording the contractile force of each ring following stimulation with 50 mM KCl, the baths were washed out with KRB. Each ring was allowed to equilibrate at a resting tension of 1.5 g for 20 min. The contractile responses in the following test experiments were expressed relative to that measured for the 50 mM KCl-induced contraction (Harada et al., 2001).

2.3. Studies on the effect of NO-1886

Test experiments involving NO-1886 were conducted on aortic rings in which contraction was induced by phenylephrine or KCl at the indicated concentrations. The concentration of NO-1886 was 0.1, 1 or 10 μM. NO-1886 was added to the incubation system 20 min prior to or during the contraction induced by each vasoconstrictor. Vehicle (DMSO)-treated rings were used as controls. In some experiments, endothelium-denuded aortic ring preparations were contracted by phenylephrine in the presence of sodium nitroprusside (1 nM) without NO-1886 administration. In some other experiments, aortic contraction was performed in the absence of extracellular Ca^{2+} using Ca^{2+}-free KRB containing 1 mM EGTA.

2.4. Inhibitor experiments

Several inhibitors and antagonists were used to examine the role of intracellular signaling pathways in the action of NO-1886. The concentrations of inhibitors/antagonists used in this study were as follows: L-NNA [NO synthase inhibitor; 30 μM], verapamil [L-type voltage dependent Ca^{2+} channels (L-type Ca^{2+} channels) blocker; 55 μM], staurosporine [protein kinase C (PKC) inhibitor; 100 nM], H-1152 [Rho-kinase (ROK) inhibitor; 100 nM], HA-1077 (ROK inhibitor; 10 μM), W7 [calmodulin inhibitor; 100 μM], PD98059 [extracellular signal-regulated kinase 1 and 2 (ERK) kinase inhibitor; 30 μM], ML-9

[myosin light chain kinase (MLCK) inhibitor; 30 μM], ryanodine [ryanodine receptors blocker; 30 μM] and nicotinamide (ADP-ribosyl cyclase inhibitor; 6 mM). The aortic rings were pre-treated with each inhibitor for 20 min and then contraction was induced by phenylephrine (1 μM) or KCl (80 mM). Each inhibitor was present in the incubation system during contraction.

2.5. Exogenous Ca^{2+}-dependent contraction

The procedure was essentially the same as that described by Leung FP et al. (Leung et al., 2005) with minor modifications. The aortic rings without endothelium were washed 3 times with Ca^{2+}-free KRB containing 1 mM EGTA, and then exposed to the same buffer for 10 min. The rings were pre-treated with 10 μM NO-1886 for 20 min and then stimulated with 80 mM KCl. After 20 min incubation, $CaCl_2$ (10^{-5} M to $10^{-2.5}$ M)-induced contraction was measured for each aortic ring.

2.6. Cell culture and measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$)

An aortic smooth muscle cell-line, A10, was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 0.1% gentamycin. $[Ca^{2+}]_i$ was measured in fura-2 acetoxymethyl ester (AM)-loaded cells using a fluorescence ratio imaging system (Meta Fluor, Molecular Devices, Sunnyvale, CA). Fura-2 fluorescence was measured by alternating excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 510 nm using an automation control system (MAC 5000, Ludl Electronic Products, Hawthorne, NY). The cells were loaded with 2 μM fura-2 AM in HEPES solution (10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (HEPES), 5 mM KCl, 1 mM $MgCl_2$, 145 mM NaCl, 1 mM $CaCl_2$, 10 mM glucose, pH 7.4) at 37 °C for 60 min. Coverslips with attached cells were transferred to an experimental chamber with HEPES buffer, mounted on the stage of an inverted fluorescence microscope (IX 71, OLYMPUS, Tokyo, Japan). Ca^{2+}-free HEPES solution (10 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, 145 mM NaCl, 1 mM EGTA, 10 mM glucose, pH 7.4) was used in some of the experiments.

2.7. Statistical analysis

Data are expressed as the means ± S.D. and were analyzed by ANOVA plus Bonferroni multiple comparison tests. A P-value <0.05 was regarded as statistically significant.

3. Results

3.1. Pre-treatment of NO-1886 attenuates phenylephrine- and KCl-induced contraction in rat aorta

The effect of NO-1886 on vasoconstriction induced by phenylephrine or by high environmental K^+ was examined in rat thoracic aorta. As shown in Fig. 1A, pre-treatment with NO-1886 (10 μM) attenuated subsequent phenylephrine (1 μM)-induced maximum contraction of aortic rings, regardless of the presence (29.3 ± 9.0%) or absence (27.9 ± 14.5%) of endothelium. Likewise, pre-treatment with NO-1886 attenuated KCl (80 mM)-induced contraction of aortic rings regardless of the presence (32.9 ± 5.0%) or absence (25.2 ± 4.1%) of endothelium (Fig. 1B). While sodium nitroprusside (1 nM), a donor of NO, inhibited phenylephrine (1 μM)-induced contraction, L-NNA, an inhibitor of NO synthesis, had little effect on the inhibitory effect of NO-1886 on phenylephrine-induced contraction (Fig. 1A). These data suggest that NO-1886 attenuated vascular contractility independent of generation of NO by the endothelial. Endothelium-denuded aortic rings were therefore used in the following experiments. NO-1886 at a concentration of 10 μM significantly inhibited, not only low (30 nM = $10^{-7.5}$ M), but also high (10 μM) concentration phenylephrine-induced vasoconstriction (Fig. 1C). The effect of NO-1886 at concentration over 10 μM was not determined since at high concentrations (>10 μM) the drug was not fully dissolved in our incubation buffer.

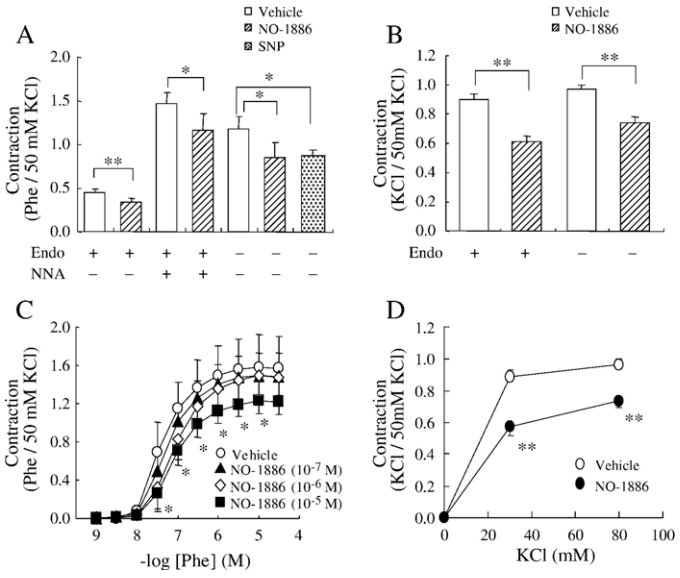

Fig. 1. NO-1886 attenuated phenylephrine (Phe)- or KCl-induced vasoconstriction in rat aorta. The effect of NO-1886 on phenylephrine (A) or KCl (B)-induced contraction in rat aorta in the presence or absence of aortic endothelium. The aortic ring preparations were pre-incubated with (10 μM) or without (vehicle) NO-1886 for 20 min followed by stimulation with phenylephrine (1 μM) or KCl (80 mM). In some ring preparations with aortic endothelium (Endo), L-NNA (30 μM) was incubated during phenylephrine-induced contraction. In some other ring preparations without aortic endothelium, sodium nitroprusside (SNP: 1 nM) was incubated during phenylephrine-induced contraction in the absence of NO-1886. (C) Concentration-response curves for phenylephrine-induced contraction of endothelium-denuded aortic rings in the presence (100 nM–10 μM) or absence (vehicle) of NO-1886 (pre-treatment for 20 min). (D) Concentration-response curves for KCl-induced contraction of endothelium-denuded aortic rings in the presence (10 μM) or absence (vehicle) of NO-1886 (pre-treatment for 20 min). Each contractile value was standardized against the contraction level induced by 50 mM KCl in the absence of pre-treatment. Data are expressed as means ± S.D. for 4 aortic rings in each group. * $P<0.05$ and ** $P<0.01$, compared with vehicle group. Similar results were obtained from at least two independent experiments.

Pre-treatment with 10 μM NO-1886 also caused similar attenuation of both 30 mM and 80 mM KCl-induced contraction in aortic rings without endothelium (Fig. 1D). This result suggested that the effect of NO-1886 was not mediated by the opening of K^+ channels.

3.2. NO-1886 relaxed phenylephrine- and KCl-induced contraction in rat aorta

Administration of NO-1886 (10 μM) caused vasodilation in aortic rings that had been contracted previously by 1 μM phenylephrine, (Fig. 2A and B). The relaxant effect lasted for 40 min after NO-1886 administration. Likewise, NO-1886-induced vasodilation in aortic rings pre-contracted by 80 mM KCl (Fig. 2C).

3.3. Lack of extracellular Ca^{2+} or inhibition of L-type Ca^{2+} channels blocked the effect of NO-1886

To examine the involvement of extracellular Ca^{2+} in the inhibitory effect of NO-1886 on vasoconstriction, aortic ring preparations were washed and incubated with Ca^{2+}-free KRB solution. A lack of extracellular Ca^{2+} almost completely attenuated phenylephrine-(94.1±1.6%) and KCl-(78.3±2.2%) induced contractions (Fig. 3A and B compared with Fig. 1C and D). Under these environmental conditions, NO-1886 failed to inhibit vasoconstriction (Fig. 3A and B). As shown in Fig. 3C and D, NO-1886 did not relax either phenylephrine- or KCl-induced contraction in the presence of verapamil (55 μM), a L-type Ca^{2+} channel blocker. The attenuation of vasoconstriction by NO-1886 was also absent in the presence of verapamil (Fig. 3E).

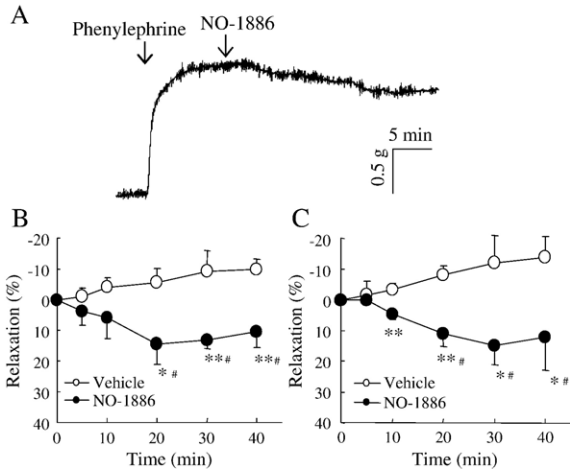

Fig. 2. NO-1886 relaxed the endothelium-denuded aortic rings which had been contracted previously by either phenylephrine or KCl. (A) Original recording of phenylephrine (1 μM)-induced contraction. After the contraction had reached a plateau level, NO-1886 (10 μM) was added to the incubation baths. (B) Time course of vasodilation induced by NO-1886 (10 μM) in aortic rings pre-contracted by phenylephrine (1 μM). (C) Time course of vasodilation by NO-1886 (10 μM) in aortic rings pre-contracted by 80 mM KCl. Data are expressed as means±S.D. for 4 aortic rings in each group. * $P<0.05$ and ** $P<0.01$, compared with vehicle group. # $P<0.05$, compared with time of zero. Similar results were obtained from at least two independent experiments.

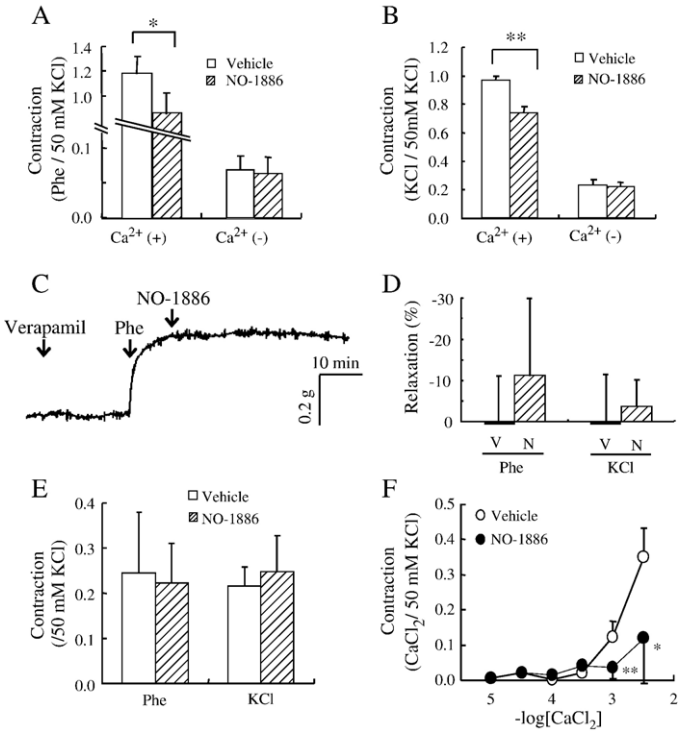

Fig. 3. Involvement of extracellular Ca^{2+} influx in the effect of NO-1886. (A) and (B) The effects of NO-1886 on phenylephrine (Phe) (A)-or KCl (B)-induced contraction in endothelium-denuded aortic rings in the presence [Ca^{2+} (+)]or absence [Ca^{2+} (−)] of extracellular Ca^{2+}. The aortic ring preparations were pre-incubated with (10 μM) or without (vehicle) NO-1886 in Ca^{2+}-free KRB for 20 min, followed by stimulation with phenylephrine (1 μM) or KCl (80 mM) for the indicated times. (C) Original recording of phenylephrine (1 μM)-induced contraction of endothelium-denuded aortic rings in the presence of verapamil (55 μM, pre-treatment for 20 min). After the contraction had reached a plateau level, NO-1886 (10 μM) was added to the incubation baths. (D) Percentage of vasodilation induced by NO-1886 (N) or vehicle (V) during phenylephrine (1 μM)-or KCl (80 mM)-induced contraction in endothelium-denuded aortic rings with verapamil pre-treatment (20 min). (E) Effect of NO-1886 on phenylephrine-or KCl-induced contractile level in endothelium-denuded aortic rings with verapamil pre-treatment (20 min). The aortic ring preparations were pre-incubated with (10 μM) or without (vehicle) NO-1886 for 20 min, followed by stimulation with phenylephrine (1 μM) or KCl (80 mM). (F) Effect of NO-1886 on $CaCl_2$-induced contraction in endothelium-denuded aortic rings pre-stimulated by 80 mM KCl. The aortic ring preparation were pre-incubated with (10 μM) or without (Vehicle) NO-1886 in Ca^{2+}-free KRB for 20 min, followed by stimulation with KCl (80 mM). The rings were then stimulated with cumulative addition of $CaCl_2$ and the contractile force of each concentration recorded. Each contractile value was standardized against the contraction level induced by 50 mM KCl in the presence of Ca^{2+} and in the absence of treatment. Data are expressed as means±S.D. for 4 aortic rings in each group. * $P<0.05$ and ** $P<0.01$, compared with vehicle group. Similar results were obtained from at least two independent experiments.

To examine the effect of NO-1886 on Ca^{2+} influx-induced vasoconstriction, $CaCl_2$ was administrated cumulatively [10 μM (=10^{-5} M)–3 mM (=$10^{-2.5}$ M)] to the aortic rings which had been incubated in Ca^{2+}-free KRB. To activate Ca^{2+} influx into the smooth muscle cells, the aortic rings were stimulated with 80 mM KCl prior to the cumulative addition of $CaCl_2$ (Leung et al., 2005). Addition of $CaCl_2$ increased the contractile force of the aortic rings without the endothelium in a concentration-dependent manner (Fig. 3F). Pre-treatment with

NO-1886 attenuated the contraction induced by the cumulative addition of $CaCl_2$ (Fig. 3F).

3.4. Effect of NO-1886 on $[Ca^{2+}]_i$

In order to clarify whether NO-1886 affected $[Ca^{2+}]_i$, we measured $[Ca^{2+}]_i$ in A10 cells, a cell-line of rat aortic smooth muscle cells. The levels of $[Ca^{2+}]_i$ were monitored during depolarization with 80 mM KCl in the presence of NO-1886 or its vehicle DMSO. As shown in Fig. 4A, pre-treatment with NO-1886 inhibited the transient $[Ca^{2+}]_i$ peak that developed immediately after KCl administration. However, this attenuation of the $[Ca^{2+}]_i$ peak was not seen in the absence of extracellular Ca^{2+} (Fig. 4B). Surprisingly, NO-1886 did not decrease $[Ca^{2+}]_i$ when it was administrated after 80 mM KCl-induced $[Ca^{2+}]_i$ had reached a plateau level (Fig. 4C). Subsequent addition of 55 μM verapamil induced a reduction in $[Ca^{2+}]_i$ (Fig. 4C). Thus, NO-1886 targets not only $[Ca^{2+}]_i$, but also other contractile components with Ca^{2+}-dependency. These actions act to maintain the Ca^{2+} sensitivity of smooth muscle contraction.

3.5. Effects of inhibitors/antagonists for signaling molecules

The signal transduction pathways involved in NO-1886-induced vasorelaxation or inhibition of vasoconstriction were investigated using specific inhibitors and antagonists (Fig. 5).

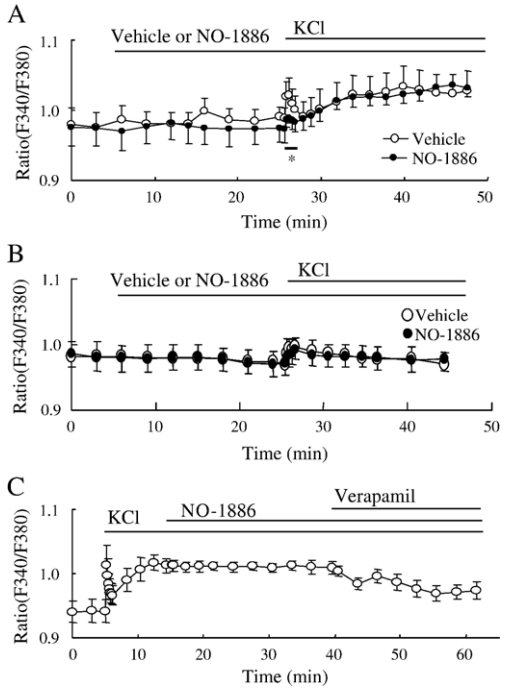

Fig. 4. Time course of the changes in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in A10 cells. A10 cells were stimulated with KCl (80 mM) and/or NO-1886 (10 μM) for the indicated times in the presence (A and C) or absence (B) of extracellular Ca^{2+}. In (C), verapamil (55 μM) was applied to the cells following NO-1886 treatment. Data are expressed as means ± S.D. for 8 cells in each group. * $P<0.05$ compared with vehicle group. Similar results were obtained from three independent experiments.

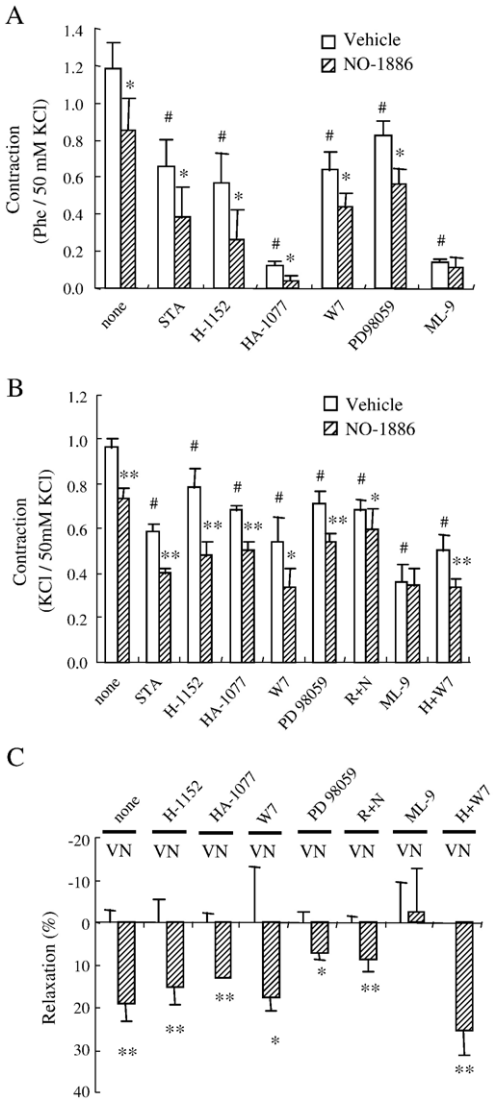

Fig. 5. Effects of several inhibitors and antagonists on the inhibitory effect of NO-1886 on 1 μM phenylephrine (Phe) (A)- and 80 mM KCl (B)-induced contraction in aortic rings without endothelium. The inhibitors and antagonists used were: Staurosporine (STA, 100 nM), H-1152 (100 nM), HA-1077 (10 μM), W7 (100 μM), PD98059 (30 μM), ryanodine+nicotinamide (R+N, 30 μM ryanodine+6 mM nicotinamide), ML-9 (30 μM) and H-1152+W7 (H+W7, 100 nM H-1152+100 μM W7). Each inhibitor or antagonist and NO-1886 were administrated to the aortic rings 20 min prior to the subsequent vasoconstriction. Each contractile value was standardized against the contraction level induced by 50 mM KCl in the absence of the pre-treatment. (C) Effects of several inhibitors and antagonists on the vasorelaxant effect of NO-1886 (N) or vehicle (V) on 80 mM KCl-induced contraction in aortic rings without endothelium. Each inhibitor or antagonist was administrated to the aortic rings 20 min prior to the subsequent KCl-induced vasoconstriction. After the contraction had reached a plateau level, NO-1886 (10 μM) was added to the incubation baths. Data are expressed as means ± S.D. for 4 aortic rings in each group. * $P<0.05$, ** $P<0.01$, compared with vehicle group. # $P<0.05$, compared with non-treated (none) vehicle group. Similar results were obtained from at least two independent experiments.

We first targeted PKC, ROK, calmodulin, Ca^{2+}-induced Ca^{2+} release, and MLCK, all of which are known to be responsible for the development of vascular contractile tone (Evans et al., 2005; Horowitz et al., 1996; Ratz et al., 2005; Wier and Morgan, 2003).

As shown in Fig. 5A and B, pre-treatment with either staurosporine (PKC inhibitor), H-1152 (ROK inhibitor), HA-1077 (ROK inhibitor) or W7 (calmodulin inhibitor) attenuated phenylephrine- or KCl-induced contraction (open column). Each inhibitor did not influence the inhibitory effect of NO-1886 on phenylephrine- and KCl-induced contraction (Fig. 5A and B). Combination of W7 and H-1152 also failed to prevent the inhibitory effect of NO-1886 on KCl-induced vasoconstriction (Fig. 5B). Blocking the Ca^{2+}-induced Ca^{2+} release pathway with ryanodine and nicotinamide also had no influence on the effect of NO-1886 in KCl-stimulated aortic rings (Fig. 5B). On the other hand, pre-treatment with ML-9, an MLCK inhibitor, completely blocked the inhibitory effect of NO-1886 on stimulant-induced vasoconstriction (Fig. 5A and B).

Fig. 5C summarizes the effect of each inhibitor on NO-1886-induced vasodilation in rings pre-contracted by 80 mM KCl. Since KCl-stimulated rings could not sustain their plateau contractile level in the presence of staurosporine, and as phenylephrine-stimulated rings could also not sustain their plateau contraction level in the presence of each inhibitor or antagonist, we were unable to evaluate NO-1886-induced vasodilation in these rings. On the other hand, KCl-stimulated rings could sustain their plateau contractile level in the presence of either H-1152, HA-1077, W7, or ryanodine+nicotinamide. As shown in Fig. 5C, these inhibitors failed to affect NO-1886-induced vasodilation (Fig. 5C). On the other hand, NO-1886 did not relax the aortic rings in the presence of ML-9 (Fig. 5C), indicating a dependency on MLCK pathways.

Several other enzymes including ERK have been reported to be activated by membrane depolarization (Zubkov et al., 2002). We therefore investigated the effect of ERK inhibition on vasoconstriction. The inhibition of ERK by PD98059 significantly attenuated phenylephrine- or KCl-induced contraction (Fig. 5A and B). However, PD98059 failed to inhibit the NO-1886-induced vasorelaxation or inhibition of vasoconstriction (Fig. 5A and C).

4. Discussion

NO-1886 belongs to the class of lipoprotein lipase activators (Tsutsumi et al., 1993), with treatment of the drug in experimental animals resulting in activation of tissue lipoprotein lipase and a reduction in the levels of plasma triacylglycerol (Chiba et al., 1997; Tsutsumi et al., 1993, 1995). This lipid-lowering action of NO-1886 has been investigated widely on the expectation that it may provide a strategy for treating metabolic disorders such as diabetes and obesity (Kusunoki et al., 2000, 2002). In this regard, treatment of experimental animals with NO-1886 increased whole body insulin sensitivity (Kusunoki et al., 2000). However, the underlying mechanisms responsible for the NO-1886-mediated improvement of insulin resistance remain to be clarified. Because the reduction of vascular resistance is closely associated with the improvement of insulin sensitivity (Rattigan et al., 1997) and because chronic treatment of rats with NO-1886 resulted in the amelioration of vascular endothelial function (Hara et al., 1998), we investigated the direct effect of NO-1886 on vascular contractility in order to further characterize the biological effects of NO-1886.

Our results showed that NO-1886 attenuated and relaxed both phenylephrine- and KCl-induced aortic contraction regardless of the presence or absence of aortic endothelium. These findings seem different from those of the previous report of Hara et al. who demonstrated a three month treatment of rats with NO-1886 enhanced endothelium-dependent vasodilation (Hara et al., 1998). Thus, their findings (Hara et al., 1998) might be attributed to the chronic effect of the drug that would influence the levels of metabolic parameters and/or gene expression both of which might affect endothelial function. In fact, Hara et al. (Hara et al., 1998) did not examine the effect of chronic NO-1886 treatment on the vasoconstrictor-induced contractile force of aortic smooth muscle itself.

Hence, our findings suggested a common mechanism exists in the targets within the smooth muscle contraction system that are affected by acute NO-1886 treatment. In general, phenylephrine binds to α_1-adrenoceptor on the plasma membrane, resulting in the activation of phospholipase C, which generates inositol 1,4,5-triphosphate (IP_3) and diacylglycerol through the degradation of phosphatidyl-inositol 4, 5-diphosphate (Wier and Morgan, 2003). Vascular smooth muscle contraction is then triggered by an increase in $[Ca^{2+}]_i$ induced by Ca^{2+} mobilization from the sarcoplasmic reticulum and a membrane depolarization-induced Ca^{2+} inflow from the extracellular spaces (Horowitz et al., 1996; Salamanca and Khalil, 2005). This vasoconstrictor-stimulated Ca^{2+} influx from the extracellular spaces is mainly through L-type Ca^{2+} channels (Horowitz et al., 1996). On the other hand, various types of K^+ channels exist in smooth muscle cells, including voltage dependent K^+ channels, Ca^{2+}-activated K^+ channels, ATP-sensitive K^+ channels, and inward rectifier K^+ channels (Nelson and Quayle, 1995). These K^+ currents hyperpolarize the smooth muscle cell membrane and prevent the entry of Ca^{2+} by closing the L-type Ca^{2+} channels, leading to vasorelaxation (Nelson and Quayle, 1995). Thus, high environmental K^+ levels result in membrane depolarization accelerating Ca^{2+} entry from extracellular spaces (Ratz et al., 2005). The lack of a blocking effect of high environmental K^+ levels on the inhibitory effect of NO-1886 on vasoconstriction suggests potassium currents are not involved in NO-1886 action.

Within the contractile machinery, release of Ca^{2+} from sarcoplasmic reticulum is activated by IP_3 receptors and ryanodine receptors, both of which contribute to the initial transient increase in $[Ca^{2+}]_i$ (Evans et al., 2005; Horowitz et al., 1996; Wier and Morgan, 2003). It is also known that ryanodine receptors are activated by influx of Ca^{2+} from the extracellular spaces, generally called Ca^{2+}-induced Ca^{2+} release (Evans et al., 2005; Wier and Morgan, 2003), during which process cyclic adenosine diphosphate-ribose (cADPR) is generated (Evans et al., 2005). As high K^+-induced membrane depolarization can activate Ca^{2+}-induced Ca^{2+} release without affecting IP_3 generation (Ratz et al., 2005), and given the fact that increases in $[Ca^{2+}]_i$ form the molecular basis of vascular contractility, we examined the involvement of Ca^{2+} in the actions of NO-1886.

Our experiments showed that the degree of aortic contraction was largely decreased in the absence of extracellular Ca^{2+} and/

or the presence of the L-type Ca^{2+} channel inhibitor, verapamil, or Ca^{2+}-induced Ca^{2+} release inhibitors, ryanodine and nicotinamide. Our results showed that treatment with ryanodine and nicotinamide had little effect on the action of NO-1886, whereas blockade of extracellular Ca^{2+} entry in Ca^{2+}-free medium or use of verapamil abolished the effects of NO-1886. Furthermore, pre-treatment with NO-1886 attenuated contractions induced by the cumulative addition of $CaCl_2$. These results suggested that the inhibitory effect of NO-1886 on vascular contraction was dependent on extracellular Ca^{2+} influx, but not on the release of Ca^{2+} from the sarcoplasmic reticulum.

The dependence of NO-1886-induced attenuation of vasoconstriction on extracellular Ca^{2+} led us to examine the effect of NO-1886 on $[Ca^{2+}]_i$. Our results showed that pre-treatment with NO-1886 attenuated the transient increase in $[Ca^{2+}]_i$ induced by KCl administration in cultured A10 smooth muscle cells. On the other hand, NO-1886 had no effect in the absence of extracellular Ca^{2+}. We therefore conclude that NO-1886 caused transient inhibition of Ca^{2+} influx from extracellular spaces. Given the fact that verapamil blocked the effect of NO-1886 on contractile force (Fig. 3C–E), we assumed that NO-1886 inhibited L-type Ca^{2+} channels. However, administration of NO-1886 to A10 cells during the KCl-induced sustained phase of $[Ca^{2+}]_i$ increase failed to decrease $[Ca^{2+}]_i$, suggesting that the action of NO-1886 was independent of L-type Ca^{2+} channels. Although the involvement of other Ca^{2+} channels or exchangers remains unclear, the observed decline in the transient increase in $[Ca^{2+}]_i$ induced by NO-1886 may delay the development of contraction in the smooth muscle cells. On the other hand, NO-1886 relaxed smooth muscle without changing $[Ca^{2+}]_i$ in KCl-induced aortic rings, indicating that the change in $[Ca^{2+}]_i$ itself does not fully explain the vasorelaxant effect of NO-1886. Taken together these results suggested that another contractile component with Ca^{2+}-dependency, namely the Ca^{2+} sensitivity (Ratz et al., 2005), may also be involved in the inhibitory effect of NO-1886 on vascular contractility.

Intracellular Ca^{2+} binds calmodulin to form a Ca^{2+}-calmodulin complex, which activates MLCK (Horowitz et al., 1996; Wier and Morgan, 2003). The activation of other kinases such as PKC and ROK inhibit MLC phosphatase activity, leading to Ca^{2+}-independent increases in the MLCK/MLC phosphatase ratio thereby facilitating MLC phosphorylation (Horowitz et al., 1996; Ratz et al., 2005; Somlyo and Somlyo, 2003). The phosphorylated MLC then interacts with actin leading to the generation of contractile force (Horowitz et al., 1996; Wier and Morgan, 2003). In the present study, we investigated whether these molecules had a role in the actions of NO-1886. Although inhibition of MLCK with ML-9 almost completely blocked the inhibitory effect of NO-1886 on vasoconstriction, the inhibitors of the other signaling molecules including calmodulin, PKC or ROK did not block the effects of NO-1886. In addition, inhibition of ERK by PD98059, which has been reported to be activated by elevation in $[Ca^{2+}]_i$ (Zubkov et al., 2002), had no effect on the action of NO-1886. Several other signaling components also participate in the phosphorylation of MLC in cells. For example, kinases such as integrin-linked kinase and zip kinase phosphorylate MLC and induce smooth muscle contraction (Haystead, 2005; Wilson et al., 2005). However, these types of MLC phosphorylation occur independent of Ca^{2+} signals (Haystead, 2005; Wilson et al., 2005). The involvement of these kinases in NO-1886-induced inhibition of smooth muscle contraction was not investigated in our study.

The phosphorylation of MLC represents the final stage of smooth muscle contraction (Horowitz et al., 1996; Salamanca and Khalil, 2005). Somlyo et al. (Somlyo et al., 2004) reported that MLC phosphorylation could be observed even in cultured aortic cells from MLCK knock out mice (MLCK −/−). However, this phosphorylation was only detected following the treatment of cells with lysophosphatidic acid, an activator of ROK (Somlyo et al., 2004). Whether other kinases also contributed to the phosphorylation of MLC in the MLCK −/− mice remains unclear (Somlyo et al., 2004). In our study, the level of aortic contraction was weak but detectable in the presence of ML-9. NO-1886 failed to inhibit the phenylephrine- or KCl-induced contraction in these ML-9-treated ring preparations. Therefore, it is unlikely that NO-1886 would attenuate MLCK-independent smooth muscle contraction. Taken together, our study suggested that NO-1886 inhibited MLCK-dependent vascular smooth muscle contraction by extracellular Ca^{2+}-dependent mechanisms. The identification of the related enzymes or components which mediate this effect of NO-1886 is currently under-researched.

In summary, we showed that NO-1886 inhibited stimulant-induced contraction and induced vasodilation in rat vascular smooth muscle. NO-1886 has two actions, namely inhibition of the initial transient $[Ca^{2+}]_i$ elevation induced by membrane depolarization, and inhibition of Ca^{2+} influx-dependent contractile components in vascular smooth muscle cells. Hara et al. (Hara et al., 1998) showed that treatment with NO-1886 attenuated the age-related decline in endothelium-dependent relaxation in the rat aorta. However, on the basis of our experiments we conclude that the acute inhibitory effect of NO-1886 on vascular contraction is endothelium-independent. Thus, contractile system of the blood vessels is one of several pharmacological targets of the lipoprotein lipase activator NO-1886.

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