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JTV-506, a new K_{ATP} channel opener, relaxes pulmonary artery isolated from monocrotaline-treated pulmonary hypertensive rats

Yohsuke Tsutsumi[1], Tetsuji Makita[2], Koji Yamaguchi[2], Osamu Shibata[1], and Koji Sumikawa[1]

[1]Department of Anesthesiology, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
[2]Division of Intensive Care Unit, Nagasaki University Hospital, Nagasaki, Japan

Abstract
Purpose. Vasodilators are considered effective in the treatment of pulmonary hypertension if the vascular reactivity remains reversible. This study was designed to investigate the possibility that JTV-506, a new adenosine trisphoshate-sensitive potassium channel opener, may serve as a useful vasodilator in the treatment of pulmonary hypertension.
Methods. After approval by the animal care committee, with the use of the isometric-force recording method, the effects of JTV-506 (1 nM–100 μM) on the contractile response to norepinephrine (0.1 μM) were examined in pulmonary arteries isolated from monocrotaline (MCT)-treated (i.e., presumed pulmonary hypertensive) and age-matched control rats. The experiments were performed in the presence of endothelium with or without treatment with N^G-nitro-L-arginine (L-NAME, 100 μM), and in its absence.
Results. JTV-506 relaxed ($P < 0.05$) norepinephrine-preconstricted, endothelium-intact arteries from MCT-treated and control rats. However, the vasorelaxation was greater ($P < 0.05$) in the arteries from MCT-treated rats than in controls. L-NAME treatment attenuated ($P < 0.05$) vasorelaxation in the arteries from both MCT-treated and control rats. However, endothelial removal attenuated ($P < 0.05$) vasorelaxation only in the arteries from MCT-treated rats and not in control arteries.
Conclusion. JTV-506 may possibly attenuate pulmonary vascular tone through its direct action on vascular smooth muscle cells. In the presence of MCT-induced pulmonary hypertension, JTV-506 may further attenuate pulmonary vascular tone through its direct action on endothelial cells, possibly by stimulating the endothelial release of NO.

Key words JTV-506 · Monocrotaline · Rat · Pulmonary hypertension

Address correspondence to: Y. Tsutsumi
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Introduction

There is growing evidence that adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channel opener has a vasodilating effect on multiple tissues [1–4]. K_{ATP} channels are believed to play an important physiological role in mediating vascular responses to a variety of endogenous vasodilators, such as calcitonin-gene related peptide or NO, as well as to changes in metabolic activity that can directly influence blood flow in various tissues. In addition, activation of the K_{ATP} channels appears to be responsible for vasodilation observed in some pathophysiological states, such as shock or hypoxia. In contrast, inhibition of K_{ATP} channels may be involved in vasoconstrictor responses to endothelin, vasopressin, or angiotensin. Over the last two decades, a number of pharmacological compounds have been found to activate the K_{ATP} channels in a variety of vascular beds, and some of those K_{ATP} channel openers have been suggested to be effective in treating systemic hypertension, angina pectoris, or cerebral ischemia. However, less information seems to be available regarding its potential efficacy in pulmonary hypertension [2,5,6].

Monocrotaline (MCT) is a toxic pyrrolizidine alkaloid extracted from the plant *Crotalaria spectabilis* [7]. A single administration of MCT to rats can induce pulmonary edema, pulmonary arteritis, pulmonary hypertension, and right ventricular hypertrophy similar to that which occurs in response to chronic hypoxia. MCT-treated rats have been widely used as experimental models of primary pulmonary hypertension [8–11]. In previous pathological studies, the pulmonary arterial endothelial cells have been shown to be significantly injured as early as 4 days after treatment with MCT, and eventually exfoliated 4 weeks after the treatment. Increases in interstitial cells and medial hypertrophy have been reported in vascular smooth muscle [12]. JTV-506 ((-)-(3S,4R)-2.2-bis(methoxymethyl)-4-[(1,6-dihydro-l-methyl-6-oxo-3-pyridazinyl)amino]-3-

hydroxychroman+++-6-carbonitrile hemihydrate), a newly synthesized benzopyran-derivative K_{ATP} channel opener, has been shown to cause vasodilation in the coronary circulation [13–15]. However, less information is available regarding the effects of JTV-506 on vascular reactivity in the pulmonary circulation. In this study, we therefore examined the ability of JTV-506 to cause vasodilation in the pulmonary circulation. Specifically, we tested the effects of JTV-506 on the contractile response to norepinephrine in pulmonary arteries isolated from MCT-treated (i.e., presumed pulmonary hypertensive) and age-matched control rats. Since JTV-506 caused pulmonary vasodilation in the presence of endothelium in both MCT-treated and control rats, in order to gain access to its underlying mechanisms, we further investigated the effects of endothelial removal and inhibition of NO synthesis on JTV-506-induced vasodilation.

Methods

The protocol for this study was approved by our institutional animal care committee.

Treatment with monocrotaline

Forty-five male Wistar rats weighing 200–250 g (7–8 weeks old) were anesthetized with an intraperitoneal injection of sodium pentobarbital 50 mg·kg^{-1} and then treated with a subcutaneous injection of a single dose of either MCT [105 mg·kg^{-1} (= 1 ml·kg^{-1}) or saline (1 ml·kg^{-1})] 3 weeks prior to the experiments. To determine the extent of MCT-induced right ventricular hypertrophy, the ventricles were separated from the vessels and atria. The right ventricular free wall (RV) was cut from the left ventricle and septum (LV + S) and weighed separately. The ventricular weight ratio was determined from RV/(LV + S).

Isometric force recording experiments

After the Wistar rats pretreated with either MCT or saline were anesthetized by intraperitoneal injection of sodium pentobarbital 50 mg·kg^{-1}, a median sternotomy was performed, and then heparin 100 U was injected directly into the right ventricle. After the rats were exsanguinated, the extrahilar pulmonary artery was isolated and then cut into two or three rings 2–3 mm in width. The pulmonary artery rings were set up for the isometric recording of tension changes in Krebs-Henseleit solution (pH 7.35–7.45) maintained at 37°C and bubbled with 95% O_2 and 5% CO_2.

The resting tension of 750 mg defined by the preliminary study was progressively applied, and the rings were allowed to equilibrate for 30 min.

The rings were exposed to 80 mM KCl to confirm the integrity of the smooth muscle cells. If the rings did not constrict up to 750 mg, they were discarded. The rings were then washed with Krebs-Henseleit solution three times followed by a second equilibration period of at least 30 min. After the second equilibration period, the rings were constricted with norepinephrine (NE) 0.1 μM at a final concentration that caused a submaximal contraction (75%–80% of the maximal constriction induced by NE) in the preliminary study. Fifteen minutes later, acetylcholine (ACh), 1 μM, was added to confirm the function of the endothelium pharmacologically. If the rings were not dilated by ACh, they were discarded. Then the rings were washed with Krebs-Henseleit solution several times, followed by a third equilibration period of at least 90 min.

After the pulmonary artery rings had been precontracted with NE 0.1 μM, JTV-506 was added in a cumulative fashion from 1 nM to 100 μM, and a cumulative concentration-response curve was constructed.

Using the same protocol as that used in the above series of experiments, we also examined the effects of JTV-506 on the contractile response to NE in the endothelium-intact rings treated with an NO synthesis inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), 100 μM, or in the endothelium-denuded rings. The endothelium was removed by inserting forceps into the vessel lumen and rolling the ring over damp filter paper. Endothelial removal was confirmed by the inability of ACh 1 μM to cause significant relaxation (>5%) during contractions induced by NE 0.1 μM.

Drugs

The following drugs were used: monocrotaline, norepinephrine, acetylcholine, and N^G-nitro-L-arginine methyl ester (Sigma Chemical, St. Louis, MO, USA). JTV-506 was a gift of the Japan Tobacco Central Pharmaceutical Research Institute (Fig. 1).

We expressed the concentration of the drugs as the final molar concentration in the bath solution. The stock solution of JTV-506 was dissolved in ethanol and then diluted in distilled water.

Monocrotaline was dissolved in HCl 1.0 N, and the pH was adjusted to 7.4 with NaOH 1.0 N. The other substances were prepared in distilled water. The final concentration of the solvents in the organ bath was less than 1.0%.

Data and statistical analysis

The relaxation caused by JTV-506 was expressed as the percent decrease in the tension of the vessel rings preconstricted by NE. All values were presented as mean ± SD. A two-way analysis of variance was used

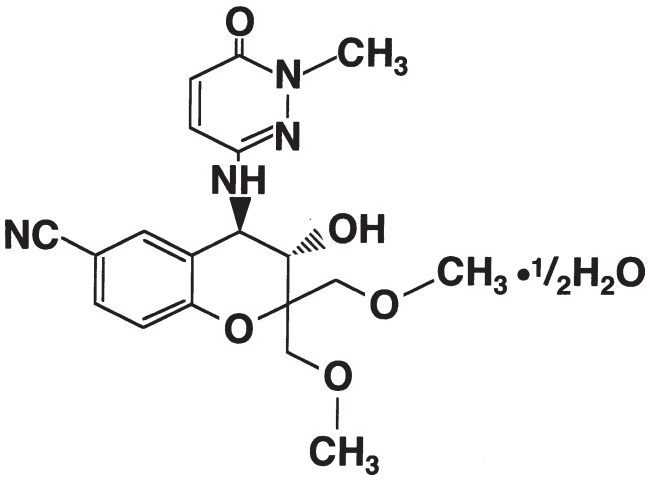

Fig. 1. Chemical structure of JTV-506

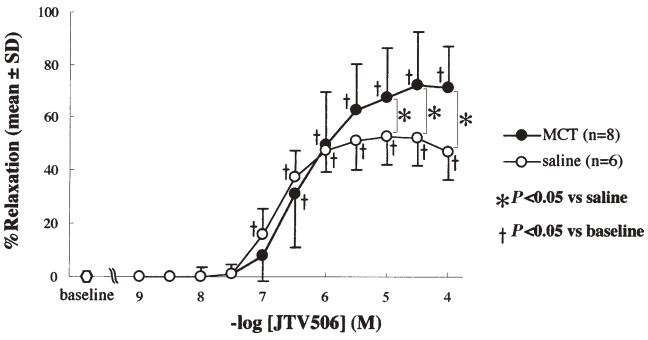

Fig. 2. Dose-response curve to JTV-506 in saline and monocrotaline (*MCT*)-treated rats. Relaxation is expressed as the percent decrease in tension of the contraction induced by norepinephrine

for intergroup comparisons. Intragroup comparisons were evaluated using a repeated-measure analysis of variance. When significance was found, Scheffé's test was used for post-hoc testing. Significant differences were determined at $P < 0.05$. In each experiment, the concentration of JTV-506 required to inhibit the NE response by 50% (IC_{50} value) was calculated as the mean ± SD of the individual IC_{50} values, using the sigmoid equation of the GraphPad curve-fitting program (GraphPad Software, San Diego, CA, USA). In all experiments, *n* represents the number of rats from which the vessel rings were obtained.

Results

The mean weight ratio of the right ventricle to the left ventricle and septum (LV + S) was significantly greater in MCT-treated rats (0.38 ± 0.14, $n = 25$) than in rats receiving saline (0.25 ± 0.03, $n = 20$).

The KCl-induced contraction of pulmonary arteries from MCT-treated and saline-treated rats was 0.61 ± 0.56 g and 0.95 ± 0.63 g, respectively. The NE-induced contraction of pulmonary arteries from MCT-treated and saline-treated rats was 0.58 ± 0.32 g and 0.92 ± 0.56 g, respectively. The mean ACh-induced dilation of pulmonary arteries from MCT-treated rats was significantly less than that of arteries from saline-treated rats (saline-treated, 67.5% ± 23.7%; $n = 6$; MCT-treated, 25.4% ± 22.8%, $n = 8$, $P < 0.05$).

JTV-506 significantly inhibited the contractile response to NE in a concentration-dependent manner in the endothelium-intact pulmonary artery rings from either the saline-treated or the MCT-treated rats. The inhibition was significantly enhanced between 10 and 100 μM JTV-506 in the rings from MCT-treated rats compared with those from saline-treated rats (saline-treated, $IC_{50} = 0.164 \pm 0.022$ μM, $n = 6$; MCT-treated, $IC_{50} = 0.458 \pm 0.067$ μM, $n = 8$, $P < 0.001$), and the maximal relaxation was significantly greater in the rings from MCT-treated rats than in those from saline-treated rats (saline-treated, 72.6% ± 1.7%, $n = 6$; MCT-treated, 52.6% ± 1.0%, $n = 8$, $P < 0.001$) (Fig. 2).

The addition of L-NAME (100 μM) led to a significant enhancement of the NE response in both the saline-treated and the MCT-treated pulmonary arteries compared with the previous response obtained in the absence of L-NAME (saline-treated arteries, 1.05 ± 0.46 g, $n = 6$, and 1.54 ± 0.56 g, $n = 8$, $P < 0.05$, respectively; MCT-treated arteries, 0.56 ± 0.16 g, $n = 8$, and 1.15 ± 0.40 g, $n = 9$, $P < 0.01$, respectively). The JTV-506-induced relaxation was attenuated by L-NAME treatment in both the saline- and the MCT-treated pulmonary arteries [saline-treated arteries before exposure to L-NAME, $IC_{50} = 0.164 \pm 0.022$ μM, $n = 6$, versus $IC_{50} = 0.253 \pm 0.047$ μM, $n = 8$, after exposure to L-NAME, $P < 0.05$ (Fig. 3); MCT-treated arteries before exposure to L-NAME, $IC_{50} = 0.458 \pm 0.067$ μM, $n = 8$, versus $IC_{50} = 0.279 \pm 0.047$ μM, $n = 9$, after exposure to L-NAME, $P < 0.05$ (Fig. 4)]. However, the JTV-506-induced relaxation was attenuated by the removal of endothelium in the MCT-treated arteries but not in the saline-treated arteries [saline-treated arteries in the presence of endothelium, $IC_{50} = 0.164 \pm 0.022$ μM, $n = 6$, versus $IC_{50} = 0.217 \pm 0.055$ μM, $n = 6$, in the absence of endothelium $P = 0.24$ (Fig. 3); MCT-treated arteries in the presence of endothelium, $IC_{50} = 0.458 \pm 0.067$ μM, $n = 8$, versus $IC_{50} = 0.372 \pm 0.084$ μM, $n = 8$, in the absence of endothelium, $P = 0.46$ (Fig. 4)].

Discussion

The results of the contraction experiments indicate that JTV-506 inhibits the contractile response to NE

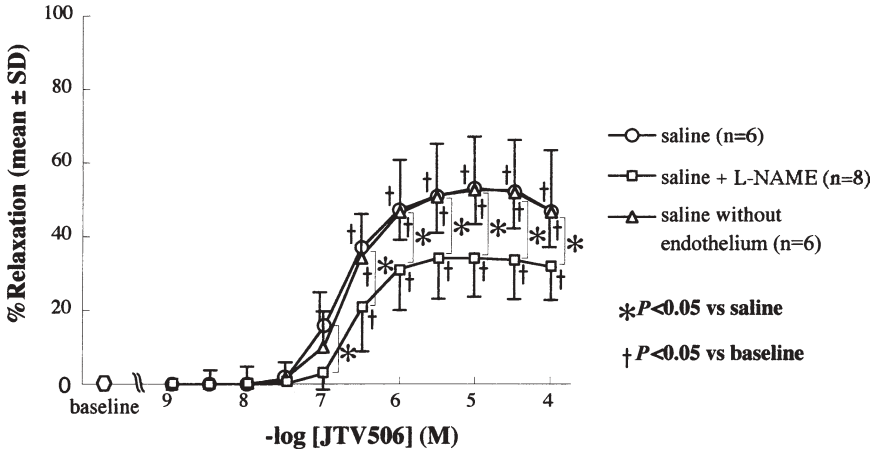

Fig. 3. Effects of pretreatment with N^G-nitro-L-arginine (*L-NAME*) and removal of endothelium on the dose-response curve to JTV-506 in saline-treated rats. Relaxation is expressed as the percent decrease in tension of the contraction induced by norepinephrine

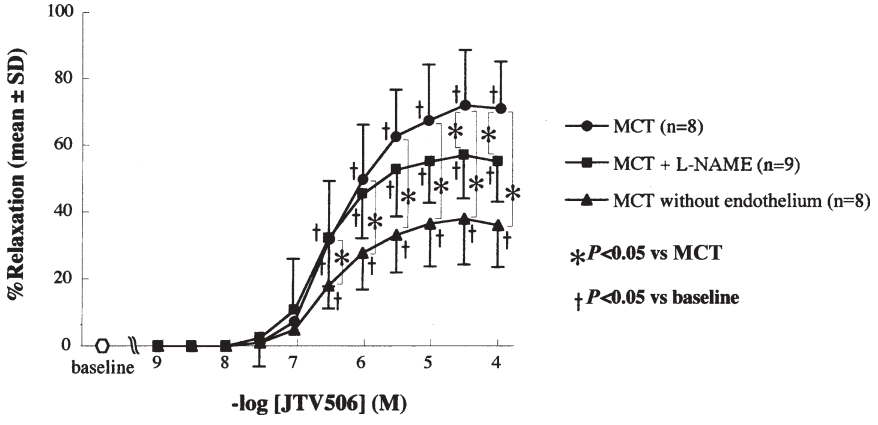

Fig. 4. Effects of pretreatment with L-NAME and removal of endothelium on the dose-response curve to JTV-506 in MCT-treated rats. Relaxation is expressed as the percent decrease in tension of the contraction induced by norepinephrine

through its direct action on vascular smooth muscular cells in both MCT-treated (i.e., presumed pulmonary hypertensive) and saline-treated rats (age-matched control rats). The observed effects of L-NAME treatment in the presence of endothelium indicated that JTV-506-induced vasorelaxation is mediated, at least in part, by NO. Since endothelial denudation significantly attenuated JTV-506-induced vasorelaxation in MCT-treated rats, endothelial NO appears to be involved in JTV-506-induced vasorelaxation in the presence of MCT-treated pulmonary hypertension. However, endothelial denudation had little influence on JTV-506-induced vasorelaxation in saline-treated rats, suggesting that nonendothelial NO may play a role in JTV-506-induced vasorelaxation.

Barron et al. [16] previously reported sustained NO production after removal of the endothelium in isolated porcine carotid arteries, suggesting that NO can be continuously produced in the absence of endothelium. Thus, JTV-506 might stimulate nonendothelial NO release in the pulmonary arteries used in this study. Barnes and Liu stated in their review that nonadrenergic, noncholinergic (NANC) nerves are believed to play a role in the control of pulmonary vascular tone, and there is convincing evidence to indicate involvement of NO in the vasodilator response mediated by NANC nerves [17]. Since the neural form of NO synthase has been shown to localize in the nerves innervating the smooth muscle of the pulmonary vessels, NO is probably released from the NANC nerve endings in the pulmonary vascular bed. JTV-506 might stimulate the release of NO from NANC nerve endings. It was previously reported that, in the lung exposed to chronic hypoxia or MCT, arterial endothelial NO synthesis and endothelial NO-dependent arterial dilation were upregulated and augmented, respectively, presumably because of altered vascular mechanical forces associated with pulmonary hypertension. Thus, it might be possible that JTV-506 exerts vasodilating action by stimulating endothelial NO production only in the presence of pulmonary hypertension. Further investigations would be necessary to clarify the underlying mechanisms behind JTV-506-induced, endothelial NO-mediated vasorelaxtion.

Previous studies have reported the existence of K_{ATP} channels in endothelial cells [18–21], suggesting that the K_{ATP} channel openers would hyperpolarize the endothelial cell membrane as well. Because endothelial cells

lack voltage-gated Ca^{2+} channels, the membrane potential is primarily involved in controlling Ca^{2+} movements across the cellular membrane by adjusting the electrochemical gradient driving Ca^{2+} entry [18,19]. In other words, endothelial cell membrane hyperpolarization would enhance the transmembrane Ca^{2+} influx into endothelial cells, which is considered essential for the endothelial synthesis and release of various vasoactive substances, including NO. Thus, the K_{ATP} channel opener might possibly enhance the vasodilator response to receptor agonists that activate transmembrane Ca^{2+} influx into endothelial cells and thereby stimulate endothelial release of NO [22,23].

Pulmonary arterial hypertension, or altered vascular mechanical forces associated with hypertension, may be responsible for the augmented endothelial NO-dependent arterial dilation and up-regulation of arterial endothelial NO synthase in lungs exposed to chronic hypoxia or MCT [24]. Robert et al. [25] demonstrated that endothelial NO synthase mRNA, but not endothelial NO synthase protein or NO production, increased in MCT-treated rat lungs. It has been reported that patients with primary pulmonary hypertension have an elevated level of NO in the breath, whereas patients with pulmonary hypertension associated with fenfluramine (an anorectic agent) do not [26,27]. Although we did not show either the expression of endothelial NO synthase mRNA and endothelial NO synthase protein or the level of NO in the pulmonary arteries in this study, MCT might contribute to the alteration of the NO pathway in the pulmonary artery.

JTV-506 has a benzopyran skeleton. The benzopyran skeleton of JTV-506 has two methoxymethyl groups instead of methyl groups at position 2. It is speculated that the two methoxymethyl groups play an important role in coronary selective action [13]. Atwal et al. [28] also reported a series of compounds with a benzopyran ring that have cardioselective actions. The benzopyranylcyanoguanidine type K_{ATP} channel opener has antiischemic efficacy in animal models of myocardial ischemia without an effect on peripheral hemodynamic variables. Atwal et al. speculate that receptor subtypes exist in smooth muscle and cardiac tissue. We also consider that some receptor subtypes might be expressed in the pulmonary arteries under various pathophysiological conditions. Whether K_{ATP} channel openers act solely by opening K_{ATP} channels is still debatable [29]. Further investigation is required to identify the binding protein for K_{ATP} channel openers and probe their relation with K_{ATP} channels in different vascular tissues.

Neither pulmonary arterial nor right ventricular pressure was measured in this study. Thus, it is not clear whether pulmonary hypertension developed in the MCT-treated rats used in this study. However, our comparison of the ventricular weight ratio [RV/(LV + S)] in MCT-treated and saline-treated rats indicated that right ventricular hypertrophy had indeed developed in our MCT-treated rats. In addition, previous studies [7,12] have demonstrated that pulmonary arterial hypertension occurs 3 weeks after treatment with MCT in rats. Thus, we believe that pulmonary hypertension developed in our MCT-treated rats.

We did not examine the effects of glibenclamide on JTV-506-induced vasodilation. Ando et al. [14] reported that JTV-506 caused an inhibition of histamine-induced contraction in guinea-pig isolated tracheal smooth muscle, and was antagonized by glibenclamide. Hirata et al. [15] found that glibenclamide caused a rightward shift of the concentration-response curve for JTV-506 in porcine isolated coronary arteries without endothelium. Therefore, we postulate that, in this study, JTV-506 caused pulmonary vasodilation mainly by opening K_{ATP} channels.

In conclusion, JTV-506 may possibly attenuate pulmonary vascular tone through its direct action on vascular smooth muscular cells. In the presence of MCT-induced pulmonary hypertension, JTV-506 may further attenuate pulmonary vascular tone through its direct action on endothelial cells, possibly stimulating the endothelial release of NO. It would be worthwhile to further investigate the possible usefulness of JTV-506 in the treatment of pulmonary hypertension.

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