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Involvement of mitochondrial fission in calcium sensing receptor-mediated vascular smooth muscle cells proliferation during hypertension

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ABSTRACT

Hyperproliferation of vascular smooth muscle cells (VSMC) is a major risk factor for cardiovascular diseases. Proper mitochondrial fission and fusion is involved with VSMC function. However, the role and mechanism of mitochondrial morphological changes in VSMC proliferation are not well understood. Here, we found that calcium sensing receptor (CaSR) was increased in the aortas from spontaneous hypertensive rats (SHRs) compared with age-matched Wistar Kyoto (WKY) rats. There was also an increase in mitochondrial fission and VSMC proliferation, which was attenuated by Calhex231. In primary rat VMSC, angiotensin II (Ang II) stimulation induced cytosolic $[Ca^{2+}]_i$ increase, mitochondrial shortening and proliferation, all of which could be attenuated by pretreatment with mitochondrial division inhibitor-1 (Mdivi-1) and Calhex231. Our data indicate that CaSR-mediated mitochondrial fission could be a therapeutic target for hyperproliferative disorders.

1. Introduction

Hypertension is one of the most common diseases worldwide. It can lead to a number of adverse health events, including myocardial infarction, heart failure, stroke, and chronic kidney disease. The cause of hypertension remains unclear, despite important advances in understanding the genetic and epigenetic basis of this syndrome [1]. This suggests the need for a new treatment paradigm. Excessive vascular smooth muscle cells (VSMC) proliferation plays an important role in hypertension and the damage of subsequent target organs [2–4], suggesting that antiproliferative therapies are needed.

Increased mitochondrial fission in VSMC is a major trigger for vasoconstriction and an important stimulus for VSMC proliferation leading to vascular remodeling [5,6]. Mitochondria not only generate ATP but also play a key role in regulating cell survival and death [7]. Mitochondrial morphology reportedly changed in response to cell proliferation, migration, and apoptosis [8–10]. The balance of mitochondrial fission and fusion determines mitochondrial morphology. In mammals, dynamin-related peptide 1 (Drp1) mediates mitochondrial fission, whereas mitofusin 2 (MFN2) mediates mitochondrial fusion. A previous study showed that mitochondrial fission contributed to the closure and constriction of ductus arteriosus [11]. An additional study reported that decreased MFN2 correlated to essential hypertension patients [12]. However, the role and mechanism of mitochondrial fission in hypertension requires further clarification.

Calcium-sensing receptor (CaSR), a G protein-coupled receptor, has been identified as a Ca^{2+} agonist and a regulator of blood pressure, as well as VSMC contraction [13,14]. However, how CaSR modulates vascular tone is not fully understood. We speculate that interfering with CaSR could affect the process of mitochondrial fission and fusion. In the present study, we found that inhibition of CaSR greatly decreased mitochondrial fission and VSMC proliferation in the aortas of spontaneous hypertensive rats (SHRs). VSMC induced by angiotensin II (Ang II) also diminished, indicating a requisite role of CaSR in mitochondrial fission.

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2. Materials and methods

2.1. Materials and reagents

Ang II (A9525), Calhex231 hydrochloride (SML0668),mitochondrial division inhibitor-1 (Mdivi-1, M0199), and BAPTA/AM (A1076) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calindol hydrochloride (CAS729610-18-8) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An antibody against CaSR (ACR-004) was purchased from Alomone labs, Ltd. (Hadassah Ein Kerem. Jerusalem). An antibody against proliferating cell nuclear antigen (PCNA, D3H8P) was purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against Ki67 (ab15580), Drp1 (ab56788), and MFN2 (ab56889) were purchased from Abcam Inc. (Cambridge, MA, USA). Antibodies against cyclin D1 (60186-1-Ig) and cyclin dependent kinase-1 (CDK1, 19532-1-AP) were purchased from Proteintech Group. Inc (Wuhan, Hubei, China). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, TA-08) and all secondary antibodies were obtained from ZSGB-Bio (Beijing, China). Dulbecco modified Eagle medium (DMEM) was obtained from Gibco (Gaithersburg, MD, USA). Fetal bovine serum (FBS) was obtained from Clark Bioscience. All other chemicals and reagents were of analytical grade.

2.2. Animals and blood pressure measurement

All animal care was performed in accordance with the protocols approved by Animal Management Rule of the People's Republic of China (Ministry of Health, P. R. China, document no. 55, 2001) and the Institutional Animal Care and Use Committee of Harbin Medical University. Adult male SHRs and age-matched Wistar Kyoto (WKY) rats were purchased from Vial River Laboratories (Beijing, China). They were maintained on a 12:12 h light-dark cycle with both rat chow and water *ad libitum*. For pharmacological inhibition of CaSR in SHRs, animals were 20 weeks of age and divided into 3 groups: WKY rats and SHRs were treated with saline injections (vehicle, intraperitoneally, 28 days, n = 10), and Calhex231-treated group (Calhex231, 10 μmol/kg per day, intraperitoneally, 28 days, n = 10). Tail arterial blood pressure was measured in non-anesthetized animals by tail cuff plethysmography using a BP2010A blood pressure system (Softron Biotechnology. Tokyo, Japan). Arterial blood pressure averages from at least 10 cycles were taken from each animal and then averaged within the group.

2.3. Cell culture

Primary rat VSMC were obtained from the thoracic aorta of male Wistar rats using a previously described explant method [6]. VSMC were seeded in DMEM+10% FBS+1% antibiotic solution (penicillin 100 U/mL, streptomycin 100 μg/mL) in a humidified 5% CO_2 at 37 °C. Media was changed every 2 days. Experiments used VSMC from passages 3–7 at 70%–90% confluence. Cell growth was arrested using serum-free DMEM to incubate the cells for 24 h prior to use. VSMC were divided into five groups and treated as follows: (1) control; (2) Ang II; VSMC were treated with 100 nmol/L Ang II for 24 h; (3) Calindol + Ang II; VSMC were preincubated with calindol hydrochloride 5 μmol/L (a specific agonist of CaSR) for 1 h and then treated with Ang II; (4) Calhex231 + Ang II; VSMC were preincubated with Calhex231 hydrochloride 5 μmol/L (a specific inhibitor of CaSR) for 30 min before adding Ang II; (5) Mdivi-1 + Ang II, VSMC were preincubated with Mdivi-1 10 μmol/L for 30 min before adding Ang II.

2.4. Western blot analysis

Proteins were extracted from all experimental samples and separated by electrophoresis on 10% or 12% poly acrylamide gel containing sodium-dodecyl sulfate gel followed by transferred onto PVDF membranes. Membranes were blocked in 5% nonfat dry milk for 1 h at room temperature, then incubated overnight at 4 °C in primary antibodies diluted in Tris-buffered saline with Tween and 5% bovine serum albumin. Antibodies and dilutions used for western blot analysis are as follows: CaSR (1:500), PCNA (1:1000), cyclin D1 (1:1000), CKD1 (1:1000), Drp1 (1:500), MFN2 (1:500), GAPDH (1:2000). Primary antibody incubation was followed by incubating with secondary antibody for 1 h at room temperature, then developing with the ECL detection system. Films were scanned and analyzed using Bio-Rad Image Lab software. All experiments were repeated more than three times.

2.5. Immunohistochemistry staining

To determine the expression of Ki67, immunohistochemistry staining was performed using formalin-fixed and paraffin-embedded sections (3 μm). The sections were incubated with Ki67 (1:100 dilution) antibody at 4 °C overnight. Cells with brown-strained particles in their nuclei were denoted as positive using microscope imaging (Zeiss, Germany). Quantitative assessments were performed with Image-pro Plus Software.

2.6. Transmission electron microscopy analysis

Transmission electron microscopy (TEM) was routinely performed to observe changes in mitochondrial morphology. Approximately 1 mm samples of aorta were excised from the vessel and placed in medium containing 2.5% glutaraldehyde at 4 °C for more than 24 h. Samples were post-fixed with osmium tetroxide, dehydrated, and embedded in epoxy resin for conventional use. Ultrathin sections (0.1 μm) were cut from the samples under an ultramicrotome, then mounted on copper grids, and contrasted with uranyl acetate and lead citrate. Discs were analyzed and documented using a JEM-1010 transmission electron microscope.

2.7. Cytosolic $[Ca^{2+}]_i$ measurement

Cultured VSMC were loaded with 10 μM fluo-3/AM for 15 min (37 °C) and washed with PBS for 4 times as previously described [9]. Fluorescence was measured using a confocal microscope (Olympus IX-70). The excitation and emission wavelength for signal detection was 488 nm and 530 nm respectively. The levels of cytosolic $[Ca^{2+}]_i$ were represented by increased fluorescence intensity in selected regions of the cytosol.

2.8. Mitochondrial networks measurement

Cultured VSMC were incubated with Mito-Tracker Green (50 nM) for 30 min at 37 °C and then viewed under a Zeiss microscope. The image was observed with a 40X objective. The mitochondrial lengths were used to elevate mitochondrial networks.

2.9. Statistical analysis

All experiments were repeated at least three times. Data are expressed as the mean ± standard deviation. Significance between multiple groups was performed by one-way analysis of variance, followed by the Tukey *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

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