ORIGINAL ARTICLE

Arterial relaxation is coupled to inhibition of mitochondrial fission in arterial smooth muscle cells: comparison of vasorelaxant effects of verapamil and phentolamine

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Abstract Mitochondria are morphologically dynamic organelles which undergo fission and fusion processes. Our previous study found that arterial constriction was always accompanied by increased mitochondrial fission in smooth muscle cells, whereas inhibition of mitochondrial fission in smooth muscle cells was associated with arterial relaxation. Here, we used the typical vasorelaxants, verapamil and phentolamine, to further confirm the coupling between arterial constriction and mitochondrial fission in rat aorta. Results showed that phentolamine but not verapamil induced vasorelaxation in phenylephrine (PE)-induced rat thoracic aorta constriction. Verapamil, but not phentolamine, induced vasorelaxation in high K⁺ (KPSS)-induced rat thoracic aorta constriction. Pre-treatment with phentolamine prevented PE- but not KPSS-induced aorta constriction and pre-treatment with verapamil prevented both PE- and KPSS-induced aorta constriction. Transmission electron microscopy (TEM) results showed that verapamil but not phentolamine inhibited KPSS-induced excessive mitochondrial fission in aortic smooth muscle cells, and verapamil prevented both PE- and KPSS-induced excessive mitochondrial fission in aortic smooth muscle cells.
muscle cells. Verapamil inhibited KPSS-induced excessive mitochondrial fission in cultured vascular smooth muscle cells (A10). These results further demonstrate that arterial relaxation is coupled to inhibition of mitochondrial fission in arterial smooth muscle cells.

1. Introduction

Mitochondria are morphologically dynamic organelles which undergo fission and fusion dynamic processes. Mitochondrial dynamics are mainly regulated by mitochondrial fusion-related proteins including the outer mitochondrial membrane (OMM) proteins, mitofusin 1 (MFN1), mitofusin 2 (MFN2), the inner mitochondrial membrane ( IMM) protein, optic atrophy factor 1 (OPA1), and fission-related proteins including dynamin-related protein 1 (DRP1), human fission factor-1 (Fis1), mitochondrial fission factor (MFF), MiD49 and MiD51.

Mitochondrial fission has been reported to be involved in apoptosis, autophagy, mitochondrial transport, cell differentiation, embryonic development and metabolism. Disorders of mitochondrial fission contribute to a variety of pathological processes. Mitochondrial fission has been implicated in diabetes, cardiomyocyte hypertrophy, myocardial ischemia/reperfusion injury, heart failure and neurodegenerative disease.

Recently, some literature showed that regulation of mitochondrial fission might be a novel target to prevent cardiovascular diseases including hypertension, pulmonary arterial hypertension, atherosclerosis, and intimal hyperplasia. Our previous study found that phenylephrine (PE)- and KPSS-induced vasoconstriction was accompanied by increased mitochondrial fission in smooth muscle cells, and mitochondrial fission inhibitors (mdivi-1 and dynasore) both inhibited vasoconstriction induced by PE or KPSS. Furthermore, Y27632 (a ROCK inhibitor) and nitroglycerin relaxed KPSS-induced vasoconstriction and inhibited KPSS-induced mitochondrial fission. These results indicated that there might be a coupling between arterial constriction and mitochondrial fission in smooth muscle cells. In order to confirm the hypothesis, we also used other typical vasorelaxants, verapamil and phentolamine, to examine the relationship between arterial constriction and mitochondrial fission in smooth muscle cells from rat thoracic aorta. Here, the effects of verapamil and phentolamine in vasoconstriction models induced by PE or KPSS further demonstrate that arterial relaxation is coupled to inhibition of mitochondrial fission in arterial smooth muscle cells.

2. Materials and methods

2.1. Agents and animals

Acetylcholine chloride (Ach) was purchased from Sigma-Aldrich Chemistry (Saint Louis, MO, USA). Mito-Tracker Green and Hoechst were purchased from Life Technology (Invitrogen, OR, USA). PE and verapamil were purchased from Harvest Pharmaceu-tical Co., Ltd. (Shanghai, China). Phentolamine was purchased from Santa Cruz Biotechnology, Inc. (Shanghai, China). Arterial smooth muscle cells (A10) were purchased from ATCC (VA, USA). Adult male Sprague-Dawley rats 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 and 50 mmol/L K+ were used for treating arterial tissues and smooth muscle cells respectively. The KPSS (60 mmol/L K+ solution) was composed of (mmol/L): NaCl, 74.7; KCl, 60; MgSO4·7H2O, 1.17; KH2PO4, 1.18; NaHCO3, 14.9; CaCl2, 1.6; d-glucose, 5.5; EDTA, 0.026. The KPSS (50 mmol/L K+) solution was composed of (mmol/L): NaCl, 84.7; KCl, 50; MgSO4·7H2O, 1.17; KH2PO4, 1.18; NaHCO3, 14.9; CaCl2, 1.6; d-glucose, 5.5; EDTA, 0.026.

2.2. Aorta tension measurement

The experiments were carried out according to our previous work. Adult male Sprague-Dawley rats were sacrificed after anesthesia with sodium pentobarbitone. The thorax was cut to expose the aorta, and the descending thoracic aorta was rapidly dissected and transferred 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 and then transferred to an organ bath with 10 mL fresh PSS solution oxygenated with 95% O2 and 5% CO2 (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).

2.3. Measurements of mitochondrial networks

The experiments were carried out according to our previous work. Cultured arterial smooth muscle cells (A10) were loaded with Mito-Tracker Green (50 nmol/L) for 20 min and Hoescht (1 µg/mL) for 15 min at 37 °C. 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 fragmentation was analyzed according to literature. Mitochondrial length was determined by use of Image-Pro Plus software.

2.4. Transmission electron microscopy (TEM)

The experiments were carried out according to our previous work. Samples were rinsed in buffer, and then fixed in 2.5%
glutaraldehyde in PBS (pH 7.4) for 2–3 days. Specimens were then 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 ultrathin sections were electron stained and observed under an electron microscope (JEM-1220, JEOL Ltd., Tokyo, Japan).

2.5. Data analysis

Data are presented as mean±SEM. Significance was determined by using Student’s t-test or one-way ANOVA followed by Holm-Sidak. *P<0.05 was considered significant.

3. Results and discussion

3.1. Phentolamine induces vasorelaxation in PE-constricted rat thoracic aorta

PE induces vasoconstriction through stimulating α₁-adrenergic receptors in plasma membrane of smooth muscle cells and the subsequent activation of inositol 1,4,5-trisphosphate receptors (IP₃Rs) on sarcoplasmic reticulum. As shown in Fig. 1, phentolamine but not verapamil induced vasorelaxation in PE-induced rat thoracic aorta constriction. Phentolamine is an α₁-adrenergic receptor antagonist, and induced vasorelaxation via inhibition of PE-induced activation of α₁-adrenergic receptors.
3.2. Verapamil induces vasorelaxation in KPSS-constricted rat thoracic aorta

KPSS induces vasoconstriction by depolarizing membrane potential of smooth muscle cells and the subsequent activation of L-type Ca\(^{2+}\) channels. In the KPSS-induced rat thoracic aorta constriction model, verapamil but not phentolamine induced vasorelaxation (Fig. 2). Due to the different mechanisms of PE- and KPSS-induced vasoconstriction, \(\alpha_1\)-adrenergic receptor antagonist and Ca\(^{2+}\) channel blocker showed distinct effects in these models.

3.3. The effect of pretreatment of phentolamine or verapamil on PE- or KPSS-induced aorta constrictions

We further examined the preventive effects of phentolamine and verapamil on PE- and KPSS-induced aorta constriction. As shown in Fig. 3, pretreatment with phentolamine prevented PE- but not KPSS-induced aorta constriction. However, pretreatment with verapamil prevented both PE- and KPSS-induced aorta constrictions (Fig. 4). Accordingly, we suggest that the primary origin of intracellular Ca\(^{2+}\) can be traced to extracellular Ca\(^{2+}\) via Ca\(^{2+}\) channels in vascular smooth muscle cells; hence the storage of intracellular Ca\(^{2+}\) is suppressed by
inhibiting Ca^{2+} channel. Thus, pre-treatment with verapamil, which reduced the store of intracellular Ca^{2+}, could weaken the effect of Ca^{2+} release from sarcoplasmic reticulum induced by PE in smooth muscle cells in aorta, thereby antagonizing constriction of the aorta. A previous study reported that verapamil pretreatment reduced the rise of intracellular Ca^{2+} induced by PI(3,5)P2 in isolated aortic smooth muscle cells and accompanied by reductions in PI(3,5)P2-induced constriction\(^\text{19}\). Such findings are in accord with the present findings.

### 3.4. The effect of pretreatment of phentolamine or verapamil on PE- or KPSS-induced excessive mitochondrial fission of aortic smooth muscle cells

Since we postulated that vasoconstriction is coupled with mitochondrial fission in vascular smooth muscle cells\(^\text{17}\), we used TEM to characterize mitochondrial morphology of smooth muscle cell in aorta treated with verapamil or phentolamine, followed by treatment of PE or KPSS. As shown in Fig. 5A and C, verapamil (but not phentolamine) pretreatment inhibited KPSS-induced excessive mitochondrial fission in smooth muscle cells of aorta. Phentolamine pretreatment inhibited PE-induced excessive mitochondrial fission in smooth muscle cells of aorta. Moreover, verapamil pretreatment prevented both PE- and KPSS-induced excessive mitochondrial fission of aortic smooth muscle cells. The statistical results are shown in Fig. 5B and D. The findings that verapamil pretreatment prevented both PE- and KPSS-induced increases in mitochondrial fission of smooth muscle cells are in accord with data showing that verapamil pretreatment inhibited both PE- and KPSS-induced vasoconstriction.
Our previous work demonstrated that the initial increase of cytosolic \([\text{Ca}^{2+}]_i\) triggered mitochondrial fission in vascular smooth muscle cells\(^\text{17}\). Verapamil inhibited KPSS-induced increases in cytosolic \([\text{Ca}^{2+}]_i\) through blocking L-type \([\text{Ca}^{2+}]_i\) channels, and phentolamine inhibited PE-induced increase of cytosolic \([\text{Ca}^{2+}]_i\), through blockade of \(\alpha_1\)-adrenergic receptor. As discussed above, the primary origin of intracellular \([\text{Ca}^{2+}]_i\) comes from extracellular \([\text{Ca}^{2+}]_i\) via \([\text{Ca}^{2+}]_i\) channels in vascular smooth muscle cells; verapamil pretreatment reduces the storage of intracellular \([\text{Ca}^{2+}]_i\) via inhibiting \([\text{Ca}^{2+}]_i\) channel, so it will weaken the effect of \([\text{Ca}^{2+}]_i\) release from sarcoplasmic reticulum induced by PE, thereby inhibiting PE-induced increase of cytosolic \([\text{Ca}^{2+}]_i\). These results further demonstrate that arterial relaxation is coupled to inhibition of mitochondrial fission in arterial smooth muscle cells.

**Figure 7** Verapamil inhibited KPSS-induced excessive mitochondrial fission in cultured vascular smooth muscle cells (A10) accessed by laser confocal microscopy. The mitochondria of cultured vascular smooth muscle cells were stained with mitochondria-specific probe mitoTracker Green. The typical time-lapse images of vascular smooth muscle cells exposed to normal PSS were revealed in (A) and to KPSS (50 mmol/L \(\text{K}^+\)) were revealed in (B). The enlarged images of the framed area exhibited clear mitochondria fragmentation after treatment with KPSS (50 mmol/L \(\text{K}^+\)). (C) The time-lapse images of vascular smooth muscle cells pretreated with verapamil (5 \(\mu\text{mol/L}\)) for 30 min, and then exposed to KPSS (50 mmol/L \(\text{K}^+\)). The enlarged images of the framed area revealed that the integrity of mitochondria was not affected by KPSS (50 mmol/L \(\text{K}^+\)) in the presence of verapamil (5 \(\mu\text{mol/L}\)). (D) The time-lapse images of vascular smooth muscle cell pre-treated with phentolamine (5 \(\mu\text{mol/L}\)) for 30 min, and then exposed to KPSS (50 mmol/L \(\text{K}^+\)). The enlarged images of the framed area showed clear mitochondria fragmentation after treatment with KPSS (50 mmol/L \(\text{K}^+\)) in the presence of phentolamine (5 \(\mu\text{mol/L}\)).
3.5. Verapamil application after PE treatment has no effect on PE-induced excessive mitochondrial fission

Verapamil did not show vasorelaxant effects in aorta which was pre-contracted with PE (Fig. 1A). We then characterized mitochondrial morphology in smooth muscle cells from aorta in this state. As shown in Fig. 6, the mitochondrial fission of smooth muscle cells in aorta was increased after PE treatment. Verapamil did not inhibit this excessive mitochondrial fission in smooth muscle cells of PE-treated aorta. These results indicate that verapamil showed no inhibitory effect on mitochondrial fission when PE had induced intracellular Ca²⁺ release and excessive mitochondrial fission. However, verapamil pretreatment reduced the storage of intracellular Ca²⁺ in vascular smooth muscle cells, thereby inhibiting PE-induced vasoconstriction and PE-induced excessive mitochondrial fission.

3.6. Verapamil inhibits KPSS-induced excessive mitochondrial fission of cultured vascular smooth muscle cells (A10)

Mitochondrial fission dynamics were observed in the live cells by use of real-time confocal microscopy with mito-Tracker staining. As shown in Fig. 7, KPSS treatment induced mitochondrial fragmentation in cultured vascular smooth muscle cells (A10). Verapamil but not phentolamine inhibited KPSS-induced excessive mitochondrial fission. Nevertheless, phentolamine had no inhibitory effect on KPSS-induced excessive mitochondrial fission. Our previous study found that vascular smooth muscle cells lost sensitivity to PE after culturing17, which even occurred in primary arterial smooth muscle cells. Therefore, we did not use phentolamine to treat the cultured vascular smooth muscle cells to observe the effects of phentolamine on PE-induced excessive mitochondrial fission.

4. Conclusions

Based on our previous work, we used other two typical vasorelaxants, verapamil and phentolamine, to further prove the coupling between arterial constriction and mitochondrial fission in rat aorta. The present results demonstrate that arterial relaxation was completely congruent with the inhibition of mitochondrial fission in arterial smooth muscle cells. Together with our previous results17, these findings show that studies with six different types of drugs (mdivi-1, dynasore, Y27632, nitroglycerin, verapamil and phentolamine) confirm the tight coupling between arterial constriction and mitochondrial fission in vascular smooth muscle cells. We suggest the existence of a novel physiological process “mitochondrial fission–contraction coupling” in arterial smooth muscle cells. Based on this hypothesis, pharmacological targeting of mitochondrial fission could be a novel approach to dilate arteries and lower blood pressure.

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