Mitochondrial Fission of Smooth Muscle Cells Is Involved in Artery Constriction

Ming-Yu Liu, Jing Jin, Shan-Liang Li, Jie Yan, Chang-Lin Zhen, Jin-Lai Gao, Yong-Hui Zhang, Yan-Qiu Zhang, Xin Shen, Liang-Shuan Zhang, Yuan-Yuan Wei, Yu Zhao, Chen-Guang Wang, Yun-Long Bai, De-Li Dong

Abstract—Mitochondria are dynamic organelles and continuously undergo fission and fusion processes. Mitochondrial fission is involved in multiple physiological or pathological processes, but the role of mitochondrial fission of smooth muscle cells in artery constriction is unknown. The role of mitochondrial fission of smooth muscle cells in arterial function was investigated by measuring the tension of rat mesenteric and thoracic aorta and by evaluating mitochondrial fission, mitochondrial reactive oxygen species, and cytosolic [Ca2+]i in rat vascular smooth muscle cells. Mitochondrial fission inhibitors mdivi-1 and dynasore antagonized phenylephrine- and high K+–induced constriction of rat mesenteric arteries. Mdivi-1 relaxed phenylephrine-induced constriction, and mdivi-1 pretreatment prevented phenylephrine-induced constriction in mice, rat aorta, and human mesenteric arteries. Phenylephrine- and high K+–induced increase of mitochondrial fission in smooth muscle cells of rat aorta and the increase was inhibited by mdivi-1. Mdivi-1 inhibited high K+–induced increases of mitochondrial fission, mitochondrial reactive oxygen species, and cytosolic [Ca2+]i, in rat vascular smooth muscle cells. Prechelation of cytosolic Ca2+ prevented high K+–induced cytosolic [Ca2+]i increase, mitochondrial fission, and mitochondrial reactive oxygen species overproduction. Mitochondria-targeted antioxidant mito-TEMPO antagonized phenylephrine- and high K+–induced constriction of rat mesenteric arteries. Nitroglycerin and ROCK (Rho-associated protein kinase) inhibitor Y27632, the 2 vasodilators with different vasorelaxant mechanisms, relaxed high K+–induced vasoconstriction and inhibited high K+–induced mitochondrial fission. In conclusion, the mitochondrial fission of smooth muscle cells is involved in artery constriction. (Hypertension. 2016;68:1245–1254. DOI: 10.1161/HYPERTENSIONAHA.116.07974.) • Online Data Supplement

Key Words: arteries ■ hypertension ■ mitochondrial dynamics ■ myocytes, smooth muscle ■ vasodilation

Hypertension is one of the most common worldwide diseases and is a major risk factor for a variety of cardiovascular and renal events, including myocardial infarction, stroke, heart failure, and end-stage renal disease.1 The latest survey shows that hypertension accounts for 7% of global disability adjusted life years and 9.4 million deaths in 2010.2 Therefore, the molecular mechanisms underlying hypertension and the antihypertensive therapies have always been the topics in cardiovascular fields.

Mitochondria are dynamic organelles and change their morphology through fission and fusion processes named as mitochondrial dynamics.3 Defects in mitochondrial dynamics are implicated in multiple cardiovascular diseases, for instance, the increased mitochondrial fission contributes to the impairment of endothelial function in diabetes mellitus and the hyperproliferation of pulmonary artery smooth muscle cells in pulmonary arterial hypertension.4,5 More interestingly, Hong et al6 show that mitochondrial fission is crucial for O2−-induced ductus arteriosus constriction and closure at birth in human and rabbits.

By using novel digital image processing/single particle tracking techniques, Giedt et al7 show that mitochondria in endothelial cells continuously undergo fusion/fission, indicating that the onset of biological function related to mitochondrial dynamics should be prompt without needing the transcription and translation processes of mitochondrial dynamic-related proteins. Therefore, we speculate that interfering mitochondrial dynamics could show acute effects on the vascular function. In the present work, we investigate the effects of acute inhibition of mitochondrial fission on the constriction and relaxation of arteries and the underlying mechanisms. We find for the first time that mitochondrial fission of smooth muscle cells is involved in artery constriction, revealing a novel mechanism for vasoconstriction and providing a potential therapeutic target for hypertension.
Methods

Agents and Animals
Additional details of agents and animals are included in the online-only Data Supplement.

Mesenteric Artery and Aorta Tension Measurement
The tension measurement of mesenteric arteries and aorta was performed as in our previous works. Additional details are included in the online-only Data Supplement.

Measurements for Mitochondrial Networks
The mitochondrial fragmentation was analyzed according to the literature. The details other Methods are included in the online-only Data Supplement.

Statistical Analysis
Data are presented as mean±SEM. Significance was determined by using Student t test or 1-way ANOVA followed by Holm–Sidak. P<0.05 was considered significant.

Results

Drp1 Selective Inhibitor Mdivi-1 Antagonizes Phenylephrine- and High K+-Induced Constriction of Rat Mesenteric Arteries
Mdivi-1 is widely used as a pharmacological tool to inhibit mitochondrial fission process. In rat mesenteric arteries with intact endothelium (Figure 1A), mdivi-1 (10–100 μmol/L) showed no effect on the basal tension (Figure 1B); however, mdivi-1 (5–100 μmol/L) induced concentration-dependent relaxation in endothelium-intact rat mesenteric arteries precontracted with phenylephrine and high K+ (KPSS). The original recordings are shown in Figure 1C and 1E, and the summary data are shown in Figure 1D and 1F.

Next, we investigated whether mdivi-1 pretreatment could prevent phenylephrine- and KPSS-induced vasoconstriction. As shown in Figure S1A in the online-only Data Supplement, mdivi-1 pretreatment for 30 minutes significantly prevented phenylephrine-induced constriction of rat mesenteric arteries with intact endothelium, and the inhibition was relieved after mdivi-1 washout. The inhibition by mdivi-1 (10 μmol/L) showed different patterns, for example, the transient contraction then complete loss of contractility in some artery samples (frame in Figure S1A). In KPSS-induced vasoconstriction model, mdivi-1 pretreatment showed similar prevention as in phenylephrine-induced vasoconstriction model (Figure S1B). Phenylephrine induces vasoconstriction through stimulating α1-adrenergic receptors in plasma membrane of smooth muscle cells and the subsequent IP3 activation on sarcoplasmic reticulum. KPSS induces vasoconstriction through depolarizing membrane potential and the subsequent activation of L-type Ca2+ channels in smooth muscle cells. The different mechanisms of these models suggest that mdivi-1 might act at the general joint of artery constriction complex.

To examine whether the effects of mdivi-1 were endothelium dependent or endothelium independent, the mechanical removal of the endothelium of rat mesenteric arteries was performed and the endothelium denudation was confirmed by the absence of acetylcholine-induced relaxation (Figure 2A). In both phenylephrine- and KPSS-induced vasoconstriction models, mdivi-1 relaxed the precontraction and mdivi-1 pretreatment prevented the constriction of rat mesenteric arteries with denuded endothelium in a dose-dependent manner (Figure 2B through 2E), indicating that the vasoactivity of mdivi-1 was endothelium independent.

Figure 1. Drp1 selective inhibitor Mdivi-1 relaxes phenylephrine (PE)- and high K+ (KPSS)-induced constriction of rat mesenteric arteries. A, Original trace demonstrating endothelium-intact rat mesenteric artery. The artery was precontracted by PE (5 μmol/L) and relaxed by acetylcholine (Ach; 1 μmol/L). B, Original trace showing that mdivi-1 showed no effect on the basal tension of rat mesenteric artery. C and D, Mdivi-1 induced concentration-dependent relaxation in endothelium-intact rat mesenteric arteries precontracted with phenylephrine and high K+ (KPSS). The original recordings are shown in Figure 1C and 1E, and the summary data are shown in Figure 1D and 1F.
Mdivi-1 Relaxes Phenylephrine-Induced Constriction, and Mdivi-1 Pretreatment Prevents Phenylephrine-Induced Constriction in Mice, Rat Aorta, and Human Mesenteric Arteries

To test whether mitochondrial fission inhibitor showed the similar effect on the vasoconstriction in large conduit arteries, we examined the effects of mdivi-1 in mice and rat thoracic aorta. As shown in Figure 3A and 3B, mdivi-1 relaxed phenylephrine-induced constriction and mdivi-1 pretreatment prevented phenylephrine-induced constriction in mice thoracic aorta. Similarly, mdivi-1 pretreatment prevented phenylephrine-induced constriction in rat thoracic aorta (Figure 3C). These results indicated that the mitochondrial fission inhibition had similar inhibitory effect on the vasoconstriction in both conduit and resistance arteries. In addition, in human mesenteric arteries, mdivi-1 relaxed KPSS-induced constriction and mdivi-1 pretreatment prevented KPSS-induced constriction (Figure S2).

Phenylephrine and KPSS Induce Excessive Mitochondrial Fission in Smooth Muscle Cells of Rat Aorta

Because mitochondrial fission suppression inhibited phenylephrine- and KPSS-induced vasoconstriction, we asked whether both phenylephrine and KPSS could induce mitochondrial fission in arteries in situ. The rat thoracic aorta were isolated and cut into pieces that were treated with phenylephrine (5 μmol/L) and KPSS for 15 minutes or preincubated with mdivi-1 (30 μmol/L) for 30 minutes and then treated with phenylephrine (5 μmol/L) and KPSS for 15 minutes. The mitochondrial morphology of smooth muscle cells in these aortas was observed by using transmission electron microscope (TEM). As shown in Figure 3D, the mitochondria showed strip shape and scattering distribution in control arteries. Both phenylephrine and KPSS treatments induced the elongated mitochondria to be much shorter and rounder, and the mitochondria undergoing division were frequently observed. Meanwhile, the mitochondria showed clustered distribution, and the smooth muscle cells were in contractive state in phenylephrine and KPSS treatment groups. However, pretreatment with mdivi-1 significantly attenuated the properties of excessive fission and smooth muscle contraction induced by phenylephrine and KPSS treatments. In these aortas, mitochondria showed elongated shape, less division, and less clustered distribution, which were similar to that observed in control arteries. The quantity analysis of mitochondrial fission is shown in Figure 3E.

The above results showed the mitochondrial morphology of smooth muscle cells of aorta at 15 minutes after KPSS treatment when the aorta has been in the sustained constriction state. We further examined the mitochondrial fission at the early stage (30 s and 1 minute after KPSS treatment) when the aorta constriction just started (Figure S3A). The TEM images showed that the excessive mitochondrial fission of smooth muscle cells had occurred at 30 s and 1 minute after KPSS treatment when the aorta started to constrict (Figure S3B).

Dynasore Relaxes Phenylephrine- and KPSS-Induced Constriction, and Dynasore Pretreatment Prevents Phenylephrine- and KPSS-Induced Constriction of Rat Mesenteric Arteries

We further used another Drp1 inhibitor dynasore12 to confirm the role of mitochondrial fission in artery constriction. Similar
to the effect of mdivi-1, dynasore relaxed the precontraction, and dynasore pretreatment prevented the constriction of rat mesenteric arteries in both phenylephrine- and KPSS-induced vasoconstriction models (Figure S4).

**Mdivi-1 Inhibits KPSS-Induced Increase of Mitochondrial Fission, Mitochondrial Reactive Oxygen Species, and Cytosolic [Ca2+]i in Rat Vascular Smooth Muscle Cells (A10)**

Because both phenylephrine and KPSS induced mitochondrial fission in arteries, we then used mitochondria-specific probes mitoTracker and mito-SOX and Ca2+ probe Fluo-3/AM to examine the effects of vasoconstriction stimuli on mitochondrial fragmentation, mitochondrial reactive oxygen species (mito-ROS) production, and cytosolic [Ca2+]i in cultured arterial smooth muscle cells (A10) by the case of high K+-induced model.

First, the merged real-time confocal images showed that mitoTracker green fluorescence colocalized with mito-SOX red fluorescence, confirming the detection of mitochondrial source of ROS by mito-SOX staining (Figure S5). Then the dynamic mitochondrial morphology was observed in the live cells by using real-time confocal microscopy. As shown in the representative images of dynamic mitochondrial morphology (Figure 4A through 4C), KPSS treatment induced mitochondrial fragmentation, but the excessive fragmentation was inhibited when the cells were pretreated with mdivi-1. We also analyzed the quantitative fragmentation of mitochondria in KPSS and KPSS plus mdivi-1 groups, and the results are shown in Figure 4D. These data were consistent with the results of the above TEM observations.

Because mitochondrial fission induced elevated production of ROS,13 we further examined mito-ROS production in cells exposed to KPSS or mdivi-1 plus KPSS by using real-time confocal microscopy. KPSS treatment induced a marked increase in mito-SOX fluorescence, which was inhibited by pretreatment of mdivi-1. The representative images and the analyzed data are shown in Figure 5A and 5B.
We further examined the effect of mitochondrial fission inhibition on cytosolic [Ca\textsuperscript{2+}] in smooth muscle cells. As shown in Figure 5C and 5D, KPSS treatment induced a marked increase of cytosolic [Ca\textsuperscript{2+}] as evidenced by increased fluo-3 fluorescence. However, pretreatment of mdivi-1 abolished the KPSS-induced increase of cytosolic [Ca\textsuperscript{2+}].

As shown in Figure 5D, there existed a rapid increase of cytosolic [Ca\textsuperscript{2+}], in KPSS and mdivi-1 plus KPSS groups initially. We speculated that the initial increase of cytosolic [Ca\textsuperscript{2+}], triggered mitochondrial fission, leading to the sustained increase of cytosolic [Ca\textsuperscript{2+}], and vasoconstriction; mdivi-1 inhibited the initiation of mitochondrial fission and then inhibited the subsequent increase of cytosolic [Ca\textsuperscript{2+}], and vasoconstriction. Therefore, we used BAPTA/AM to chelate the intracellular free Ca\textsuperscript{2+} and examined the changes of cytosolic [Ca\textsuperscript{2+}], mitochondrial fragmentation, and mito-ROS production in smooth muscle cells. Results showed that the prechelation of cytosolic Ca\textsuperscript{2+} prevented KPSS-induced cytosolic [Ca\textsuperscript{2+}] increase, mitochondrial fission, and mito-ROS overproduction (Figure S6), which supported our hypothesis.

**Mito-TEMPO Antagonizes Phenylephrine- and KPSS-Induced Constriction of Rat Mesenteric Arteries**

Because mito-ROS production increased in smooth muscle cells exposed to high K\textsuperscript{+}, we further examined mito-TEMPO, a mitochondria-targeted antioxidant, on phenylephrine- and KPSS-induced constriction of rat mesenteric arteries. As shown in Figure S7A, mito-TEMPO significantly inhibited phenylephrine-induced constriction of rat mesenteric arteries with intact and denuded endothelium; however, tempol and apocynin, the antioxidants without targeting mitochondria, showed no significant effect on phenylephrine-induced constriction. Similarly, mito-TEMPO inhibited KPSS-induced constriction of rat mesenteric arteries.

**Figure 4.** KPSS induces increase of mitochondrial fragmentations that are inhibited by mdivi-1 in arterial smooth muscle cells (A10) evaluated by real-time confocal microscopy. The mitochondria were stained with mitochondrial-specific probe mitoTracker Green. The representative time-lapse images of smooth muscle cells exposed to normal physiological salt solution (PSS) are shown in A and to KPSS (50 mmol/L K\textsuperscript{+}) are shown in B. The enlarged images of the boxed area showed clear mitochondrial fragmentation after KPSS treatment. C. The time-lapse images of smooth muscle cell preincubated with mdivi-1 for 30 min and then followed by exposure to KPSS. The enlarged images of the boxed area showed the integrity of mitochondria was not affected by KPSS in the presence of mdivi-1. D. The summary data showed that mdivi-1 inhibited KPSS-induced increase of mitochondrial fragmentations. Mitochondrial length was analyzed by using Image-Pro Plus software and normalized to the mean of mitochondrial length at 0 min.
constriction of rat mesenteric arteries (Figure S7B). These results indicate that mito-ROS plays important role in mediating phenylephrine- and KPSS-induced vasoconstriction.

We further observed the mitochondrial morphology of smooth muscle cells in rat aorta pretreated with mito-TEMPO (20 μmol/L) followed by phenylephrine stimulation by using TEM. As shown in Figure S8, mito-TEMPO treatment did not prevent phenylephrine-induced excessive mitochondrial fission in smooth muscle cells, indicating that the mito-ROS–mediating vasoconstriction was a downstream effector of mitochondrial fission.

Nitroglycerin and Y27632 Relaxes KPSS-Induced Vasoconstriction and Inhibits KPSS-Induced Mitochondrial Fission

Because suppression of mitochondrial fission relaxed the vasoconstriction, we asked whether the known vasorelaxants could inhibit mitochondrial fission in arterial smooth muscle. Nitroglycerin shows vasorelaxant effect through providing nitric oxide and Rho-associated protein kinase (ROCK) inhibitor Y27632 through decreasing Ca²⁺ sensitization. Both nitroglycerin and Y27632 antagonized KPSS-induced aorta constriction (Figure 6A through 6D). Interestingly, both nitroglycerin and Y27632 also inhibited KPSS-induced mitochondrial fission in smooth muscle cells of rat aorta (Figure 6E and 6F). In cultured arterial smooth muscle cells (A10), both nitroglycerin and Y27632 inhibited KPSS-induced excessive mitochondrial fragmentation (Figure S9).

Pharmacological DRP1 Inhibition Antagonizes Phenylephrine- and KPSS-Induced Constriction of Mesenteric Arteries in Spontaneously Hypertensive Rats

The mesenteric arteries from spontaneously hypertensive rats (SHRs) showed not only morphological remodeling but also functional remodeling, such as the increased inflammatory molecule expression and the impaired acetylcholine-induced relaxation. We wondered whether the responses of mesenteric arteries to phenylephrine and KPSS stimuli were different between SHRs and Wistar Kyoto (WKY) rats after mitochondrial fission inhibition. SHRs showed increased systolic blood pressure, diastolic blood pressure, and heart body ratio (Figure S10A and S10B). Mdivi-1 showed similar relaxing effects on phenylephrine- and KPSS-induced constriction of mesenteric

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Figure 5. Effects of mdivi-1 on KPSS-induced mitochondrial reactive oxygen species (mito-ROS) overproduction and increase of cytosolic [Ca²⁺]i in arterial smooth muscle cells (A10) evaluated by real-time confocal microscopy. The mito-ROS was stained with mitochondrial-specific probe mito-SOX Red, and the cytosolic [Ca²⁺]i was stained with Fluo-3/AM. The levels of mito-ROS and cytosolic [Ca²⁺]i were represented by the relative intensity of fluorescence. A, The representative time-lapse images of smooth muscle cells exposed to normal physiological salt solution (PSS), KPSS (50 mmol/L K⁺), and preincubation with mdivi-1 for 30 min followed by exposure to KPSS. B, The analyzed data of dynamic changes of mito-ROS production. The numbers of analyzed cells in control, KPSS, and mdivi-1 plus KPSS were 87, 15, and 25 respectively. **P<0.01 vs control; ##P<0.01 vs KPSS. C, The representative time-lapse images of smooth muscle cells exposed to normal PSS, KPSS (50 mmol/L K⁺), and preincubation with mdivi-1 for 30 min followed by exposure to KPSS. D, The analyzed data of dynamic changes of cytosolic [Ca²⁺]i indicated that mdivi-1 inhibited KPSS-induced increase of cytosolic [Ca²⁺]i. The numbers of analyzed cells in control, KPSS, and mdivi-1 plus KPSS were 64, 100, and 100, respectively. **P<0.01 vs control; ##P<0.01 vs KPSS.
arteries from SHR and WKY rats (Figure S10C), and mdivi-1 pretreatment showed similar preventive effect on phenylephrine- and KPSS-induced constriction of mesenteric arteries from SHR and WKY rats (Figure S10D), indicating that drugs inhibiting mitochondrial fission would have similar efficacy in hypertensive subjects. We further compared the Drp1 protein expression in aorta form SHRs and WKY rats. Results showed that there was no significant difference of p-Drp1 (ser616) and total Drp1 protein expression between SHRs and WKY rats (Figure S10E).

We further compared the mitochondrial morphology of smooth muscle cells in aorta from WKY and SHR rats by using TEM. The basal mitochondrial fragmentation was more in smooth muscle cells of aorta from SHR than that from WKY rats (Figure S11A through S11C). Both phenylephrine and KPSS treatment induced increase of mitochondrial fission in smooth muscle cells of aorta from WKY and SHR rats, and pretreatment with mdivi-1 significantly attenuated the excessive fission induced by phenylephrine and KPSS treatments (Figure S11A through S11E). Mdivi-1 is water insoluble and has to be dissolved in dimethyl sulfoxide. Because it is still unknown about pharmacokinetics of mdivi-1 and acute injection of the solvent dimethyl sulfoxide-induced fluctuation of blood pressure, we did not further study the effect of mdivi-1 in vivo.

**Discussion**

In this study, we have investigated the role of mitochondrial fission of smooth muscle cells in vascular constriction and relaxation by using phenylephrine- and KPSS-induced artery constriction models. We find that mitochondrial fission of smooth muscle cells is involved in artery constriction.

Based on our findings, we put forward a novel mechanism of vascular constriction, and the schematic diagram is shown in Figure S12. In this model, it is speculated that the initial extracellular Ca²⁺ influx in smooth muscle cells triggers mitochondrial fission; mitochondrial fission induces mito-ROS overproduction that leads to contraction of smooth muscle cells. To evidence this model, we designed the elaborated experiments. First, we used 2 mitochondrial fission inhibitors, mdivi-1 and dynasore. Mdivi-1 is a selective and dynasore is a nonselective drp1 inhibitor. Both of them antagonized phenylephrine- and KPSS-induced artery constriction. Our previous study showed that KPSS- but not phenylephrine-induced artery constriction was sensitive to L-type Ca²⁺ blocker. Mdivi-1 and dynasore relaxed both phenylephrine- and KPSS-induced artery constriction, indicating that they acted at the general joint of artery constriction complex. Then, by using the approaches of TEM and mitochondrial network staining, we found that phenylephrine and KPSS-induced mitochondrial...
fission in smooth muscle cells of aorta and in cultured vascular smooth muscle cells, and the increased mitochondrial fission was inhibited by mdivi-1. On the contrary, the known vasorelaxants nitroglycerin and ROCK inhibitor Y27632 not only relaxed KPSS-induced aorta constriction but also inhibited KPSS-induced increase of mitochondrial fission in smooth muscle cells of rat aorta and cultured arterial smooth muscle cells. It was reported that NO inhibited mitochondrial fission and ROCK activation induced mitochondrial fission. Although nitroglycerin and Y27632 have different mechanisms of vasorelaxant effects, both of them inhibited mitochondrial fission. In a way, we have used 4 different types of mitochondrial fission inhibitors, mdivi-1, dynasore, nitroglycerin, and Y27632, to demonstrate that mitochondrial fission of smooth muscle cells is involved in artery constriction. It was noticeable that, as shown in Figures 3D and 6E, the excessive mitochondrial fission and smooth muscle contraction always occurred simultaneously. When the smooth muscle was in contractive state, the excessive mitochondrial fission is increased; when the smooth muscle was relaxed, the excessive mitochondrial fission was decreased. Comprehensively analyzing of above data, we propose that the mitochondrial fission of smooth muscle cells is coupled with the smooth muscle contraction. Further experiments showed that KPSS treatment increased mito-ROS generation, and mitochondria-targeted antioxidant mito-TEMPO inhibited phenylephrine- and KPSS-induced artery constriction, indicating that mito-ROS might be the link of this coupling. Previous study reported that targeted mitochondrial ROS scavenging inhibited adrenergic vasoconstriction, which is consistent with our findings. We noticed that mito-TEMPO at 20 μmol/L completely inhibited phenylephrine-induced vasoconstriction, but the inhibition ratio was no more than 40% in KPSS-induced model (Figure S7). These results indicated that, although mito-ROS was involved in both phenylephrine- and KPSS-induced vasoconstriction models, the weighing of mito-ROS in these models was different.

Our study showed that both high K+ and phenylephrine induced mitochondrial fission in smooth muscle cells of aorta, indicating that mitochondrial fission of smooth muscle cells might be the common consequences of different vasoconstriction stimuli. A previous work reported that Ca2+ influx through voltage-dependent Ca2+ channels induced by high K+ led to mitochondrial fission in neurons and Hela cells; a recent work demonstrated that norepinephrine induced mitochondrial fission in cultured cardiomyocytes, which was attributed to the stimulation of α1-adrenergic receptors and the consequent increase of intracellular [Ca2+]. These reports were similar to the present finding in smooth muscle cells. We prechelated the cytosolic Ca2+ with BAPTA-AM in smooth muscle cells and found that the mitochondrial fission and mito-ROS generation induced by high K+ was prevented. These results suggest that the initial increase of intracellular Ca2+ plays a triggering role for inducing mitochondrial fission in vascular smooth muscle cells.

Mitochondrial fission-induced mito-ROS generation has been extensively proved. Although mito-ROS plays an important role in normal physiological cell signaling, excessive mito-ROS production leads to pathological states. In angiotensin II–treated endothelial cells, the production of mito-ROS was increased and mito-ROS scavenger mito-TEMPO attenuated the angiotensin II–induced hypertension through improving endothelial-dependent relaxation of arteries, indicating that mito-ROS in endothelial cells was involved in regulation of vascular function. In the present study, we found that mito-TEMPO but not tempol inhibited both high K+- and phenylephrine-induced constriction in endothelium-intact and endothelium-denuded mesenteric arteries, indicating that mitochondrial ROS in smooth muscle cells plays more important role in vasoconstriction.

The cross talk between mito-ROS and Ca2+ signaling has long been studied and discussed in multiple cell types. For instance, in cardiomyocytes, mitochondria-derived ROS have been implicated in the modulation of RyR2, (ryanodine receptor 2)-mediated Ca2+-induced Ca2+ release, thus the regulation of cardiac contraction; in pulmonary arterial smooth muscle cells, mito-ROS trigger calcium increases, which induces pulmonary vasoconstriction during hypoxia. However, to our knowledge, the regulation of mito-ROS on Ca2+, and contractility in systemic vascular smooth muscle cells are not completely understood. In cerebral arteries, mitochondrial-derived ROS activated Ca2+ sparks in smooth muscle cells, but the consequence was cerebral artery dilation because that Ca2+ spark activated large-conductance Ca2+-activated K+ channels. Another work showed that mito-ROS stimulated nuclear factor-κB-dependent Ca,1,2 channel expression in arterial myocytes, thereby modulating cerebral arterial contractility, but this finding could not explain our present results. High K+ and phenylephrine induce mitochondrial fission and vasoconstriction within several minutes, such short period is not enough for the process Ca1,2 transcription and translation. A recent work reported that superoxide enhanced calcium entry through L-type channels activated by high K+ in rat cortical afferent arterioles; they showed that the effects of superoxide in cortical afferent arterioles were sensitive to tempol. Another mechanism of vasoconstriction is through increasing Ca2+ sensitization by inhibition of myosin light chain phosphatase without dependence on [Ca2+]. Activated ROCK results in myosin light chain phosphatase inhibition and vasoconstriction. Mito-ROS activates ROCK, thus induces vasoconstriction.

We used phenylephrine- and high K+-induced vasoconstriction models in the tissue level, but only used high K+-induced model in the cellular level because we found that the A10 cells lost sensitivity to phenylephrine after culturing. In fact, even in primary arterial smooth muscle cells, the response to phenylephrine was also lost after cell culturing. Therefore, we only summarized the role of mitochondrial fission in high K+ (membrane depolarization)–induced vasoconstriction in Figure S12.

Mitochondrial dynamics were linked to mitochondrial metabolism, and mitochondrial inhibition induced metabolic vascular relaxation. Therefore, we further examined the cell oxygen consumption of A10 cells. KPSS and KPSS plus Mdivi-1 treatments showed no significant effect on oxygen consumption of cultured A10 cells (Figure S13).

In addition to using 4 different types of drugs, mdivi-1, dynasore, nitroglycerin and Y27632, we further performed
the Drp1 knockdown experiments. Knockdown of Drp1 was used previously in studying the role of mitochondrial fission in pathological processes. However, it should be noticed that all mitochondrial fission, vasoconstriction, and vasorelaxation activities are dynamic physiological processes; inhibition of mitochondrial fission by knockdown of Drp1 has its own disrupted the basal physiological process, we thought that this technique was not matched with our aim. Even so, we used Ad-siRNA-Drp1 to knockdown Drp1 expression to inhibit mitochondrial fission in A10 cells. However, the cells with Drp1 knockdown were intolerant to KPSS stimulation and disrupted when treated with KPSS (data not shown). We have used the methods currently available to prove the relationship between mitochondrial fission and vasorelaxation.

**Perspectives**

Based on the phenomena of mitochondria continuously undergoing fusion/fission activities, we speculate that interfering mitochondrial dynamics could show acute effects on the vascular function, and indeed, we find that vasoconstriction stimuli induce increase of mitochondrial fission of smooth muscle cells in arteries; acute inhibition of mitochondrial fission of smooth muscle cells antagonizes artery constriction. Furthermore, based on our findings, we put forward a possibility that there exists a mitochondrial fission–contraction coupling in arterial smooth muscle cells. Our work reveals a novel mechanism for vasorelaxation.

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**Disclosures**

None.

**References**

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Novelty and Significance

What Is New?

- Vasoconstriction stimuli induce increase of mitochondrial fission of smooth muscle cells in arteries.
- Inhibition of mitochondrial fission of smooth muscle cells inhibits artery constriction.
- Mitochondrial fission of smooth muscle cells is involved in artery constriction.

What Is Relevant?

- Mitochondria are dynamic organelles and continuously undergo fission and fusion processes.
- Defects in mitochondrial dynamics are implicated in multiple cardiovascular diseases.

Summary

Vasoconstriction stimuli induce increase of mitochondrial fission of smooth muscle cells, and inhibition of mitochondrial fission of smooth muscle cells inhibits artery constriction. The present findings propose a novel physiological concept mitochondrial fission–contraction coupling in arterial smooth muscle cells and find a novel role of mitochondrial fission in artery tension regulation.
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Methods

Agents and Animals
Acetylcholine chloride (Ach), apocynin and mitoTEMPO were purchased from Sigma Aldrich Chemistry (Saint Louis, MO, USA). Mdivi-1 and dynasore were purchased from Selleck Chemicals (Shanghai, China). Tempol was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mito-Tracker Green, mitoSOX, fluo-3/AM, and hoechst were purchased from life technology (invitrogen, Oregon, USA). Phenylephrine (PE), Acetylcholine chloride (Ach) and Apocynin were dissolved in double distilled water, and others were dissolved in DMSO (Tianjin Fuyu Fine Chemical Co., Ltd). Arterial smooth muscle cells (A10) were purchased from ATCC. Adult male Sprague-Dawley rats and Kunming mice 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. High K\(^+\) salt solutions containing 60 mM and 50 mM K\(^+\) were used for treating artery tissues and smooth muscle cells respectively. The KPSS (60 mM K\(^+\)) solution was composed of (mM): NaCl, 74.7; KCl, 60; MgSO\(_4\).
\(^{7}\)H\(_2\)O, 1.17; KH\(_2\)PO\(_4\), 1.18; NaHCO\(_3\), 14.9; CaCl\(_2\), 1.6; D-glucose, 5.5; EDTA, 0.026. The KPSS(50mM K\(^+\)) solution was composed of (mM): NaCl, 84.7; KCl, 50; MgSO\(_4\).
\(^{7}\)H\(_2\)O, 1.17; KH\(_2\)PO\(_4\), 1.18; NaHCO\(_3\), 14.9; CaCl\(_2\), 1.6; D-glucose, 5.5; EDTA, 0.026.

Mesenteric Artery Tension Measurement
Briefly, adult male Sprague-Dawley rats 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 composed of (mM): NaCl, 130; KCl, 4.7; MgSO\(_4\).
\(^{7}\)H\(_2\)O, 1.17; KH\(_2\)PO\(_4\), 1.18; NaHCO\(_3\), 14.9; CaCl\(_2\), 1.6; D-glucose, 5.5 and aerated with 95% O\(_2\) and 5% CO\(_2\) (pH 7.4 at 37°C). Mesenteric arteries (3th branch) were separated from venous and fat tissue in a dish containing PSS solution. The vessels were dissected into 2 mm rings and moved to an organ bath filled with 5 mL fresh PSS solution oxygenated with 95% O\(_2\) and 5% CO\(_2\) (pH 7.4 at 37°C). Mesenteric arteries were mounted in a multi wire myograph system (DMT620, Danish Myo Technology, Aarhus, Denmark). The tension measurement of human mesenteric arteries was performed similarly.

Aorta Tension Measurement
Adult male Sprague-Dawley rats or Kunming mice 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 4mm 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\(_2\) and 5% CO\(_2\) (pH 7.4 at 37°C). After equilibration, the tension was measured by using a multichannel acquisition and analysis system (Model BL-420E, Taimeng Technology Instrument, Chengdu, China).

Human Mesenteric Artery Specimens
The procedures were approved by the Clinical Research Ethics Committee of Harbin Medical
University. Human mesenteric arteries were harvested from the removed tissues of colon cancer resection from a patient after obtaining informed consent. The patient (male) was 52 years, without cardiovascular diseases such as hypertension and diabetes.

**Measurements for Mitochondrial Networks**
Cultured arterial smooth muscle cells (A10) were loaded with Mito-Tracker Green (50 nM) for 20 min and Hoechst (1 µg/mL) for 15 min at 37 ºC. The cells were imaged by using the Zeiss LSM 700 confocal microscope (Carl Zeiss, Jena, Germany). All imaging was observed with a 40 × oil immersion objective lens. Mitochondrial length was analyzed by using Image-Pro Plus software.

**Measurement of Mitochondrial Reactive Oxygen Species**
Cultured arterial smooth muscle cells (A10) were loaded with MitoSOX (5 µM) for 20 min and Hoechst (1µg/mL) for 15 min at 37ºC and then the fluorescence was measured by using confocal microscopy. Confocal microscope images were collected by using a Zeiss LSM 700 with the Zeiss LSM software. Images of mitoSOX fluorescence were obtained using a 40× oil objective with an excitation at 555 nm and an emission of 585 nm. Images of Hoechst staining were obtained by using excitation at 405 nm and an emission of 435 nm. The levels of mitochondria ROS were represented by the relative intensity of fluorescence.

**Measurement of Cytosolic [Ca^{2+}]_{i} of Smooth Muscle Cells**
Cultured arterial smooth muscle cells (A10) were loaded with 5 µM fluo-3/AM for 15 min (37°C) and rinsed four times with warm PBS (37°C). The fluorescence was measured by using confocal microscopy (Zeiss LSM 700; Zeiss; Oberkochen, Germany). The excitation and emission wavelength for signal detection was 488 nm and 518 nm respectively. The levels of cytosolic [Ca^{2+}]_{i} were represented by the relative intensity of fluorescence in selected regions of cytosolic parts.

**Transmission Electron Microscopy (TEM)**
Selected samples (aorta tissue) were incubated with mdivi-1 (30 µM) or PSS (Control) for 30 min, then added constriction agents (PE, KPSS) 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% OsO4 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).

**Oxygen Consumption Rate Assay**
Oxygen consumption was assessed using an oxygen consumption rate assay kit (MitoXpress-Xtra HS method) (Cayman Chemical) according to the manufacturer’s instructions (Luxcel Biosciences Ltd.). A10 cells (4×10^{4}) were seeded on a black 96-well plate with clear bottom and cells were incubated for 24 h. A10 cells were incubated with 10 µl of MitoXpress probe immediately before measuring the fluorescence intensity. Fluorescence intensity was measured with time-resolved fluorescence plate reader (Infinite M200 PRO, Tecan) with optimal filter wavelengths of 380 nm for excitation and 650 nm for emission. Measurement was performed under a sealed environment (by overlaying each well with HS Mineral Oil), in which the exchange of O_{2} was limited.
Results

Figure S1. Drp1 Selective Inhibitor Mdivi-1 Prevents Phenylephrine (PE)- and High K⁺ (KPSS)-Induced Constriction of Rat Mesenteric Arteries. (A) Mdivi-1 pre-treatment for 30 min prevented PE (5 µM)-induced constriction of rat mesenteric arteries with intact endothelium. The different patterns of inhibition were shown in the framed box. The inhibitory effect of mdivi-1 was relieved after washout of mdivi-1. **P<0.01 vs DMSO (control). (B) Mdivi-1 pretreatment for 30 min prevented KPSS (60 mM K⁺)-induced constriction of rat mesenteric arteries with intact endothelium. The inhibitory effect of mdivi-1 was relieved after washout of mdivi-1. **P<0.01 vs DMSO (control).
Figure S2. Mdivi-1 Antagonizes KPSS-Induced Constriction of Human Mesenteric Arteries. (A,B) Mdivi-1 induced concentration-dependent relaxation in human mesenteric arteries pre-contracted with KPSS. The relaxation ratio of mdivi-1 was calculated by subtracting the relaxation ratio of corresponding control (DMSO) to avoid the error induced by natural rundown of the artery tension. **P<0.01 vs control. (C,D) Mdivi-1 pretreatment for 30 min prevented KPSS-induced constriction of human mesenteric arteries. **P<0.01 vs control.
Figure. S3. KPSS induces mitochondrial fission at the early stage of vasoconstriction. 
(A) The original recording of KPSS-induced vasoconstriction showed that the contraction had occurred at 30s and 1min after KPSS treatment. (B) The TEM images showed that the excessive mitochondrial fission of smooth muscle cells had occurred at 30s and 1min after KPSS treatment when the aorta started to constrict. The analyzed data were shown in right panel. **P<0.01 vs control. The numbers of mitochondria was 671, 286, and 522 in control, 30s and 1min after KPSS.
Figure S4. Dynasore Relaxes PE- and KPSS-Induced Constriction, and Dynasore Pretreatment Antagonizes PE- and KPSS-Induced Constriction of Rat Mesenteric Arteries. (A,B) Dynasore relaxed PE-induced constriction, and dynasore pretreatment antagonized PE-induced constriction of rat mesenteric arteries. The relaxation ratio of mdivi-1 was calculated by subtracting the relaxation ratio of corresponding control (DMSO) in (A). *P<0.05; **P<0.01 vs Control (DMSO). (C,D) Dynasore relaxed KPSS-induced constriction, and dynasore pretreatment antagonized KPSS-induced constriction of rat mesenteric arteries. The relaxation ratio of mdivi-1 was calculated by subtracting the relaxation ratio of corresponding control (DMSO) in (C). **P<0.01 vs Control(DMSO).
Figure S5. The Merged Real-Time Confocal Image shows that MitoTracker Green Fluorescence Colocalizes with MitoSox Red Fluorescence. Mitochondrial network was stained in green and mitochondrial source of ROS stained in red.
Figure S6. BAPTA/AM(5µM) Pretreatment Inhibits KPSS-Induced Increase of Cytosolic [Ca^{2+}], Mitochondrial Fission and MitoROS in Arterial Smooth Muscle Cells(A10).

(A) The cytosolic [Ca^{2+}] was stained with Fluo-3/AM. The levels of cytosolic [Ca^{2+}] were represented by the relative intensity of fluorescence. **P<0.01 vs KPSS.

(B-D) The representative time-lapse images showed that BAPTA/AM(5 µM) pretreatment inhibited KPSS-induced increase of mitochondria fragmentation in arterial smooth muscle cells (A10). The mitochondria were stained with mitochondria-specific probe mitoTracker Green. The framed areas were enlarged.

(E) The mitochondria ROS was stained with mitochondria-specific probe mitoSOX Red and the levels of mitochondria ROS were represented by the relative intensity of fluorescence. **P<0.01 vs KPSS.
Figure S7. MitoTEMPO Antagonizes PE- and KPSS-Induced Constriction of Rat Mesenteric Arteries. (A) MitoTEMPO antagonized PE-induced constriction of rat mesenteric arteries with intact endothelium. (a) The original recordings showed that mitoTEMPO dose-dependently inhibited PE-induced constriction of rat mesenteric arteries with intact endothelium, but tempol and apocynin showed no significant effects. (b) The original recordings showed that mitoTEMPO inhibited PE-induced constriction of rat mesenteric arteries with denuded endothelium. (c) The summarized data showed that mitoTEMPO antagonized PE-induced constriction of rat mesenteric arteries with intact endothelium. **P<0.01 vs Control. (B) MitoTEMPO antagonized KPSS-induced constriction of rat mesenteric arteries with intact endothelium. Tempol and apocynin showed no significant effects. **P<0.01 vs Control.
Figure S8. The mitochondria morphology of smooth muscle cells in rat aorta pretreated with mitoTEMPO followed by PE stimulation. (A) The representative TEM images showed that mitoTEMPO (20 µM) treatment did not prevent PE-induced excessive mitochondrial fission in smooth muscle cells, but additional treatment with midivi-1(30 µM) inhibited PE-induced excessive mitochondrial fission. (B) The analyzed data of mitochondrial fission. \( **P<0.01 \) vs control. \( ##P<0.01 \) vs PE. The number of mitochondria was 671,409,306, and 82 in control, PE, mitoTEMPO+PE, mitoTEMPO+midivi-1+PE groups.
The representative images of smooth muscle cells stained with mitochondria-specific probe mitoTracker Green showed that both nitroglycerin (10μM) and Y27632 (5μM) inhibited KPSS (50 mM K⁺)-induced excessive mitochondrial fragmentations, and the summary data were shown in (E). Mitochondrial length was analyzed by using Image-Pro Plus software and normalized to the mean of mitochondrial length at 0 min.
Figure S10. The Effects of Mdivi-1 on Vasoactivity and Drp1 Expression of Arteries from SHR and WKY Rats. (A,B) SHR rats showed increased systolic blood pressure, diastolic blood pressure, and heart body ratio. **P<0.01 vs WKY. LV, left ventricle; SP, septum; BW, body weight. (C) Mdivi-1 showed similar relaxing effects on PE- and KPSS-induced
constriction of mesenteric arteries from SHR and WKY rats. (D) Mdivi-1 pretreatment showed similar antagonizing effects on PE- and KPSS-induced constriction of mesenteric arteries from SHR and WKY rats. (E) There was no difference of p-Drp1(ser616) and total Drp1 protein expressions between SHRs and WKY rats.
Figure S11. The mitochondria morphology of smooth muscle cells in aorta from WKY and SHR rats. (A-B) The representative images observed by using transmission electron microscope (TEM). Both PE (5 µM) and KPSS (60 mM K⁺) treatments induced increase of mitochondrial fission in smooth muscle cells of aorta from WKY and SHR rats, and
pre-treatment with mdivi-1(30 µM) inhibited the excessive fission induced by PE and KPSS treatments. (C) The quantity analysis showed that the basal mitochondrial fragmentation was more in smooth muscle cells of aorta from SHR than that from WKY rats. **P<0.01 vs WKY. (D-E) The quantity analysis showed that both PE (5 µM) and KPSS (60 mM K+) treatments induced increase of mitochondrial fission in smooth muscle cells of aorta from WKY and SHR rats, and pre-treatment with mdivi-1(30 µM) inhibited the excessive fission induced by PE and KPSS treatments. *P<0.01 vs control; **P<0.01 vs PE; ***P<0.01 vs KPSS. The numbers of mitochondria analyzed per group were shown in the bar. Aspect ratio, ratio between major and minor axes of an ellipse is equivalent to the mitochondrion.
Figure S12. The schematic diagram of the role of mitochondrial fission in vasoconstriction. In smooth muscle cells, Ca$^{2+}$ increase induced by membrane depolarization triggers mitochondrial fission; mitochondrial fission induces mitochondrial ROS (mitoROS) overproduction, which induces smooth muscle contraction.
**Figure S13.** KPSS treatment and Mdivi-1 (30 μM) plus KPSS treatment showed no significant effect on oxygen consumption of cultured A10 cells. Antimycin A (30μM) was as a positive control which significantly inhibited the oxygen consumption in A10 cells, indicating that the measurement system was reliable. In control group, the equal volume of medium (160 μL) was added. RFU, Relative Fluorescence Unit.