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# Contribution of Chloride Channel Activation to the Elevated Muscular Tone of the Pulmonary Artery in Monocrotaline-Induced Pulmonary Hypertensive Rats

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ABSTRACT—In monocrotaline-treated rat pulmonary artery from which endothelium was removed, greater spontaneous muscular tone was observed under resting conditions than in vehicle-treated artery. The aim of the present study was to show the possible contribution of $Cl^-$ channels in the mechanism of the elevated tone. Verapamil almost completely inhibited the elevated spontaneous muscular tone by decreasing $[Ca^{2+}]_i$. The elevated muscular tone was also inhibited by 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS), a $Cl^-$ channel inhibitor. After the inhibition of muscular tone by DIDS, verapamil did not induce further relaxation. Quantitative RT-PCR analysis indicated that the mRNA levels of ClC3 and $Ca^{2+}$-activated $Cl^-$ channels did not change in the pulmonary hypertensive pulmonary artery from those of vehicle-treated rats. These results suggest that the elevated muscular tone observed in the monocrotaline-induced hypertensive pulmonary artery is due to membrane depolarization of smooth muscle cells and that this phenomenon might be mediated by the activation of DIDS-sensitive $Cl^-$ channels.

*Keywords*: $Cl^-$ channel, Pulmonary hypertension, Smooth muscle, Vasoconstriction, Monocrotaline

Pulmonary hypertension is characterized by an elevation of pulmonary blood pressure without changing the systemic circulation. Major pathophysiological changes found in patients with this disease include right ventricular hypertrophy and pulmonary vascular remodeling. Because the chronic exposure of rats to a hypoxic environment or a single injection of monocrotaline (MCT), a plant toxin pyrrolizidine alkaloid, can mimic pulmonary hypertension with pathophysiological changes similar to those in humans, these rats have been widely used as an experimental model of pulmonary hypertension (1 – 4).

Accumulating evidence indicates a potential role for endothelial dysfunction as an early event in pulmonary hypertension (5 – 7). We have recently found that NO-mediated arterial relaxation in the pulmonary artery from MCT-treated rats is impaired, although the expression of eNOS mRNA is increased in the MCT rat artery (8). We have further suggested in the report that the dissociation between eNOS expression and NO production is due to an inhibition of receptor-mediated $Ca^{2+}$ metabolism in endothelial cells, as well as to an apparent decrease in the $Ca^{2+}$ sensitivity of eNOS. The endothelium dysfunction also contributes to the induction of vascular remodeling, including increases in medial thickness and connective tissue (9 – 12).

Several pathophysiological changes in arterial smooth muscle cells have also been observed in the pulmonary hypertensive rat. The sensitivities to norepinephrine, prostaglandin and serotonin are also increased in the pulmonary artery of the hypertensive rat (13, 14). In addition, the amount of endothelin-1 in the arterial tissue has also been reported to be increased (15, 16), and daily infusion of an $ET_A$-receptor blocker, BQ-123, has been shown to inhibit cardiopulmonary changes in the MCT-induced hypertensive rat (17). In contrast, Suzuki and Twarog (18) have observed that the membrane of pulmonary arterial cells isolated from the pulmonary hypertensive rat is depolarized in the resting state. Ito et al. (19) also reported that MCT-treatment depolarized smooth muscle membrane in the pulmonary arterial tissue. However, the mechanism of the membrane depolarization due to pulmonary hypertension remains unknown. The present study was undertaken to elucidate the mechanism for elevated membrane potential in the pulmonary artery of the MCT-induced pulmonary hypertensive rat.

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## MATERIALS AND METHODS

*MCT-induced pulmonary hypertensive rats and tissue preparation*

MCT (300 mg) was dissolved in 1.8 ml of 1 M HCl, followed by the addition of 3 – 4 ml of distilled water. This solution was adjusted to pH 7.4 with 1 M NaOH and brought to a volume of 15 ml with distilled water (1). MCT (60 mg · $kg^{-1}$) or its vehicle was administered to 6-week-old male Sprague Dawley rats (180 – 210 g) as a single subcutaneous injection. Rats were housed with a 12:12-light-dark cycle and given water and standard rat chow ad libitum. All experiments were performed 21 days after the administration. Pulmonary hypertension was confirmed by right ventricular hypertension, as described by Nakazawa et al. (8). Rats were killed by a sharp blow to the neck and exsanguination. Heart and lungs were removed *en bloc*, and right and left extra-pulmonary arteries were dissected. Except as otherwise stated, endothelium was gently rubbed with a glass rod moistened with physiological salt solution (PSS).

*Solutions*

PSS, which contained 136.9 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.5 mM glucose and 5.0 mM HEPES, was saturated with 100% $O_2$ at 37°C. The pH was adjusted to 7.3 – 7.4 with 1 M NaOH at 37°C. The high $K^+$ solution (72.7 mM) was made by replacing NaCl with equimolar KCl. $Ca^{2+}$-free solution was made by omitting $CaCl_2$ and adding 0.5 mM EGTA.

*Histology*

The pulmonary arteries isolated from vehicle- or MCT-treated rats were fixed in 10% neutral buffered formalin and embedded in paraffin. Four-micrometer-thick sections were stained with hematoxylin and eosin and then examined under a light microscope.

*Measurements of muscle force*

Pulmonary arteries without endothelium were cut into rings approximately 2-mm wide. Muscle tension was recorded isometrically under a resting tension of 10 mN. At the end of each experiment, 100 $\mu$M papaverine was added to determine the basal tone (0%).

*Simultaneous measurement of intracellular $Ca^{2+}$ levels and muscle force*

Intracellular $Ca^{2+}$ levels ($[Ca^{2+}]_i$) were measured simultaneously with muscle contractions, as described by Ozaki et al. (20), using a fluorescent $Ca^{2+}$ indicator, fura-PE3. Briefly, endothelium-denuded pulmonary arterial tissue (1-mm-wide) was incubated in PSS with a 10 $\mu$M acetoxymethyl ester of fura-PE3 and 0.02% cremophor EL for 3 – 4 h at 37°C. Experiments were performed with a fluorimeter (CAF-110; Japan Spectroscopic, Tokyo), and the ratio of F340 to F380 (F340/380) was used as an indicator of $[Ca^{2+}]_i$. We took the ratio and muscle force under the resting condition to be 0% and that under the high $K^+$-stimulated condition to be 100%.

*Quantitative RT-PCR*

Total RNA extraction and quantitative RT-PCR was performed by a previously described procedure (8). The oligonucleotide primers for ClC3 designed from rat ClC3 mRNA (D17521) were ACC AGC TAT AAT GGC TTT CC (sens) and CTA CCA CAA TCT CCA TTG GG (reverse). The oligonucleotide primers for $Ca^{2+}$-activated $Cl^-$ channels (ClCA) designed from rat ClCA mRNA (AF077303) were ACT TCC GGT CTG ATA CCT AA (sens) and TTG GCC AGA ATT GCA ATG TA (reverse). The expected sizes of the PCR products for ClC3 and ClCA were 259 base pairs (bp) and 110 bp, respectively. The PCR products were electrophoresed on 2% agarose gel containing 0.1% ethidium bromide. We visualized detectable fluorescent bands with an ultraviolet (UV)-transilluminator and saved the image using FAS III (Toyobo, Tokyo).

*Drugs*

Drugs used were MCT, papaverine, verapamil, 4,4'-diisothiocyanate-stilbene-2,2'-disulfonic acid (DIDS), IAA-94 (Sigma, St. Louis, MO, USA); BQ-123 (Peptide Institute, Inc., Mino-shi); and fura-PE3 (Teflabs, Austin, TX, USA). RES-701-1 was generously donated by Kyowa Hakko Kogyo Co., Ltd. (Tokyo).

*Statistical analyses*

Results are expressed as means ± S.E.M. Comparison between the control and test groups was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Differences with $P$ values less than 0.05 were considered to be statistically significant.

## RESULTS

*Morphological change in the pulmonary artery of the MCT-induced pulmonary hypertensive rat*

Pulmonary arteries of the vehicle- and MCT-treated rats were stained with hematoxylin and eosin and examined under a light microscope (Fig. 1A). Endothelial cells of vehicle- and MCT-treated arteries were located along the inner surface, which appeared to be intact in both preparations. In contrast, in the media layer in the hypertensive pulmonary artery, smooth muscle cell bodies and nuclei seemed to be swelling. Furthermore, thickenings of media and adventitia were observed in the pulmonary artery of MCT-treated rat.

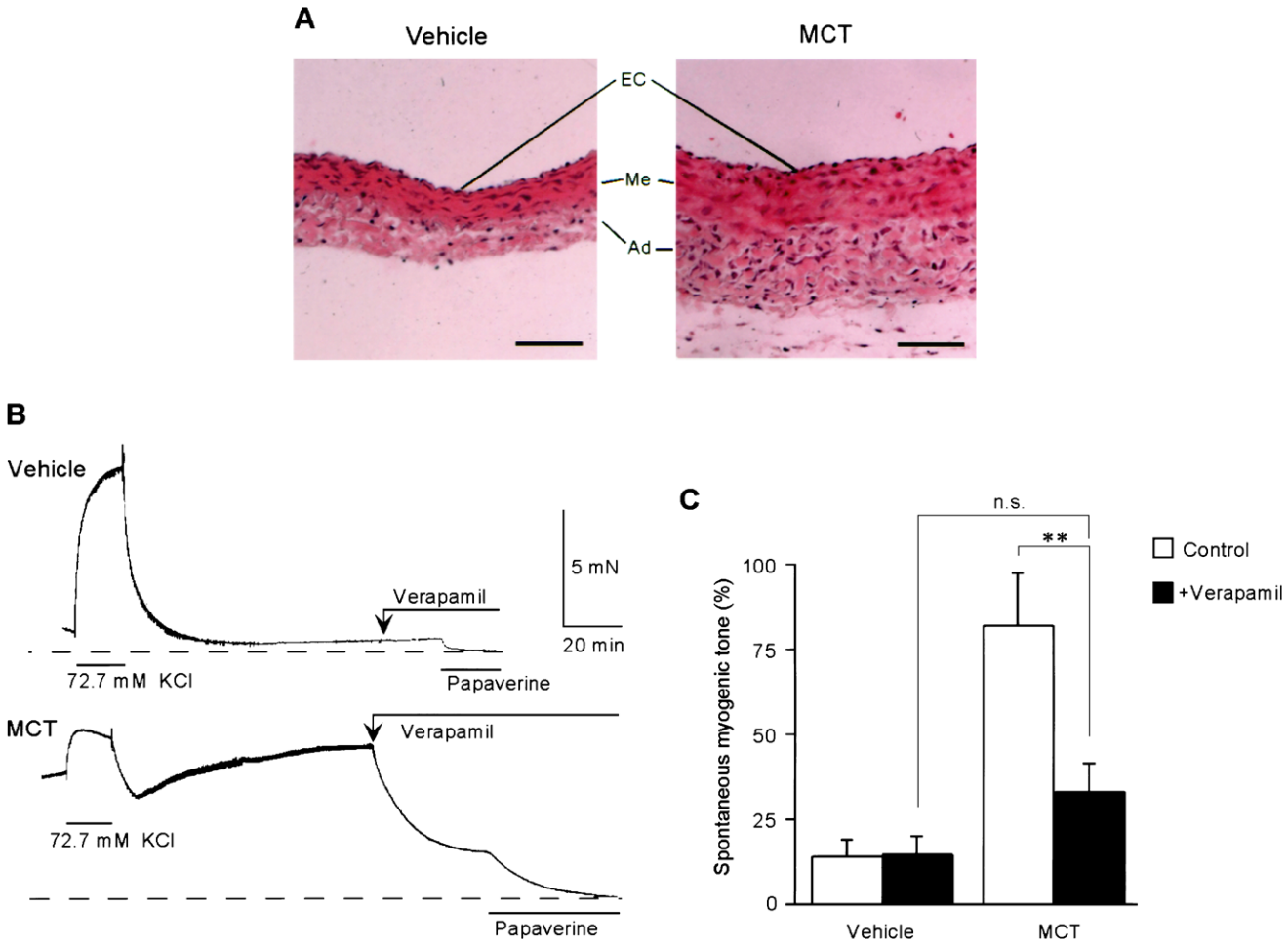


**Fig. 1.** Morphological and functional changes in pulmonary artery in the MCT-treated rat. A: Representative light micrographs of sections stained with hematoxylin and eosin in pulmonary artery. Left: Pulmonary artery isolated from the vehicle-treated rat. Right: Pulmonary artery isolated from MCT-treated rat at 21 days after injection of MCT. Scale bar = 100 $\mu$m. EC: endothelial cell, Me: media, Ad: adventitia. B: Effect of verapamil on spontaneous muscular tone in the pulmonary artery. Typical trace of the spontaneous muscular tone in the pulmonary artery of the vehicle-treated rat (upper trace) or MCT-treated rat (lower trace) is shown. C: The summarized results from panel B (n = 5 each). Contractions elicited by 72.7 mM KCl and resting tension in the presence of 10 $\mu$M verapamil and 100 $\mu$M papaverine were taken as 0% and 100%, respectively. **Significantly different from the control ($P$<0.01).

*Elevation of spontaneous active muscular tone in MCT-treated rats*

In the endothelium-denuded, vehicle-treated rat pulmonary artery, 72.7 mM KCl induced a sustained contraction (Fig. 1B). In the resting state, the addition of 10 $\mu$M verapamil had no appreciable effect on the basal tone. However, 100 $\mu$M papaverine, a non-selective and potent inhibitor of smooth muscle contraction, slightly decreased the basal active tone. In contrast, in MCT-treated, endothelium-denuded pulmonary artery, 72.7 mM KCl induced only a very small contraction compared to that in the vehicle-treated rat. Furthermore, the addition of 10 $\mu$M verapamil induced a greater decrease in the basal active tone far below the resting level, compared to that in the vehicle-treated artery. Papaverine (100 $\mu$M) further decreased the basal tone. Figure 1C provides a summary of the results shown in Fig. 1B. It is noted that the active muscular tonus in the vehicle- and MCT-treated rats was 13.6 ± 5.1% (n = 5) and 81.3 ± 15.6% (n = 5), respectively, when the muscle tonus in the presence of both verapamil and papaverine was taken as 0% and a high $K^+$-induced sustained contraction was taken as 100%, respectively. The $ET_A$-receptor antagonist (BQ-123, 3 $\mu$M) and $ET_B$-receptor antagonist (RES701-1, 10 $\mu$M) appeared to have no effect on the spontaneous muscular tone.

*Effect of verapamil on cytosolic $Ca^{2+}$ levels ($[Ca^{2+}]_i$)*

Using a fura-PE3-loaded pulmonary arterial strip, we simultaneously measured $[Ca^{2+}]_i$ and muscle contractions (Fig. 2). In this series of experiments, $[Ca^{2+}]_i$ levels at

resting and at high $K^+$-stimulation were taken as 0% and 100%, respectively, because papaverine interfered with the fluorescent intensity of fura-PE3. In the pulmonary artery from vehicle-treated rats, 10 $\mu$M verapamil decreased the resting $[Ca^{2+}]_i$ and muscle force only by 7.8 ± 2.1% and 7.4 ± 1.8% (n = 5), respectively. In contrast, in the artery from the MCT-treated rat, 10 $\mu$M verapamil decreased the resting $[Ca^{2+}]_i$ by 46.5 ± 12.3%, which was accompanied by a 27.6 ± 7.3% (n = 7) decrease in muscle force.

*Role of the $Cl^-$ channel in spontaneous muscular tone*

We examined the effects of $Cl^-$ channel blockers on spontaneous muscular tone and 24 mM KCl-induced contractions. DIDS (100 and 300 $\mu$M) did not inhibit the 24 mM KCl-induced contraction in vehicle-treated and MCT-treated arteries, as shown in Fig. 3A (upper trace) and Fig. 3B. In contrast, DIDS inhibited the spontaneous active tone in MCT-treated artery in a concentration-dependent manner (n = 7, Fig. 3A lower trace), but it had no effect on the spontaneous tone in vehicle treated artery (n = 5, data not shown). Verapamil (10 $\mu$M), added in the presence of DIDS, had no effect on the spontaneous muscular tone. However, the addition of 100 $\mu$M papaverine further decreased the spontaneous active tone, as shown in Fig. 3A (lower trace). Another $Cl^-$ channel blocker, IAA94 (100 and 300 $\mu$M), inhibited the high $K^+$-induced contraction possibly through a nonspecific direct action on L-type $Ca^{2+}$

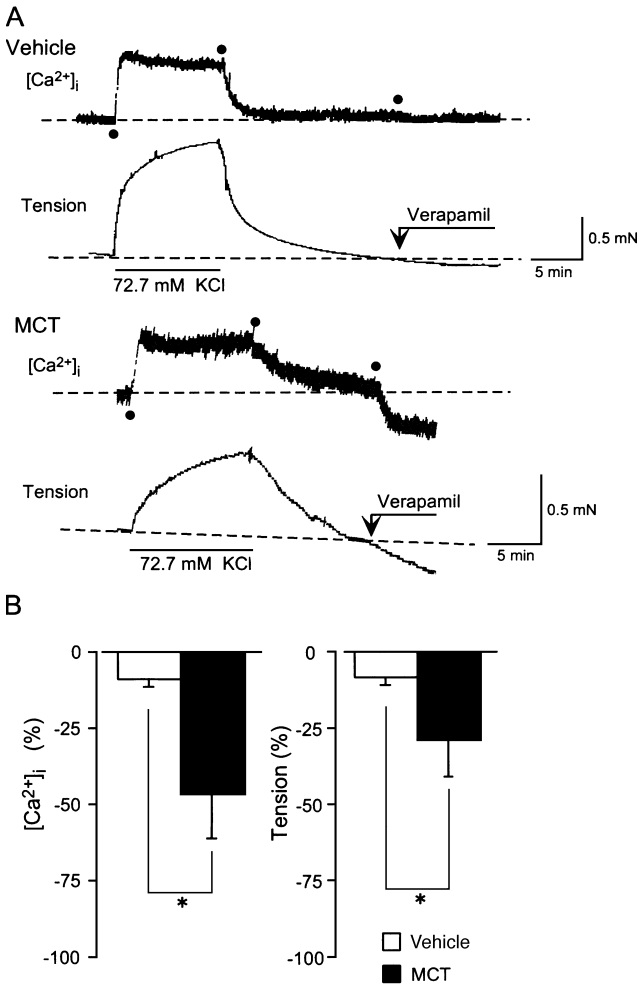


**Fig. 2.** Effect of verapamil on the muscular spontaneous tone and the cytosolic $Ca^{2+}$ levels. A: Typical trace of $[Ca^{2+}]_i$ and muscle force in pulmonary artery loaded with fura-PE3 in the vehicle- or MCT-treated rat. After measuring the effects of 72.7 mM KCl on $[Ca^{2+}]_i$ and muscle force, the effects of 10 $\mu$M verapamil on the muscular spontaneous tone and $[Ca^{2+}]_i$ were examined. A typical trace out of five experiments is shown. B: The summarized results from panel A. Responses elicited by 72.7 mM KCl and the resting state were taken as 0% and 100%, respectively. *Significantly different from vehicle-treated artery ($P$<0.05).

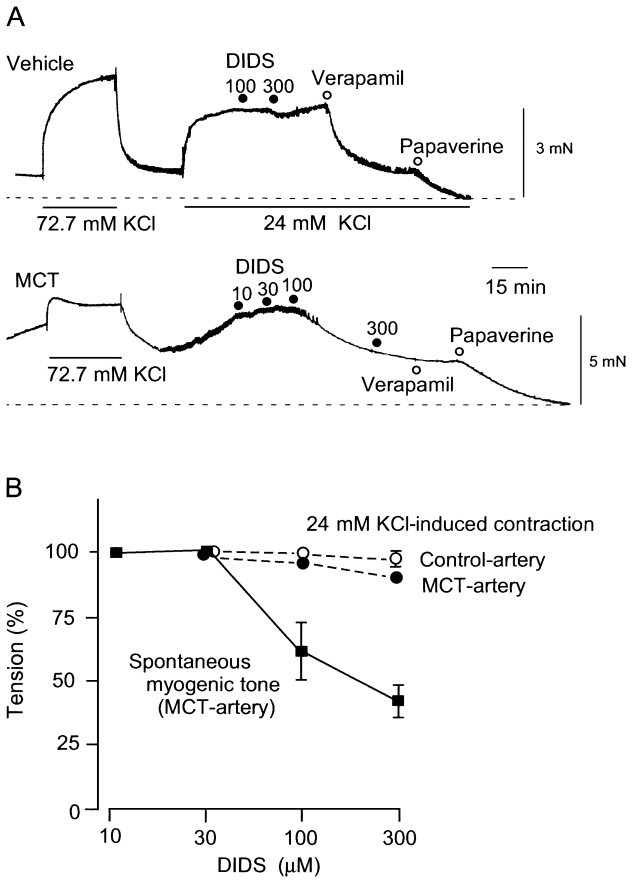


**Fig. 3.** Effect of DIDS on the 24 mM KCl-induced contraction or the spontaneous muscular tone in pulmonary artery isolated from vehicle- or MCT-treated rat. In panel A, the upper trace shows the effects of 100 – 300 $\mu$M DIDS on the 24 mM KCl-induced contraction in the pulmonary artery from vehicle-treated rats. The lower trace shows the effects of 10 – 300 $\mu$M DIDS on the spontaneous muscular tone of the hypertensive pulmonary artery from MCT-treated rat. The chart trace shows a typical trace out of five experiments. Panel B shows the concentration-response curve representing the effects of DIDS on the KCl-induced contractions in vehicle-treated and MCT-treated arteries or the spontaneous muscular tone in MCT-treated arteries (n = 5 each).

channels (n = 5, data not shown). Interference of the fura-PE3 fluorescence by DIDS and IAA94 made it impossible for us to measure the change in $[Ca^{2+}]_i$ in the arteries.

We also examined the effect of DIDS and verapamil on resting tension in the aorta. Neither DIDS (300 $\mu$M) nor verapamil (10 $\mu$M) affected the resting tension in vehicle-treated and MCT-treated aortae (n = 4 each, data not shown).

We carried out quantitative RT-PCR analysis to examine the changes in ClC3 mRNA levels in the pulmonary arteries (Fig. 4A). The expressions of the housekeeping gene, GAPDH (308 bp), were identical between the arteries from vehicle- and MCT-treated rats. The levels of ClC3 mRNA (259 bp) expression were also identical in both preparations (n = 4). We also analyzed the expression levels of ClCA, with our results indicating that the levels of ClCA mRNA expression did not differ between vehicle-treated and MCT-treated rats (n = 3) (Fig. 4B).

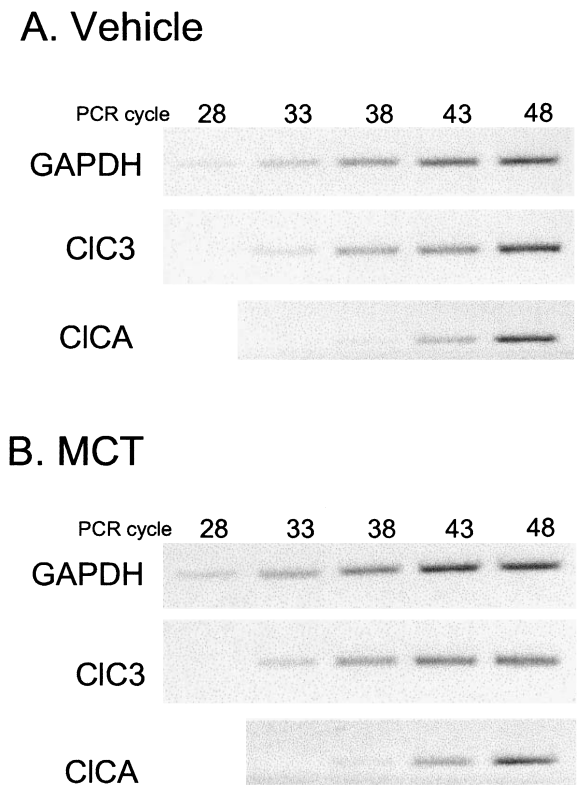


**Fig. 4.** Quantitative RT-PCR analysis of ClC3 mRNA (A) and ClCA mRNA (B) in the pulmonary artery of vehicle- and MCT-treated rat. A 1-$\mu$g sample of total RNA from pulmonary artery of the vehicle- or MCT-treated rats was used in each RT-PCR analysis. Panel A shows the detection results for the RT-PCR products for GAPDH and ClC3 from 28 PCR-cycles to 48 PCR-cycles, respectively. Panel B shows the detection results for the RT-PCR products for GAPDH and ClCA from 28 PCR-cycles to 48 PCR-cycles, respectively. Typical results from 3 – 4 experiments are shown in the figure.

## DISCUSSION

We have found in the present study that the main pulmonary artery isolated from MCT-induced pulmonary hypertensive rats exhibits greater muscular tone in the resting state. In addition, this tone is sensitive to verapamil, a voltage-sensitive $Ca^{2+}$ channel blocker, and DIDS, a $Cl^-$ channel blocker.

Suzuki and Twarog (18) have reported that the membrane potential of the rat main pulmonary artery depolarizes at 8 days after exposure to hypoxia or 20 days after the injection of MCT. Ito et al. (19) also observed the membrane depolarization in MCT-treated pulmonary arterial tissue. Consistent with these observations, we observed elevated muscular tone in the hypertensive pulmonary artery at 21 days after MCT injection. Consistent with the report of Suzuki and Twarog (18), this muscular tone is associated with an increase in $[Ca^{2+}]_i$ that is inhibited by the voltage-dependent $Ca^{2+}$-channel blocker verapamil. Membrane stretch or an increase in transmural pressure causes a change in muscular tone in vascular smooth muscle cells (21). Several other studies have also revealed that the vascular spontaneous active tone is dependent on extracellular $Ca^{2+}$, and the contraction is associated with membrane depolarization (22 – 24). Recently, Nelson and coworkers (25) have shown that pressure-induced membrane depolarization and vasoconstriction are inhibited by $Cl^-$ channel antagonists, whereas the ClCA channel inhibitor niflumic acid does not inhibit the contraction, suggesting that the activation of some types of $Cl^-$ channels, other than ClCA, may be essential to inducing membrane depolarization of the cerebral arterial cells. More recently, it has been reported in the canine pulmonary artery that ClC3 is expressed and that the cell-swelling induced by decreasing external osmolarity is accompanied by an activation of the DIDS-sensitive $Cl^-$ channel in the pulmonary artery (26). The authors postulated that the ClC3 channel might contribute to the regulation of cell volume, resting membrane potential, and muscular tone. In the present study, the verapamil-sensitive spontaneous muscular tone in the hypertensive pulmonary artery was completely inhibited by DIDS. This result strongly supports the hypothesis that activation of the ClC3 channel or other DIDS-sensitive $Cl^-$ channels may contribute to the elevated spontaneous muscular tone.

Although we could have confirmed that ClC3 and ClCA are molecularly expressed in rat pulmonary artery, the quantitative RT-PCR analysis indicated that the expression of ClC3 mRNA or ClCA mRNA levels in the hypertensive pulmonary artery did not differ from that of the vehicle-treated artery. This result suggests that the DIDS-sensitive membrane depolarization may not be mediated by the increase in the number of ClC3 or ClCA, but may be

mediated at the level of activity of these $Cl^-$ channels. Further examinations using the patch-clamp technique are necessary to clarify the change in activity of these $Cl^-$ channels.

It has also been reported that tissue endothelin-1 levels are increased in the pulmonary artery of MCT-treated rat (15, 16). In addition, it has been reported that the daily infusion of $ET_A$-antagonist inhibits the cardiopulmonary changes produced by MCT-induced pulmonary hypertension (17). In the present study, however, $ET_A$ antagonist (BQ-123) and $ET_B$-receptor antagonist (RES701-1) had no effect on the spontaneous muscular tone in vitro, indicating that elevated levels of endothelin-1 might not be related to spontaneous muscular tone.

In conclusion, the elevated spontaneous muscular tone observed in the MCT-induced hypertensive pulmonary artery is due to membrane depolarization induced by activation of DIDS-sensitive $Cl^-$ channels.

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