

Original Paper

# Mitochondrial Fission Inhibitors Suppress Endothelin-1-Induced Artery Constriction

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## Key Words

Endothelin-1 • Mdivi-1 • Mitochondrial fission • Vascular smooth muscle

## Abstract

**Background/Aims:** Endothelin-1 is implicated in the pathogenesis of hypertension, but the underlying mechanisms remained elusive. Our previous study found that inhibition of mitochondrial fission of smooth muscle cells suppressed phenylephrine- and high K<sup>+</sup>-induced artery constriction. Here, we studied the effects of mitochondrial fission inhibitors on endothelin-1-induced vasoconstriction. **Methods:** The tension of rat mesenteric arteries and thoracic aorta was measured by using a multi-wire myograph system. Mitochondrial morphology of aortic smooth muscle cells was observed by using transmission electron microscopy. **Results:** Dynamin-related protein-1 selective inhibitor mdivi-1 relaxed endothelin-1-induced constriction, and mdivi-1 pre-treatment prevented endothelin-1-induced constriction of rat mesenteric arteries with intact and denuded endothelium. Mdivi-1 had a similar inhibitory effect on rat thoracic aorta. Another mitochondrial fission inhibitor dynasore showed similar effects as mdivi-1 in rat mesenteric arteries. Mdivi-1 inhibited endothelin-1-induced increase of mitochondrial fission in smooth muscle cells of rat aorta. Rho-associated protein kinase inhibitor Y-27632 which relaxed endothelin-1-induced vasoconstriction inhibited endothelin-1-induced mitochondrial fission in smooth muscle cells of rat aorta. **Conclusion:** Endothelin-1 increases mitochondrial fission in vascular smooth muscle cells, and mitochondrial fission inhibitors suppress endothelin-1-induced vasoconstriction.

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## Introduction

Hypertension is a common risk factor for the development of diverse cardiovascular diseases. Endothelin-1 (ET-1), the most potent endogenous vasoconstrictor, is implicated in

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the pathogenesis of hypertension. Since ET-1 is first identified in 1988, numerous studies prove that ET-1 exerts its biological effects through regulating vascular tone, renal sodium and water excretion, cell growth and proliferation, and extracellular matrix accumulation [1, 2]. The effects of ET-1 are mediated by binding to ET-1 receptors, endothelin-A (ETA) receptor and endothelin-B (ETB) receptor. Presently, the selective ETA receptor antagonists and mixed ETA receptor/ ETB receptor antagonists have been shown to be therapeutic for hypertension [3-5].

Mitochondria are dynamic organelles and undergo fission and fusion processes to control their shape, number, and intracellular distribution. Under physiological conditions, fusion and fission processes are balanced [6, 7]. However, under certain pathological conditions, the fission process is increased and the excessive mitochondrial fission promotes the disease progression; for instance, the mitochondrial fission increases in endothelial cells in diabetic states and in pulmonary artery smooth muscle cells in pulmonary arterial hypertension, and the increased mitochondrial fission contributes to the impairment of endothelial function in diabetes and hyperproliferation of pulmonary artery smooth muscle cells in pulmonary arterial hypertension [8, 9].

Vascular smooth muscle cells are major players in regulating vessel tone and vascular remodeling, and are crucial targets for hypertension therapy. Because mitochondria continuously change their morphology by undergoing fusion and fission, we speculate that interfering mitochondrial dynamics could show acute effect on the vascular function. Our previous work found that mitochondrial fission inhibition by using mitochondrial fission inhibitors inhibited phenylephrine- and high K<sup>+</sup>-induced vasoconstriction [10]. We further used typical vasorelaxants verapamil and phentolamine to demonstrate that artery relaxation was coupled to inhibition of mitochondrial fission in arterial smooth muscle cells [11]. The mechanism of ET-1- induced contractile force generation in smooth muscle cells was generally thought to be through Rho-associated protein kinase (ROCK) dependent pathways [12, 13], and ROCK activation was reported to mediate mitochondrial fission [14, 15]. Therefore, we hypothesized that mitochondrial fission inhibitors would inhibit ET-1-induced vasoconstriction.

## Materials and Methods

### *Reagents*

Acetylcholine chloride (ACh), mitoTEMPO were purchased from Sigma Aldrich Chemistry (Saint Louis, MO, USA). Mdivi-1 and dynasore were purchased from Selleck Chemicals (Shanghai, China). Endothelin-1 was Tocris Bioscience product. Phenylephrine (PE) and acetylcholine chloride (ACh) were dissolved in double distilled water, and others were dissolved in DMSO (Tianjin Fuyu Fine Chemical Co., Ltd.). High K<sup>+</sup> physiological salt solutions (KPSS) containing 60 mM K<sup>+</sup> were used for treating artery tissues. The KPSS (60 mM K<sup>+</sup>) solution was composed of (mM): NaCl, 74.7; KCl, 60; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.17; KH<sub>2</sub>PO<sub>4</sub>, 1.18; NaHCO<sub>3</sub>, 14.9; CaCl<sub>2</sub>, 1.6; D-glucose, 5.5; EDTA, 0.026.

### *Animals*

Adult male Sprague-Dawley rats (male, body weight 320-350g, 8-10 weeks) were purchased from Charles River (Charles River Laboratory Animal, Beijing, China). All animal procedures and experiments were approved by the Institutional Animal Care and Use Committee of Harbin Medical University.

### *Mesenteric artery tension measurement*

The experiments were carried out according to our previous works [10, 16-18]. Briefly, animals were sacrificed after anesthetized by sodium pentobarbitone (40 mg/ kg, ip). The entire mesentery was rapidly dissected out and transferred to physiological salt solution (PSS) at room temperature. The PSS solution was aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4 at 37°C). Mesenteric arteries (3th branch) were dissected into 2 mm rings and moved to an organ bath filled with 5 mL fresh PSS solution oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4 at 37°C). The arterial rings were threaded carefully with wires through the lumen and the

isometric contractions were measured by multi wire myograph system (DMT620, Danish Myo Technology, Aarhus, Denmark).

#### *Aorta tension measurement*

The experiments were carried out according to our previous works [10, 16, 17]. The rats were sacrificed after anesthetized with sodium pentobarbitone. The thorax was cut to expose the aorta, and the descending thoracic aorta were rapidly dissected out and removed to physiological salt solution (PSS) at room temperature. After the perivascular tissue was carefully removed, aortic rings were cut approximately 4 mm in length and mounted between two stainless steel triangle hooks before moved to an organ bath with 10 mL fresh PSS solution oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4 at 37°C). After equilibration, the tension was measured by using a multichannel acquisition and analysis system (Model BL-420S, Taimeng Technology Instrument, Chengdu, China).

#### *Transmission electron microscopy (TEM)*

The experiments were carried out according to our previous works [10]. Selected samples (aorta tissue) were incubated with ET-1 (4 nM) for 15 min, or pre-incubated with mdivi-1 or Y27632 for 30 min, then treated with ET-1 (4 nM) for 15 min. Samples were rinsed in buffer, then fixed in 2.5% glutaraldehyde in PBS (pH 7.4). Following 2 - 3 days, post-fixed in PBS-buffered 1% OsO<sub>4</sub> for 1 - 2 h, stained en bloc in uranyl acetate, dehydrated in ethanol, and embedded in epoxy resin by standard procedures. The ultra-thin sections were electron stained and observed under an electron microscope (JEM-1220, JEOL Ltd., Tokyo, Japan). Three individual aorta samples in each group were harvested and each sample was taken 15 - 20 photos randomly. The mitochondrial morphology was analyzed by using image pro plus software and the parameters were presented with AR and FF. AR: aspect ratio (ratio between major and minor axes of an ellipse equivalent to the mitochondrion), AR is a measure of mitochondrial length. FF: form factor (perimeter  $2/4 \pi$  area), FF is a combined measure of both mitochondrial length and degree of branching.

#### *Data analysis*

Data are presented as mean  $\pm$  SEM. Significance was determined by using Student's *t* test for comparison. All statistical tests were performed using SigmaPlot (12.5 version). *P* < 0.05 was considered significant.

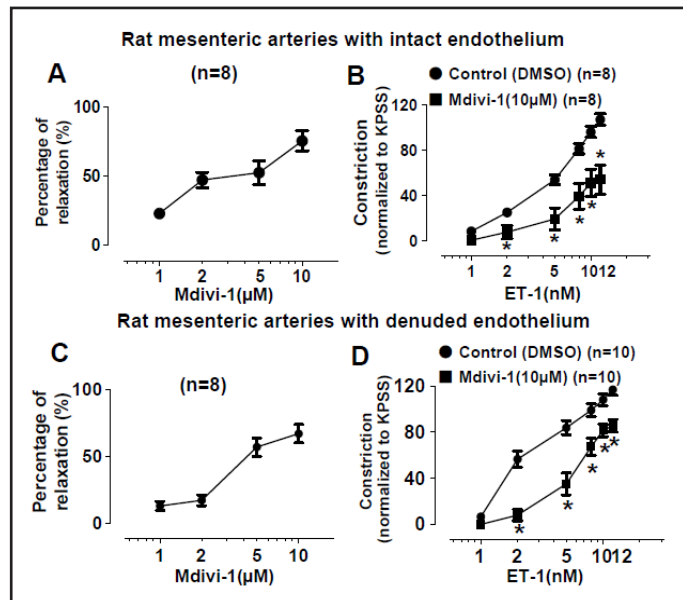
## Results

### *Drp1 selective inhibitor mdivi-1 relaxes endothelin-1-induced constriction, and mdivi-1 pretreatment inhibits endothelin-1-induced constriction in rat mesenteric arteries*

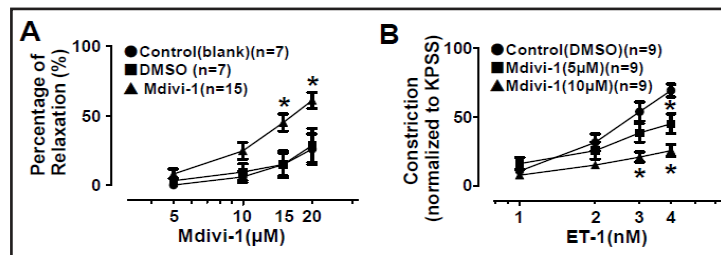
Drp1 is mitochondrial fission related protein and mdivi-1 is a Drp1 selective inhibitor which is widely used as a pharmacologic tool to inhibit mitochondrial fission process [9, 10, 19, 20]. Mdivi-1 (1-10  $\mu$ M) induced concentration-dependent relaxation in endothelium-intact rat mesenteric arteries pre-contracted with ET-1 (4 nM). The summarized data were shown in Fig. 1A. Next, we investigated whether mdivi-1 pretreatment could inhibit ET-1-induced vasoconstriction in endothelium-intact rat mesenteric arteries. A pre-contraction induced by KPSS (high K<sup>+</sup> physiological salt solution) was used to normalize the ET-1-induced contraction. Compared with the control (DMSO), mdivi-1 pretreatment for 30 min significantly prevented ET-1-induced constriction of rat mesenteric arteries with intact endothelium in a dose-dependent manner (Fig. 1B).

Then, we investigated whether mdivi-1 relaxed ET-1-induced vasoconstriction, and whether mdivi-1 pretreatment inhibited ET-1-induced vasoconstriction in rat mesenteric artery with denuded endothelium. Mdivi-1 (1-10  $\mu$ M) induced concentration-dependent relaxation in endothelium-denuded rat mesenteric arteries pre-contracted with ET-1 (Fig. 1C). Mdivi-1 pretreatment inhibited ET-1-induced vasoconstriction in endothelium-denuded rat mesenteric arteries (Fig. 1D).

**Fig. 1.** Mdivi-1 relaxes ET-1-induced constriction, and mdivi-1 pre-treatment inhibits ET-1-induced constriction in rat mesenteric arteries. (A) Mdivi-1 induced concentration-dependent relaxation in endothelium-intact rat mesenteric arteries pre-contracted with ET-1 (4 nM). The relaxation ratio of mdivi-1 was calculated by subtracting the relaxation ratio of corresponding control (DMSO). (B) Mdivi-1 (10  $\mu$ M) pretreatment inhibited ET-1-induced vasoconstriction in endothelium-intact rat mesenteric arteries.  $**P < 0.01$  vs. Control. (C) Mdivi-1 induced vasorelaxation in endothelium-denuded rat mesenteric arteries pre-contracted with ET-1 (4 nM). The relaxation ratio of mdivi-1 was calculated by subtracting the relaxation ratio of corresponding control (DMSO). (D) Mdivi-1 (10  $\mu$ M) pretreatment inhibited ET-1-induced constriction of rat mesenteric arteries with denuded endothelium.  $**P < 0.01$  vs. Control.



**Fig. 2.** Mdivi-1 relaxes ET-1-induced constriction, and mdivi-1 pretreatment inhibits ET-1-induced constriction in rat thoracic aorta. (A) Mdivi-1 induced vasorelaxation in rat thoracic aorta pre-contracted with ET-1 (4 nM).  $*P < 0.05$  vs. Control (DMSO). (B) Mdivi-1 pretreatment inhibited ET-1-induced constriction of rat thoracic aorta.  $**P < 0.01$  vs. Control (DMSO).



The above data indicated that the effects of mdivi-1 on ET-1-induced vasoconstriction were endothelium-independent.

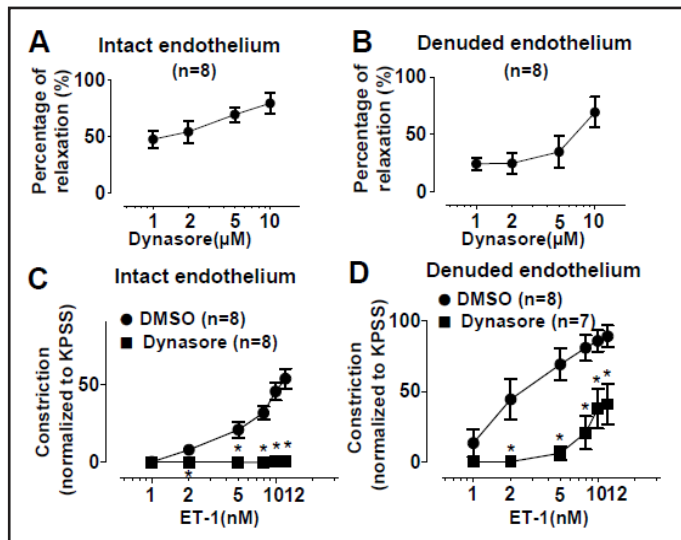
*Mdivi-1 relaxes endothelin-1-induced constriction, and mdivi-1 pretreatment inhibits endothelin-1-induced constriction in rat thoracic aorta*

In order to test whether mitochondrial fission inhibitor showed the similarly inhibitory effects on the vasoactivity in large conduit arteries, we examined the effects of mdivi-1 on rat thoracic aorta. Mdivi-1 relaxed ET-1-induced vasoconstriction, and mdivi-1 pretreatment antagonized ET-1-induced vasoconstriction in rat thoracic aorta (Fig. 2). These results indicated that the mitochondrial fission inhibition had similarly inhibitory effects on the vasoactivity in both conduit and resistance arteries.

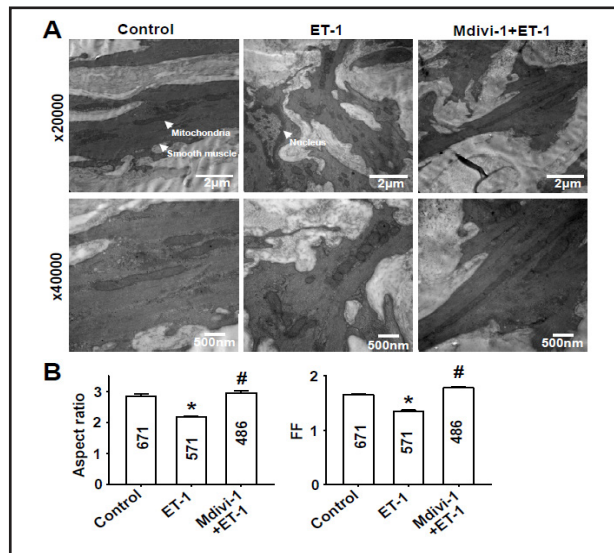
*Dynasore relaxes endothelin-1-induced constriction, and dynasore pretreatment inhibits endothelin-1-induced constriction in rat mesenteric arteries*

We further used another Drp1 inhibitor dynasore [10, 21] to confirm the role of mitochondrial fission in artery constriction. Similarly to the effects of mdivi-1, dynasore relaxed the ET-1 induced pre-contraction, and dynasore pretreatment for 30 min prevented the constriction induced by ET-1 in rat mesenteric arteries with intact and denuded endothelium (Fig. 3A-3D).

**Fig. 3.** Dynasore relaxes ET-1-induced constriction, and dynasore pretreatment inhibits ET-1-induced constriction of rat mesenteric arteries with intact and denuded endothelium. (A,B) Dynasore induced vasorelaxation in endothelium-intact and endothelium-denuded rat mesenteric arteries pre-contracted with ET-1 (4 nM). The relaxation ratio of dynasore was calculated by subtracting the relaxation ratio of corresponding control (DMSO). (C,D) Dynasore (5  $\mu$ M) pre-treatment inhibited ET-1-induced constriction of rat mesenteric arteries with intact and denuded endothelium. The vessel tension was normalized to that induced by KPSS. \*\* $P < 0.01$  vs. Control (DMSO). \* $P < 0.05$  vs. Control (DMSO).



**Fig. 4.** The mitochondria morphology of smooth muscle cells in rat thoracic aorta analyzed by transmission electron microscope (TEM). (A) The representative photos by TEM. (B) The quantity analysis of mitochondrial fission showed that mdivi-1 inhibited ET-1-induced increase of mitochondrial fission in smooth muscle cells of rat aorta. \* $P < 0.05$  vs control; # $P < 0.05$  vs ET-1. The number of mitochondria analyzed per group was showed in the bar.



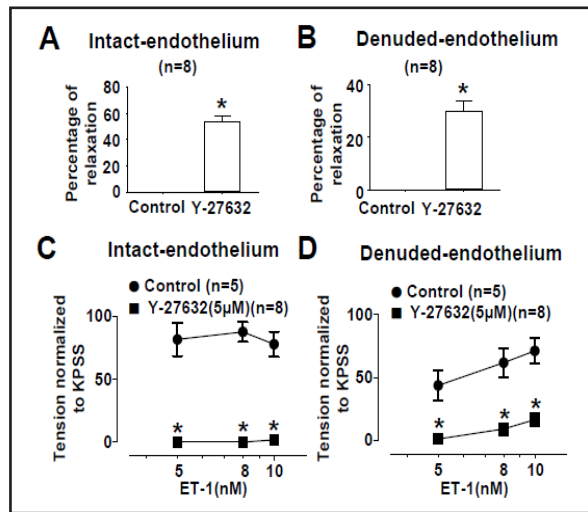
*Mdivi-1 inhibits endothelin-1-induced increase of mitochondrial fission in smooth muscle cells of rat aorta*

Since mitochondrial fission suppression inhibits ET-1-induced vasoconstriction, we asked whether ET-1 could induce mitochondrial fission in arteries *in situ*. The mitochondria morphology of smooth muscle cells in aortas were observed by using transmission electron microscope (TEM). The mitochondria showed strip shape and scattering distribution in control arteries. ET-1 treatment induced the elongated mitochondria to be much shorter and rounder. However, pre-treatment with mdivi-1 significantly attenuated the excessive fission properties induced by ET-1 (Fig. 4A). In these aortas, mitochondria showed elongated shape and less division, which were similar to that observed in control arteries. The quantity analysis of mitochondrial fission was shown in Fig. 4B.

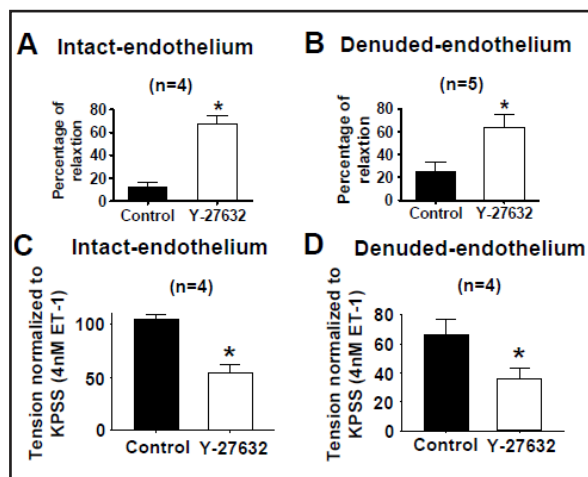
*ROCK inhibitor Y-27632 inhibits mitochondrial fission and relaxes endothelin-1-induced vasoconstriction*

Rho-associated coiled-coil protein kinase (ROCK) is the downstream signal of ET-1 receptor activation and ROCK activation contributes to multiple ET-1-mediated pathological effects [22-25]. ROCK inhibitors inhibit ET-1 induced contraction in different types of arteries

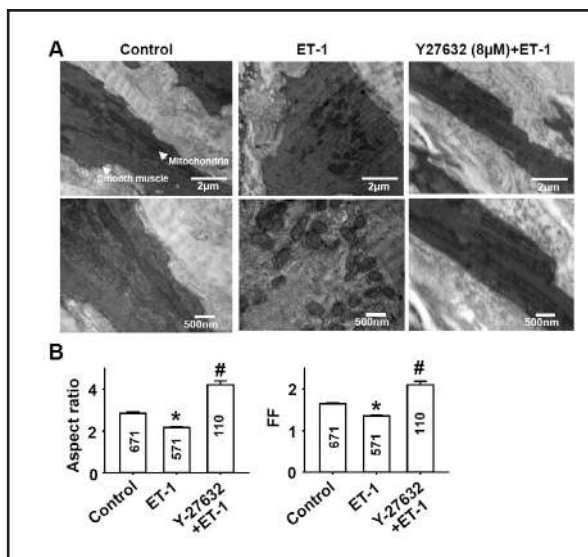
**Fig. 5.** ROCK inhibitor Y-27632 relaxes ET-1-induced constriction, and Y-27632 pretreatment inhibits ET-1-induced constriction in rat mesenteric arteries. (A,B) Y-27632 (5  $\mu$ M) relaxed ET-1-induced constriction in rat mesenteric arteries with intact and denuded endothelium. \* $P$ <0.05 vs. Control (DMSO). (C,D) Y-27632 (5  $\mu$ M) pretreatment inhibited ET-1-induced constriction of rat mesenteric arteries with intact and denuded endothelium. \* $P$ <0.05 vs. Control (DMSO).



**Fig. 6.** ROCK inhibitor Y-27632 relaxes ET-1-induced constriction, and Y-27632 pretreatment inhibits ET-1-induced constriction in rat thoracic aorta. (A,B) Y-27632 (8  $\mu$ M) relaxed ET-1-induced constriction in rat aorta with intact and denuded endothelium. \* $P$ <0.05 vs. Control (DMSO). (C,D) Y-27632 (8  $\mu$ M) pretreatment inhibited ET-1-induced constriction in rat aorta with intact and denuded endothelium. \* $P$ <0.05 vs. Control (DMSO).



**Fig. 7.** Y-27632 inhibits ET-1-induced increase of mitochondrial fission in smooth muscle cells of rat thoracic aortas. The mitochondria morphology of smooth muscle cells in rat thoracic aortas was analyzed by transmission electron microscope (TEM). (A) Representative photos by TEM. (B) The quantity analysis of mitochondrial fission in smooth muscle cells of rat aorta. \* $P$ <0.05 vs control; # $P$ <0.05 vs ET-1. The number of mitochondria analyzed per group were shown in the bar.



[26-28]. We asked whether ROCK inhibitor which inhibits ET-1-induced vasoconstriction could suppress ET-1-induced mitochondrial fission. Firstly, we evidenced that ROCK inhibitor Y-27632 inhibited ET-1-induced vasoconstriction in rat mesenteric arteries (Fig. 5) and thoracic aorta (Fig. 6). Then, we found that Y-27632 inhibited ET-1-induced mitochondrial

fission in smooth muscle cells of rat thoracic aorta (Fig. 7). ROCK1 was reported to mediate mitochondrial fission and deletion of ROCK1 prevented mitochondrial fission [14]. Y-27632 is a specific ROCK1 inhibitor. So, we thought that Y-27632 was also a type of mitochondrial fission inhibitor through inhibiting ROCK1. The effect of Y-27632, combined with that of mdivi-1 and dynasore, suggested that mitochondrial fission was involved in ET-1-induced vasoconstriction and inhibition of mitochondrial fission in smooth muscle cells suppressed ET-1-induced vasoconstriction.

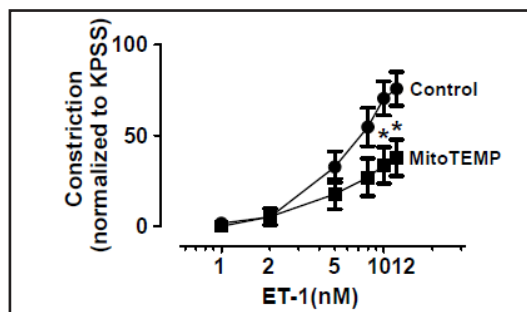
Because that ET-1 was reported to increase ROS generation [29, 30] and ROS was involved in artery constriction [31, 32], we further studied the effects of mitochondria-targeted antioxidant mitoTEMPO, on ET-1-induced constriction of rat mesenteric arteries. MitoTEMPO up to 400  $\mu\text{M}$  inhibited ET-1-induced constriction of rat mesenteric arteries (Fig. 8).

## Discussion

In the present study, we found for the first time that ET-1 induced increase of mitochondrial fission in vascular smooth muscle cells and mitochondrial fission inhibitors suppressed ET-1-induced vasoconstriction. Our findings reveal the importance of mitochondrial fission in ET-1-induced vasoconstriction and mitochondrial fission inhibitors would be a novel class of drugs inhibiting ET-1-induced vasoconstriction.

ET-1 induces contractile force generation through  $\text{Ca}^{2+}$ -dependent or  $\text{Ca}^{2+}$ -independent mechanisms in different tissues. These mechanisms may vary in different conditions or cell types. In smooth muscle cells, the  $\text{Ca}^{2+}$ -dependent mechanisms denote that, through binding to ETA receptors, ET-1 induces vasoconstriction through increases of intracellular  $\text{Ca}^{2+}$  via activation of the phospholipase C that hydrolyzes phosphatidyl inositol trisphosphate ( $\text{IP}_3$ ) and the neutral diacylglycerol (DAG), or ET-1 activates non-selective cation (NSC) channels [33], store-operated  $\text{Ca}^{2+}$  channel (SOCC) [34]. L-type  $\text{Ca}^{2+}$  channels play little role in the responses to ET-1 in vasoconstriction [35, 36]. On the other hand, the  $\text{Ca}^{2+}$ -independent mechanisms show that ET-1 induces vasoconstriction without the necessity of intracellular  $\text{Ca}^{2+}$  increase (a threshold level of  $[\text{Ca}^{2+}]_i$  was needed.), but requiring ET-1 induced RhoA/ROCK activation. The  $\text{Ca}^{2+}$ -independent mechanisms of ET-induced contractile force generation have been reported in different tissues. In intrapulmonary arteries from chronically hypoxic rats, ET-1 induced vasoconstriction without a change in  $[\text{Ca}^{2+}]_i$  but dependently of Rho kinase activation [12]. In rat hepatic stellate cells, changes in  $[\text{Ca}^{2+}]_i$  were neither necessary nor sufficient for contractile force generation and ET-1-stimulated force generation was sensitive to ROCK1 inhibitor Y-27632 [37]. In bovine trabecular meshwork, ET-1-induced contraction of trabecular meshwork was  $\text{Ca}^{2+}$ -independent, but inhibited by the ROCK inhibitor Y-27632 [38]. In airway smooth muscle, Y-27632 inhibited ET-1-induced rho/ROCK-mediated  $\text{Ca}^{2+}$  sensitization and contraction. A recent work showed that LC20 diphosphorylation (Ser19 and Thr18) contributed to sustained endothelin-1-induced vasoconstriction through ROCK-mediated inhibition of MLCP in renal afferent arterioles [13].

The role of dynamin-related protein-1 (Drp1) in mitochondrial fission has been well established. Recent works found that mitochondrial translocation of Drp1 were dependent on ROCK activation [39], and ROCK mediated mitochondrial fission by promoting dynamin-related protein-1 (Drp1) recruitment to the mitochondria [14]. These findings suggest that ROCK inhibitor Y-27632 should be a type of mitochondrial fission inhibitor. We found that



**Fig. 8.** MitoTEMPO (400  $\mu\text{M}$ ) inhibits ET-1-induced constriction of rat mesenteric arteries.  $n=4$ . \* $P<0.05$  vs Control.

Y-27632 inhibited ET-1-induced mitochondrial fission, consistent with the role of ROCK in mitochondrial fission [14]. In fact, in the present study, we have used three types of mitochondrial fission inhibitors with different action mechanisms, mdivi-1, dynasore and Y-27632, to evidence the role of mitochondrial fission of smooth muscle cells in ET-1-induced vasoconstriction.

Numerous studies have been focusing on the relationship between mitochondrial ROS and mitochondrial fission. On the one hand, mitochondrial fission induces increase of ROS, for instance, high glucose conditions induced increase of mitochondrial fission and ROS levels, and inhibiting mitochondrial fission in sustained high glucose conditions normalized cellular ROS levels in cardiomyocytes [40]. On the other hand, ROS stimulates mitochondrial fission, for instance, it was reported that mitochondrial oxidative stress caused an imbalance in mitochondrial fission-fusion, resulting in mitochondrial fragmentation [41]. The interaction between ROS and mitochondrial fission may exacerbate each other [42]. ET-1 induced ROS-dependent activation of ROCK signaling [29] and ROCK activation contributed to mitochondrial fission [14], therefore, it is conceivable that ET-1 induces mitochondrial fission through ROS/ROCK activation pathway.

ET-1 induces cellular ROS generation in smooth muscle cells [29], but the exact sources of ET-1-induced ROS in vascular smooth muscle cells were not conclusive. Most studies considered that ET-1-induced generation of ROS in the vascular smooth muscle cells were from mitochondria [43, 44]. We found that the mitochondria-targeted antioxidant, mitoTEMPO, inhibited ET-1-induced constriction of rat mesenteric arteries, confirming ET-1-induced mitochondria-derived ROS. However, it should be noticed that mitoTEMPO was used up to 400  $\mu$ M to inhibit ET-1-induced vasoconstriction. In our previous study, mitoTEMPO at 20  $\mu$ M completely inhibited PE-induced vasoconstriction, but the inhibition ratio was no more than 40% in KPSS-induced model [10]. These results indicated that the importance of mitoROS in different vasoconstriction models was varied.

## Abbreviations

Endothelin-1 (ET-1); Physiological salt solution (PSS); Rho-associated protein kinase (ROCK).

## Acknowledgments

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## Disclosure Statement

The authors declare no conflict of interest.

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