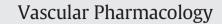
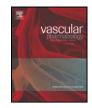
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Increase in caveolae and caveolin-1 expression modulates agonist-induced contraction and store- and receptor-operated Ca²⁺ entry in pulmonary arteries of pulmonary hypertensive rats



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ABSTRACT

Caveolin-1 (Cav-1) is a major component protein associated with caveolae in the plasma membrane and has been identified as a regulator of store-operated Ca^{2+} entry (SOCE) and receptor-operated Ca^{2+} entry (ROCE). However, the contributions of caveolae/Cav-1 of pulmonary arterial smooth muscle cells (PASMCs) to the altered Ca²⁺ signaling pathways in pulmonary arteries (PAs) during pulmonary hypertension (PH) have not been fully characterized. The present study quantified caveolae number and Cav-1 expression, and determined the effects of caveolae disruption on ET-1, cyclopiazonic acid (CPA) and 1-Oleoyl-2-acetyl-glycerol (OAG)-induced contraction in PAs and Ca²⁺ influx in PASMCs of chronic hypoxia (CH)- and monocrotaline (MCT)-induced PH rats. We found that the number of caveolae, and the Cav-1 mRNA and protein levels were increased significantly in PASMCs in both PH models. Disruption of caveolae by cholesterol depletion with methyl-\B-cyclodextrin (MBCD) significantly inhibited the contractile response to ET-1, CPA and OAG in PAs of control rats. ET-1, SOCE and ROCE-mediated contractile responses were enhanced, and their susceptibility to MBCD suppression was potentiated in the two PH models. MBCD-induced inhibition was reversed by cholesterol repletion. Introduction of Cav-1 scaffolding domain peptide to mimic Cav-1 upregulation caused significant increase in CPA- and OAG-induced Ca²⁺ entry in PASMCs of control, CH and MCT-treated groups. Our results suggest that the increase in caveolae and Cav-1 expression in PH contributes to the enhanced agonist-induced contraction of PA via modulation of SOCE and ROCE; and targeting caveolae/Cav-1 in PASMCs may provide a novel therapeutic strategy for the treatment of PH.

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1. Introduction

Pulmonary hypertension (PH) is a devastating pathological condition associated with a board spectrum of diseases of different etiologies [1–3]. Many forms of PH are deadly, and others always worsen the prognosis of the underlying diseases. It is characterized by profound pulmonary vascular remodeling, sustained vasoconstriction and enhanced vasoreactivity [4]. Intracellular Ca²⁺ signals play key roles in the regulation of a wide range of physiological and pathophysiological processes, including proliferation, migration and contraction of pulmonary arterial

¹ The authors have equal contribution to this publication.

smooth muscle cell (PASMC) [5]. Accumulating evidence suggests that increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of PASMCs is crucial for vascular remodeling and elevated pulmonary vascular tone in patients and animal models of PH.

Ca²⁺ influx in PASMCs is mainly regulated by voltage-dependent Ca²⁺ channels (VDCC) and non-selective cation channel mediated pathways, such as store-operated Ca²⁺ entry (SOCE), and receptor-operated Ca²⁺ entry (ROCE) [5–11]. Previous studies suggested that membrane depolarization activates VDCC, enhances Ca²⁺ entry, and leads to PASMC contraction, migration, and proliferation in idiopathic pulmonary arterial hypertension (IPAH) patients and PH rats [12-14]. However, antagonists of VDCC are only effective in a small fraction of IPAH patients [15–17]. In contrast, the elevated resting $[Ca^{2+}]_i$ in PASMCs and vascular tone of pulmonary arteries (PAs) of PH rats can be reduced to the control level by non-selective cation channel blockers, indicating Ca²⁺ entries via these channels are important for the altered Ca²⁺ homeostasis [6]. The canonical transient receptor potential (TRPC) channels are the major components of SOCE and ROCE in PASMCs [6,18,

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19]. SOCE and ROCE are pharmacologically and molecularly distinctive Ca²⁺ pathways gated by TRPC1 and TRPC6, respectively, in rat PASMCs [6]. Orai1 also contributes to SOCE in PASMCs [19,26,27]. Enhanced SOCE and ROCE are associated with the increase in mRNAs and proteins of TRPC1 and TRPC6 channels in PASMCs of various models of PH and patients with IPAH [6,20,21]. Deletion of *trpc1* and/or *trpc6* genes effectively attenuates hypoxia-induced PH [22–24], and a functional single nucleotide polymorphism of *TRPC6* has been identified in patients of IPAH [25]. These results suggested that TRPC1 mediated SOCE and/or TRPC6-mediated ROCE are important signaling pathways for the development of PH.

Caveolae are cholesterol and sphingolipid enriched plasma membrane lipid-raft domains found in various cell types, including smooth muscle and endothelial cells [28,29]. They act as signaling platforms, where membrane receptors, ion channels and signaling molecules sequester and interact, and play critical roles in modulating many pathways associated with cell proliferation, migration, and contraction. Caveolin-1 (Cav-1), the major protein constituent (22-kDa) of caveolae [30], is abundantly expressed in the PASMCs [31]. It has a caveolin scaffolding domain (CSD) which binds to signaling molecules including Gprotein coupled receptors, eNOS, integrins, growth factor receptors, protein kinases and ion channels [32-34]. In smooth muscle and endothelial cells, caveolae are important sites for the functional coupling of the membrane receptors and channels to the junctional sarcoplasmic reticulum (SR) [35], and are crucial for SOCE [36–38]. Previous studies showed that Cav-1 knockout causes dilated cardiomyopathy and PH [39] and affects TRPC1 and TRPC4 subcellular localization and agonistinduced Ca²⁺ entry in pulmonary endothelial cells [40]. The expression of Cav-1 and the number of caveolae are increased in PASMCs of IPAH patients; and down-regulation of Cav-1 with siRNA or disruption of caveolae with MBCD attenuates SOCE in IPAH-PASMCs [31]. These studies suggested that caveolae and Cav-1 are crucially involved in the pathogenic mechanisms of PH.

Despite the abovementioned information, the pathological roles of caveolae/Cav-1 on SOCE and ROCE of PASMCs in different forms of PH are unclear. There is no information on caveolae in relation to ROCE in PASMCs, despite some indications that TRPC6 can be regulated by lipid raft in fibroblast and T-lymphocytes [41,42]. Moreover, the contribution of caveolae to the enhanced ROCE has not been examined in any form of PH. In this study, we used the models of chronic hypoxia (CH) and monocrotaline (MCT)-induced PH to examine the contributions of caveolae/Cav-1 to the contractile responses mediated by SOCE and ROCE in PAS, and the interactions of caveolae, SOCE and ROCE in PASMCs in the development of PH.

2. Materials and methods

2.1. Animals and PH models

Adult male Sprague–Dawley rats (200–250 g) were purchased from the animal center of the Fujian Medical University (Fuzhou, China). All animal procedures were performed following the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Fujian Medical University Animal Use and Care Committee. Animals were randomly assigned to control, CH- and MCT-induced PH groups. CH- and MCT-induced PH model were produced by established method [6,43,44]. CH-induced PH was established by placing the rats in a hypoxic chamber and exposed to normobaric hypoxia $(10\% O_2)$ for 3 weeks. MCT-induced PH was generated by a single intraperitoneal injection of MCT (50 mg/kg). The rats were housed in laboratory cages and maintained in a 12 hour light-dark cycle, with food and water ad libitum. Twenty-one days after MCT injection or CH exposure, rats were anesthetized with urethane (1 g/kg). Right ventricle systolic pressure (RVSP) and mean systemic arterial pressure (MSAP) were measured by accessing the right ventricle through the jugular vein and carotid artery [6,43,44]. Right heart hypertrophy was determined by the Fulton index as the right ventricle to the left ventricle plus septum [RV/ $(\mathrm{LV}+\mathrm{S})]$ mass ratio.

2.2. Isolation of pulmonary artery and isometric contraction measurement

After hemodynamic measurements, the lungs were removed and rapidly transferred to a Petri dish filled with cold (4 °C) oxygenated modified Krebs solution containing (in mM) 118 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.18 KH₂PO₄, 25 NaHCO₃, 10 glucose, and 2 CaCl₂. Under a dissecting microscope, the third- and fourth-generation PAs were isolated and cleaned free of connective tissue, and then cut into 4-mm-length rings. The endothelium was removed by gently rubbing the lumen with a small wooden stick, and the arterial rings were suspended between two stainless steel stirrups in organ chambers filled with modified Krebs solution for isometric tension recording. The solution was gassed with 95% O₂/5% CO₂ to maintain a pH of 7.4 and a temperature at 37 °C. Isometric contraction was measured using a force transducer (JZJ01H; Chengyi, China) connected to an RM6240 system. Resting tension was adjusted to 0.8–1.0 g. Arteries were exposed three times to 60 mM KCl to establish maximum contraction, and then to phenylephrine $(3 \times 10^{-7} \text{ M})$ followed by acetylcholine (10^{-6} M) to verify complete disruption of endothelium. PAs exhibited >20% acetylcholineinduced relaxation were discarded.

2.3. Culture of pulmonary arterial smooth muscle cells

PASMCs were enzymatically isolated and transiently cultured as previously described [43,44]. Briefly, lungs were removed and transferred to a Petri dish filled with cold HEPES-buffered salt solution (HBSS) containing (in mM) 130 NaCl, 5 KCl, 1.2 MgCl₂, 1.5 CaCl₂, 10 HEPES, and 10 glucose, pH 7.4. The third- and fourth-generation PAs were isolated and cleaned free of connective tissue. The endothelium was removed by gently rubbing the luminal surface with a cotton swab. Arteries were then allowed to recover for 30 min in cold (4 °C) HBSS, followed by 20 min in reduced- Ca^{2+} (20 μ M) HBSS at room temperature. The tissue was digested at 37 °C for 20 min in 20 µM Ca²⁺ HBSS containing collagenase (Type I, 1750 U/ml), papain (9.5 U/ml), bovine serum albumin (2 mg/ml), and dithiothreitol (1 mM), then removed and washed with Ca²⁺-free HBSS to stop digestion. PASMCs were gently dispersed by trituration with a small-bore pipette in Ca²⁺-free HBSS at room temperature. The cell suspension was placed on 25 mm glass cover slips in Ham's F-12 medium (with L-glutamine) supplemented with 0.5% fetal calf serum, 100 U/ml of streptomycin, and 0.1 mg/ml of penicillin. PASMCs from chronic hypoxic and normoxic animals were transiently (~24 h) cultured inside a modular incubator chamber (Billups-Rothenberg, Inc.) under 4% O₂/5% CO₂ and 21% O₂/5% CO₂, respectively.

2.4. Transmission electron microscopy

For transmission electron microscopy (TEM) analysis of caveolae of pulmonary artery smooth muscle layer, isolated PAs were fixed with 3% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.2) for several days at 4 °C. Semi-thin and ultra-thin sections were prepared by standard procedures, and stained with uranyl acetate and lead citrate. Electron micrographs were taken using the PHILIPS EM208 TEM. A double-blind method was used to quantify the number of caveolae in the electron micrographs.

2.5. Immunofluorescent microscopy

PASMCs grown on glass coverslips were washed with PBS solution, and fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were permeabilized with 0.01% Triton X-100 in PBS solution for 10 min, and blocked with 2% BSA in PBS solution for 1 h. Following fixation and blocking, the cells were incubated overnight at 4 °C with a rabbit polyclonal Cav-1 antibody (1:400; Cell Signaling Technology)

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