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15-HETE mediates sub-acute hypoxia-induced TRPC1 expression and enhanced capacitative calcium entry in rat distal pulmonary arterial myocytes

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ABSTRACT

Sub-acute hypoxia causes pulmonary vasoconstriction (HPV) is associated with increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and contraction of pulmonary arterial smooth muscle cells (PASMCs). We previous have demonstrated that 15-hydroxyeicosatetraenoic acid (15-HETE), a metabolite of arachidonic acid by 15-lipoxygenase (15-LO), causes elevated $[Ca^{2+}]_i$ in PASMCs partly through Ca^{2+} entry via other than L-type Ca^{2+} channels. In this study, we used SKF96365/La^{3+} (SOCC antagonists) and Nordihydroguaiaretic acid (NDGA, a blockage of 15-LO) to examine the effect of 15-HETE on capacitative Ca^{2+} entry and activity/expression of store-operated Ca^{2+} channels (SOCCs) during sub-acute hypoxic procedure and the contribution of SOCCs on the maintenance of vascular tones. The results showed that the 15-HETE induced constriction of PA rings from normoxic and sub-acute hypoxic rats can be abolished by SKF96365 and La^{3+}. Capacitative Ca^{2+} entry (CCE) was also enhanced in PASMCs cultured with 15-HETE under sub-acute hypoxic condition (3% O_2, 48 h) and incubation with NDGA in PASMCs can greatly suppress this enhancement. Moreover, TRPC1, not TRPC4 and TRPC6, mRNA and protein expression were increased in PASMCs during these procedures. Meanwhile, the effect of 15-HETE on CCE and TRPC1 expression under sub-acute hypoxic cultivation were greatly suppressed in 15-LO knockdown PASMCs and PAs. These results suggest that 15-HETE mediated HPV through increased TRPC1 expression, leading to enhanced CCE, contributing to the maintenance of vascular tone.

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1. Introduction

Sub-acute exposure to hypoxia produces pulmonary vasoconstriction (HPV), which has long been recognized as a major factor leading to the pathogenesis of hypoxic pulmonary hypertension (HPH) [1–4]. Although the mechanisms underlying the sub-acute hypoxia-induced HPV are still elusive, the alteration in Ca^{2+} homeostasis is an important event in the development and maintenance of HPH [5–7]. Previous studies including ours have demonstrated that the hypoxia-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in pulmonary arterial smooth muscle cells indeed triggers the HPV [8–10].

It is well known that the hypoxia caused elevation of $[Ca^{2+}]_i$ is achieved mainly through two processes: an initial Ca^{2+} release from intracellular stores via inositol 1,4,5-triphosphate (IP_3) receptor and ryanodine receptor-operated Ca^{2+} channels [8,11–13]; or the Ca^{2+} influx through Ca^{2+}-permeable ion channels [14,15]. The channels for Ca^{2+} influx include voltage-dependent Ca^{2+} channels (VDCCs); receptor-operated Ca^{2+} channels (ROCCs) and store-operated Ca^{2+} channels (SOCCs) [16–18]. And among these channels, more and more studies provide an important role for SOCCs in the chronic hypoxia-induced increase in resting $[Ca^{2+}]_i$ which is responsible for HPV but eliminate a role for VDCCs during this procedure [19–21]. In contrast to VDCCs, the SOCCs are activated by depletion of intracellular stores and the subsequent Ca^{2+} influx by which to replenish the stores is known as capacitative Ca^{2+} entry (CCE) [22,23]. The CCE through SOCCs is present in rat PASMCs and enhanced during hypoxic exposure. Previous studies including ours also revealed a role for SOCCs in HPV [24–27].

In most studies, it has been shown that SOCCs involve the transient receptor potential (TRP) channels (TRPC), a group of cationic channels consisting of seven members [28]. Although TRPC channels are the primary candidates, the precise TRPCs isoform are still not identified and how their contribution to HPV and HPH remains unclear [29,30]. Most recently, TRPC1, TRPC4 and TRPC6 have been

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shown to be expressed in distal pulmonary arteries smooth muscle cells (PASMCs) [24,31–33], also the expression of TRPC1 and TRPC6 isoform are increased during hypoxic exposure, leading to an enhanced CCE and elevation of $[Ca^{2+}]_i$ [34]. However, the mechanism underlying the enhanced expression during hypoxia is still not understood.

15-hydroxyeicosatetraenoic acid (15-HETE), a 15-lipoxygenase (15-LO) metabolite, plays an important role in HPV as well as several pulmonary diseases. Our previous studies have shown that hypoxia activated 15-LO in the lung, resulting in increase in the production of 15-HETE and pulmonary arterial constriction in neonatal rabbit [35]. We demonstrated a critical role of 15-HETE in the Ca^{2+} signaling, ion channel functions, and enzymes activity (PKC, ERK), which may be related to the gene expression [25,36–40]. We also have found that 15-HETE can increase $[Ca^{2+}]_i$ via the Ca^{2+} release from intracellular Ca^{2+} stores and extracellular Ca^{2+} influx through SOCCs in rat PASMC under normal condition [25]. Thus, it is possible that 15-HETE is a mediator regulating Ca^{2+} homeostasis by regulating the CCE via SOCCs and plays a role in the sub-acute hypoxia-induced $[Ca^{2+}]_i$ rise in PASMCs leading to HPV. As TRPC subfamily are the likely SOCCs and the expression of TRPC is regulated by hypoxic exposure, we designed these studies to determine the effect of 15-HETE on TRPCs expression and CCE in rat PASMCs during sub-acute hypoxic procedure and characterize their involvement in the HPV.

2. Materials and methods

2.1. Reagents and instruments

15(RS)-hydroxy-eicosatetraenoic acid (15-HETE), Nordihydroguiairetic acid (NDGA) and nifedipine were purchased from Cayman Chemical Company (Ann Arbor MI, USA), SKF96365 was purchased from Enzo Life Sciences (Lausen, Switzerland), La^{3+} was purchased from Sigma–Aldrich (St. Louis, MO), Fluo 3-acetoxymethyl (fluo 3-AM) ester was purchased from Molecular Probes Eugere (OR, USA), cyclopiazonic acid (CPA) was purchased from Sigma–Aldrich Company (MO, USA). Anti-TRPC1 antibody and anti-TRPC6 antibody were purchased from Alomone Labs (Jerusalem, Israel). Anti-15-LO antibody was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Anti-β-actin antibody was purchased from Santa Cruz Biotechnology, Inc. (CA, USA). The other regular reagents were from common commercial sources. The instruments: dissecting microscope (SMZ-168, Motic); force displacement transducer (JZ100, Gaobeidian Xinhang Electrical Equipment Limited Company); signal amplifier (600 series eight-channel amplifier, Gould, Electronics); CO_2 Incubators (Thermo Scientific, Waltham, MA 02454, USA); inverted microscope (TE200, Nikon, Japan); confocal laser scanning microscope (Fluoview-FV300, Olympus, Japan); high performance liquid chromatograph (Angilent 1200, USA).

2.2. Animals and distal pulmonary arteries dissection

Adult male Wistar rats (200–250 g) were from the experimental Animal Center of Harbin Medical University. All animals' protocols were approved by the institutional Animal Care and Use Committee (IACUC). The distal pulmonary arteries (200- to 500-μM outer diameter) were dissected out from the chests and placed in cold oxygenated Krebs solution (in mM: NaCl 116, KCl 4.2, $CaCl_2$ 2.5, NaH_2PO_4 1.6, $MgSO_4$ 1.2, $NaHCO_3$ 22, and D-glucose 11, pH 7.4). Then the distal pulmonary arteries were de-endothelialized by rubbing the luminal surface with cotton after removal of connective tissue.

2.3. Tension studies of pulmonary arterial ring

Adult male Wistar rats (200–250 g) were placed in two separated chambers at the same time and exposed to either normoxic or hypoxic condition for 9 days. The normoxic chamber was continuously flushed with room air and the hypoxic chamber was flushed with a mixture of room air and N_2 to maintain oxygen level to less than 12% as previous described [35]. Tension studies were performed according to previous published manners [40]. Briefly, the distal intrapulmonary arteries were separated and cut into rings and mounted on tungsten wire before immerged in the vessels of Krebs or HBSS solution at 37 °C. HBSS (in mM: NaCl 130, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 2.5, HEPES 10 and D-glucose 10, pH 7.4) was only used in experiments with La^{3+} to avoid precipitation and chelation of La^{3+}. As previous described, these rings were initially loaded with 0.3 g tension before the studies started. Pulmonary arterial rings were treated with SKF96365/La^{3+} or 15-HETE at a final concentration of 50/30 μM and 1 μM in 2 mL normoxic/hypoxic Krebs/HBSS solution, respectively. After the removal of 15-HETE, all vessels relaxed to baseline tension. On the other hand, the vessels were incubated with SKF96365 or La^{3+} for 20 min then exposed to 15-HETE again, recorded the ring tension. Data were recorded and analyzed with CODAS software (DataQ Instruments, Inc.).

2.4. Cell isolation and culture

The method for obtaining single PASMCs has been described previously [39,40]. Briefly, the distal pulmonary arteries were de-endothelialized and gently digested with 0.1% collagenase (type II, Worthington, Shanghai, P.R. China) and 0.1% Bovine Serum Albumin in PBS solution for 1 h at 37 °C. The digested PASMCs were then cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% streptomycin and 1% penicillin for 3–5 days in a humidified incubator with 5% CO_2 at 37 °C. Before each experiment, the cells were incubated in 0.3% FBS–DMEM for 24 h to stop the growth.

2.5. Measurement of store-operated Ca^{2+} entry (CCE)

The PASMCs cultured under different conditions were gently digested with 0.25% Trypsin (Amresco Life Science Research, Solon, USA) and collected in normal Krebs solution, bubbled with 21% O_2–5% CO_2 and 92% N_2–5% CO_2, respectively for the CCE measurement. Cell viability was consistently greater than 98%.

$[Ca^{2+}]_i$ in PASMCs was monitored using membrane-permeable Ca^{2+}-sensitive fluorescent dye, fluo-3 acetoxymethyl ester (Fluo-3 AM) and recorded by confocal laser scanning microscope (CLSM). Briefly, the cells were incubated with 5–10 μM Fluo-3 AM (dissolved in DMSO with pluronic acid) for 30 min at 37 °C, in normoxic and hypoxic Krebs solution, respectively. After washing thoroughly, cells were then transferred to a recording chamber and perfused for 10 min with a Ca^{2+}-free normoxic/hypoxic Krebs solution containing 5 μM nifedipine to prevent calcium entry through VDCCs and 10 μM CPA to deplete Ca^{2+} stores. We then assessed the CCE by measuring the increase in $[Ca^{2+}]_i$ FI caused by restoration of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) to 2.5 mM in the continued presence of nifedipine and CPA. Fluo-3 was excited at 488 nm and $[Ca^{2+}]_i$ FI was measured at 30 s intervals before and after restoration of $[Ca^{2+}]_o$, while collecting emitted light at 530 nm by CLSM and recorded total of 30 min. Data analysis was performed off-line using Fluoview-FV300 (Olympus) to select cell regions and FI were extracted and analyzed with Excel (Microsoft) and OriginPro 7.5 software (OriginLab Corporation). Changes of $[Ca^{2+}]_i$ were shown according to fluorescence traces representing FI normalized to initial fluorescence intensity (FI_0) in separated experiments. Changes

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