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Original Article

Inhibition of 15-lipoxygenase (15-LOX) reverses hypoxia-induced down-regulation of potassium channels Kv1.5 and Kv2.1

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Abstract: The inhibition of voltage-gated potassium channels (Kv) plays an important role in the cerebral hypoxia-induced cell death. The activity of Kv can be inhibited by 15-hydroxyeicosatetrienoic acid (15-HETE). Therefore, as the key enzyme which catalyzed the formation of 15-HETE, 15-LOX may be involved in Kv inhibition induced by cerebral hypoxia. In our study, Wistar rats cerebral arterial smooth muscle cells (CASMCs) were placed under the condition of hypoxia and control, 15-LOX was proved involved in hypoxia-induced vasoconstriction. Furthermore, 15-LOX gene over expression under normoxic condition, as well as 15-LOX gene knockout or inhibition under hypoxic condition was performed to investigate the expression and activity of Kv1.5 and Kv2.1 in CASMCs. Results showed that both hypoxia and 15-LOX over expression could cause Kv1.5 and Kv2.1 suppression, but no suppression was observed under hypoxic condition when 15-LOX gene was knockout or inhibited, which made 15-LOX a new target for the treatment of cerebral hypoxia. In conclusion, AA/15-LOX/15-HETE induces vasoconstriction by down-regulating Kv channels, and Kv2.1/1.5 channels are the targets. Our study also suggests a therapeutic strategy to improve ischemic vascular occlusion by lowering 15-HETE level and preventing Kv channel down-regulation, which makes 15-LOX as a new target for the treatment of cerebral hypoxia.

Keywords: 15-lipoxygenase (15-LOX), 15-hydroxyeicosatetrienoic acid (15-HETE), hypoxia, Kv1.5, Kv2.1

Introduction

Cerebral vascular disease is one of diseases with high morbidity and mortality, 75%~80% of which is caused by ischemic cerebrovascular. Hypoxia-induced vascular constriction was an important pathogenesis which could lead to cell death in cerebral ischemia [1, 2]. However, the underlying mechanism is still unknown and the treatment could not achieve the desired effect. In recent years, hypoxia inhibits voltage-gated potassium (Kv) channels has been reported to be related to hypoxia-induced vascular constriction [3, 4]. The inhibition of Kv channels may be involved in hypoxic vasoconstriction through prolonging repolarization period for calcium entry. There are four subtypes of potassium channels in vascular smooth muscle cells, Kv, ATP-sensitive K^+, inward rectification and large conductance Ca^{2+}-activated K^+ [5, 6], among which Kv1.2, Kv1.5 and Kv2.1 are sensitive to hypoxia and Kv1.5 and Kv2.1 were reported to contribute to hypoxic cerebral vasoconstriction [7, 8].

15-Hydroxyeicosatetraenoic acid (15-HETE) can be oxidized by 15-lipoxygenase (15-LOX) and cause strong vasoconstriction in vascular bed of different models [9, 10], such as dog saphenous vein, rabbit aorta, canine basilar artery and femoral arteries [11]. In addition, several endothelial agents, e.g., endothelin, prostaglandin, leukotriene and cytochrome P450 metabolites [12, 13], could induce hypoxic vasoconstriction [14]. Furthermore, hypoxia induces the expression of vascular 15-LOX and increases the sensitivity of cerebral arteries to 15-HETE [15]. Now it's clear that 15-LOX is involved in cerebral ischemia reperfusion injury and other pathological processes in hypoxic brain injury.

In previous studies, we knew that 15-HETE could affect the function of internal carotid artery contraction by inhibiting voltage-gated potassium channels (Kv) in CASMCs [16]. As the key enzyme which catalyzes the production of 15-HETE, whether the level of 15-LOX would play a key role in the process of 15-HETE regulating Kv? In order to answer these questions, the role of 15-HETE in hypoxic isolated internal carotid artery (ICA) constriction by measuring its tension. RNA interference was performed to down regulate the expression of 15-LOX or nordihydroguaiaretic acid (NDGA) was used to inhibit the catalytic action of 15-LOX. The expression of Kv2.1 and Kv1.5 was examined by western-blot and RT-PCR, as well as the activity of Kv channels by whole-cell recording in cerebral arterial smooth muscle cells (CASMC) of rats. The results revealed that inhibition of 15-LOX reversed hypoxia-induced down-regulation of potassium channels Kv1.5 and Kv2.1. 15-LOX inhibitor was observed to involve in hypoxia and Kv channel level and function which was related to ischemic cerebrovascular vasoconstriction. It provides new ideas for the treatment of vascular disease.

Methods and materials

Culture of Wistar rats CASMCs and ICA rings

Wistar rats (225±25 g) were housed in The Animal Resource Center of Harbin Medical University. The procedures were approved by Institutional Animal Care and Use Committee. Wistar rats were decapitated and the heads were placed in 75% soak for 5 min. Cerebral arteries were isolated under a dissecting microscope. The isolated vascular smooth muscle cells were transferred and stirred in DMEM solution supplemented with 20% fetal bovine serum and 1% penicillin/streptomycin. The solution was centrifuged for 10 min to have cell pellets. The resuspended cells were distributed into a plate with 6 orifices and cultured in a humidified incubator (37°C, 5% CO_2) for 3~5 days. The purity of CASMCs in primary cultures was confirmed by specific monoclonal antibody for smooth muscle actin (Boehringer Mannheim). Before experiments, cell growth was stopped by adding in 0.3% FBS-DMEM for 12 h. CASMCs were divided into five groups. Group one (normoxic control) was maintained in an incubator with a 5% CO_2/95% O_2, 37°C. Group two (hypoxia group) were incubated in gas mixture composed of 3% O_2, 5% CO_2, and 92% N_2. Group three (Over-expressing 15-LOX in normoxic group) were Over-expressing 15-LOX and incubated in normoxic condition. Group four (Knockout 15-LOX in hypoxia group) were knockout 15-LOX and incubated in hypoxia condition. NDGA (50 μmol/L) was added into the fifth group in gas mixture composed of 3% O_2, 5% CO_2, and 92% N_2.

Sixteen healthy Wistar rats were randomly divided into two groups (n=8): group A with normal oxygen supply (FiO_2 21%) and group B with hypoxia (FiO_2 10%). After 9 days, the rats were killed and the ICA rings were prepared. ICA rings were cut into 1.0-1.5 mm in length, dispersed in cultural medium with 4 mg/ml papain for 18 min at 37°C [17], and transferred into the medium with collagens (1 mg/ml, Invitrogen USA) for 20 min at 37°C [18]. Each fragment was mounted onto a tungsten wire, and immersed into an oxygenized KH solution (mM: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 2.5; KH_2PO_4, 1.2; glucose, 6.0; pH 7.4) at 37°C. After equilibration for 40 min, the rings were loaded with a tension of 0.3 g. The relationship between vasoconstriction in ICA rings and dose-response for 15-HETE was assessed in normoxic and hypoxic groups (n=8). 15-HETE (Cayman Chemical Company in USA) was added into KH solution from10^{-8} M to 10^{-6} at 3 min intervals up to final concentrations.

Over-expression and knockout of 15-LOX gene

The human full-length 15-LOX cDNA was inserted into the plasmid mammalian expression vector pcDNA 3.1 (Invitrogen, Carlsbad, CA), *p*cDNA3.1/*Not*I*:* 15-LOX-*Xba*I vector was constructed and transfected the CASMCs. The CASMCs were seeded into 6-well plate with 1 × 10^5/well. After 24 h, cells were transfected with a mix containing 1 μg of plasmid *p*cDNA3.1/*Not*I*:* 15-LOX-*Xba*I and vector alone in Opti-MEM (Invitrogen, Carlsbad, CA) using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN). After 6 h, the transfection mix was replaced with complete media. Knockout 15-LOX: the gene 15-lipoxygenase (Alox15) was knocked out used RNAi kit, and the small mRNA which we are used were LV-15-LOX-RNAi (8884-1): CCAGTATATCTGACCTCCCTGTAGA, LV-15-LOX-RNAi (88851R) TGGATTGGTTCTACTGGGTTCCTAA, LV-15-LOXRNAi (8886-1) CAGACTCCTGCTTTGTTGTTTAGTT, After incubation, cells were lysed and evaluated for 15-LOX, 15-HETE,

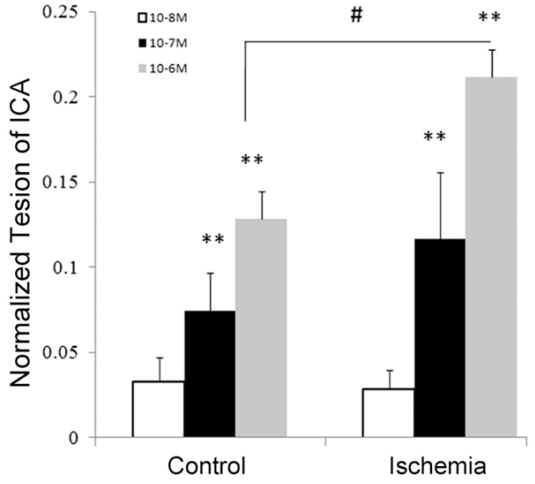

Figure 1. Hypoxic enhanced the vasoconstriction caused by 15-HETE in ICA rings. Wistar rats were randomly divided into two groups (n=8), group one with normal oxygen supply and the other with hypoxia. After 9 days, the ICA rings were prepared and 15-HETE was added into KH solution from 10^{-8} M to 10^{-6} M. ICA tension under hypoxia and control were measured. ICA tension showed a dose-dependent on 15-HETE under hypoxia and normoxia, meanwhile, hypoxia intensified vasoconstriction caused by 15-HETE. Values are means ± SEM (n=5). * and #, $P<0.05$, ** and ##, $P<0.01$ compared with corresponding untreated cells.

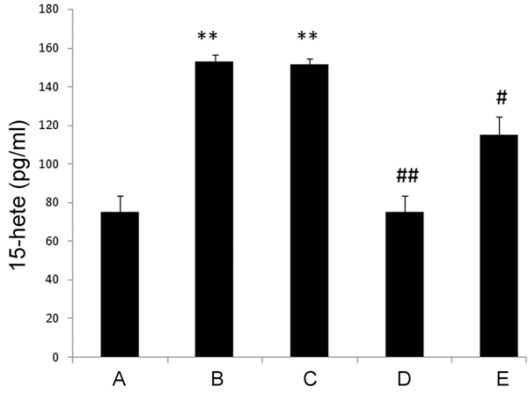

Figure 2. Inhibition of 15-LOX reversed hypoxia-induced increase of 15-HETE. CASMCs were divided into five groups and were placed under the condition of hypoxia or normoxia respectively. Level of 15-HETE was determined by western blotting. A: 15-HETE level of cell placed under normoxic condition as control; B: 15-HETE level of cells placed under hypoxia condition; C: Level of 15-HETE in cells over expressing 15-LOX under normoxic condition; D: Level of 15-HETE in 15-LOX gene knockout cells under hypoxic condition; E: 15-HETE level of cells placed under hypoxia condition with 50 μmol/L NDGA. Values are means ± SEM (n=5). * and #, $P<0.05$, ** and ##, $P<0.01$ compared with corresponding untreated cells.

Kv1.5 and Kv2.1. by western-blot analysis and RT-PCR.

RT-PCR

Sequences for rat Kv1.5 and Kv2.1 were obtained from GenBank TM database. Primers for rKv1.5a (M27158) are sense 5'-GGGC-AAGATCGTGGGTT-3' and antisense 5'-GGCTT-AAATACTCGGTGGTG-3' with 460 bp fragment. Primers for rKv2.1a (× 16476) are sense 5-CACCATCGCTCTGTCACTCA-3' and antisense 5'-GCAGGCCCAGTTCGTTGTA-3' with fragment size 395 bp. Primers for β-actin are sense 5'-CCGTAAAGACCTCTATGCCAACA-3' and antisense 5'-CGGACTCATCGTACTCCTGCT-3' with fragment size: 230 bp. Total RNAs from primary-cultured CASMCs were extracted by Trizol and reversely transcribed by cDNA synthesis kit (Fermentas). The fidelity and specificity of sense and antisense oligonucleotides were tested with BLAST. cDNA samples were amplified in DNA thermal cycler (PerkinElmer). PCR products were electrophoresed through a 1% agarose gel. cDNA bands were visualized by Gel Star gel staining (FMC BioProducts). Invariant mRNA of β-actin was used as an internal control to quantify PCR products. OD values for channel signals, measured by a Kodak electrophoresis documentation system, were normalized to OD values of β-actin signals. The ratios were expressed as arbitrary units for quantitative comparison.

Western blotting

Primary-cultured CASMCs were gently washed three times in cold PBS and placed in 200μl lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl-sulfate [SDS], then mixed with loading buffer (200 mM Tris-HCl pH 6.8, 50% glycerol, 2% SDS, 20% β-mercaptoethanol, 0.04% bromophenol blue), boiled for 5 min. The protein (100 μg) were separated on 12% SDS-PAGE, electrotransferred to a polyvinylidenedifluoride (PVDF) membrane (Bio-Rad, CA, USA), blocked with 5% nonfat milk in TBS-Tween buffer for 1.5 h at room temperature, and incubated overnight at 4°C with the primary antibody against β-actin (1:2000, ProteinTech, IL, USA), Kv1.5 (1:2000, ProteinTech, IL, USA). Kv2.1 (1:2000, ProteinTech, IL, USA). The membrane was washed with TBS-Tween buffer for 60 min (6 times for 10 min each), and then incubated for 1 h in horserad-

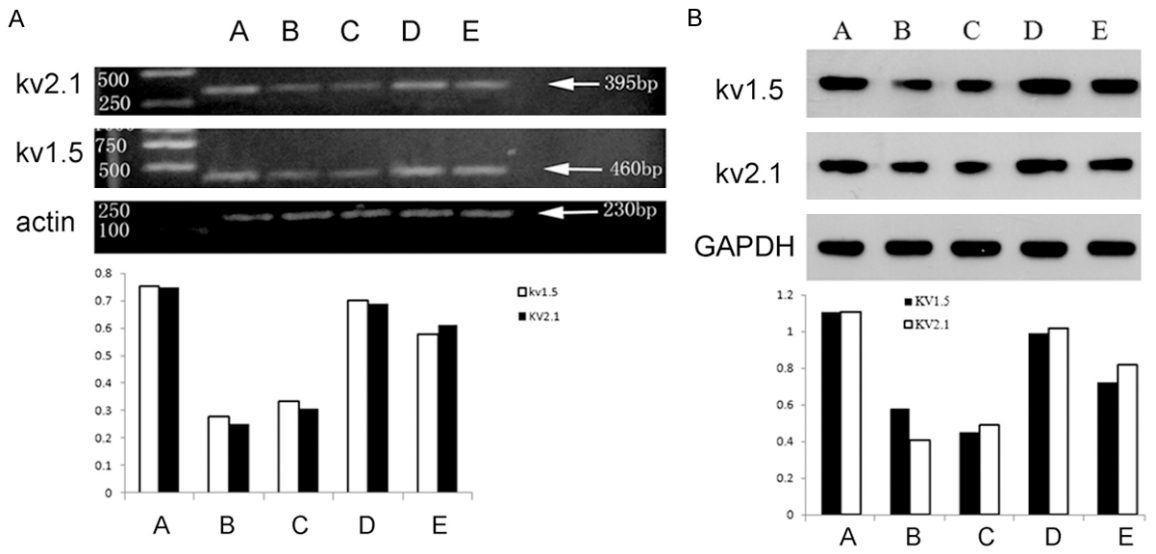

Figure 3. Inhibition of 15-LOX reversed hypoxia induced down-regulation of potassium channels Kv1.5 and Kv2.1. Different treatments. A: Cell placed under normoxic condition as control; B: Cells placed under hypoxia condition; C: Cells over expressing 15-LOX under normoxic condition; D: 15-LOX gene knockout cells under hypoxic condition; E: Cells placed under hypoxia condition with 50μmol/L NDGA. P-A: Upper panel: Agarose gels of RT-PCR products using primer sets of Kv1.5 and Kv2.1. Molecular weight markers are present on the left in each gel. Lower panel is the quantization by Kodak image analysis normalized to the β-actin band. P-B: Upper panel shows a representative western blot results of Kv1.5 and Kv2.1. The lower panel is the quantization by Kodak image analysis normalized to the GAPDH band. Shown are the means ± SEM (n=3).

ish peroxidase-conjugated goat antimouse IgG at room temperature. After extensive washing, the bands were visualized with enhanced chemiluminescence reagents (THERMO) and exposed to X-ray film. The densitometry of the bands was analyzed by Bio-Rad imaging system Quantity One®.

Electrophysiological experiments

In voltage-clamp of CASMCs, Kv channel currents were evoked by depolarization pulses, which were isolated by adding TTX and nimodipine (10 μM). An Axon-Patch 200B amplifier (Axon Instrument, Foster CA, USA) produced depolarization pulses to clamp membrane potentials to different levels and recorded outward Kv currents. Electrical signals were inputted into pClamp-9 (Axon Instrument) for data acquisition and analysis. Transient capacitance was compensated, and output band width was 3 kHz. Standard pipette solution for whole-cell recording contained (mM) 150 K-gluconate, 5 NaCl, 0.4 EGTA, 4 Mg-ATP, 0.5 Tris-GTP and 4 Na-phosphocreatine, 10 HEPES (pH 7.4 adjusted by 2 M KOH). The osmolarity of pipette solution was 295-305 mosmol, and the resistance of pipettes was 8-10 MΩ to have good access and prevent run-down in synaptic responses.

Statistical analysis

All values were presented as means ± standard error, and calculated by using two-tailed analyses of variance (ANOVA) followed by Dunnett's test to examine the significance among experimental groups. Tests were performed using a significance level of $P<0.05$ (*) or $P<0.01$ (**).

Results

Hypoxic enhanced the vasoconstriction caused by 15-HETE in ICA rings

ICA tension reflexes the vasoconstriction. In our study, two groups of rats were placed under the conditions of hypoxia and normoxia respectively, and the effect of 15-HETE on the sensitivity of ICA tension to hypoxia was tested. 15-HETE was added into KH solution from 10^{-8} M to 10^{-6} M at 3 min intervals up to final concentrations. As showed in **Figure 1**, the different concentration of 15-HETE vs. the tension of ICA rings under hypoxia and control, ICA tension is higher under hypoxia than control, and the relationship between vasoconstriction in ICA rings and dose-response for 15-HETE was assessed in normoxic and hypoxic groups (n=8). The results suggested that 15-HETE could strengthen ICA

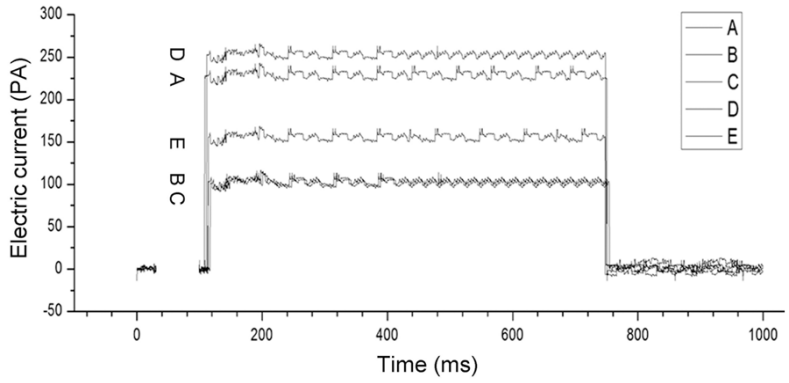

Figure 4. Inhibition of 15-LOX reversed hypoxia-induced blocks activity of voltage-gated potassium channels. Different treatments. A: cell placed under normoxic condition as control; B: cells placed under hypoxia condition; C: cells over expressing 15-LOX under normoxic condition; D: 15-LOX gene knockout cells under hypoxic condition; E: cells placed under hypoxia condition with 50 μmol/L NDGA.

vasoconstriction, while anoxia condition could enhance the effect.

Inhibition of 15-LOX reversed hypoxia-induced increase of 15-HETE in CASMCs

The expression of 15-LOX was increased under hypoxic condition [19], which catalyzed the generation of 15-HETE [20]. Here, CASMCs were divided into five groups and were placed under the condition of hypoxia or normoxia respectively (see methods and materials section 2.1). As showed in **Figure 2**, the level of 15-HETE was increased under hypoxic condition or over expressing 15-LOX which also proved that 15-LOX was increased under hypoxic condition. Besides, under hypoxic condition, the high level of 15-HETE was decreased when 15-LOX gene was knockout or 15-LOX inhibitor was added. Therefore, hypoxia may induce over expression of 15-LOX, which subsequently promote the synthesis of 15-HETE under hypoxic condition.

Inhibition of 15-LOX reversed hypoxia induced down-regulation of potassium channels Kv1.5 and Kv2.1

In terms of mechanisms underlying 15-HETE induced vasoconstriction under hypoxic condition, the Kv channels were involved, among which Kv1.5 and Kv2.1 were sensitive to hypoxia [8, 16]. If 15-HETE reduces the quantity or function of Kv, a delayed repolarization leads to dominant Ca^{2+} influx and enhances smooth muscle tension in hypoxic vasoconstriction. Western-blot and RT-PCR were used to check level of Kv1.5 and Kv2.1 in mRNA and protein expression level. Five days after cultured under different conditions as described previous, CASMCs were collected in consistent quantities from five groups. Kv protein and mRNA were readout by an enhanced chemiluminescence detection system and a computerized scanner. As showed in **Figure 3**, both 15-LOX and hypoxia could inhibit mRNA level (**Figure 3A**) and protein expression level (**Figure 3B**) of Kv1.5 and Kv2.1. Both 15-LOX gene knockout and 15-LOX inhibition by adding 50 μmol/L NDGA can increase hypoxia induced down-regulation of potassium channels Kv1.5 and Kv2.1 in mRNA expression level and protein expression level.

Inhibition of 15-LOX reversed hypoxia-induced blocks activity of voltage-gated potassium channels

In addition to expression of Kv2.1 and Kv1.5, we also examined whether the activity of Kv channels was influenced by 15-LOX. Whole-cell Kv-currents on CASMCs were evoked by depolarization pulses and recorded by voltage-clamp, which was isolated by tetrodotoxin (TTX) to block sodium channels and nimodipine to block calcium channels. Kv currents under different conditions described previously were recorded and shown in **Figure 4**. We can observe that hypoxia and 15-LOX over expression blocked Kv currents significantly, but 15-LOX gene knockout and inhibition can reverse this phenomenon, besides, gene knockdown has more obvious effect. This result indicated that hypoxiainduced blocks the activity of voltage-gated potassium channels can be reversed by 15-LOX down-regulation.

Discussion

The previous studies showed that Kv channels in type I of carotid body cells were inhibited during hypoxia [21], which suggested the inhibition of Kv plays an important role in the cerebral

hypoxia-induced vasoconstriction. However, which subtypes of Kv channel in CASMCs are down regulated during hypoxia, or which molecule is responsible for this process are remained unclear. Kv1.2, Kv1.5 and Kv2.1 are presumably sensitive to hypoxia. Kv1.5 and Kv2.1 transcripts and proteins are present in cerebral arterial smooth muscle. In addition, a substantial component of the voltage dependence and kinetics of Kv currents in cerebral arterial myocytes is consistent with Kv1 and Kv 2 channels [8, 16]. Thus, we examined the expression of Kv 1.5 and Kv2.1.

The activity of Kv can be inhibited by 15-HETE, under the catalysis of 15-LOX. The previous studies showed that 15-LOX in ICA endothelia and smooth muscles higher in hypoxia than normoxia [16]. 15-HETE enhances the sensitivity of ICA rings to hypoxia (**Figure 1**), conversely, down-regulating 15-LOX by inhibitor NDGA can decease the sensitivity of ICA rings to hypoxia [22-24]. When ATP production reduces during hypoxia, cellular membrane may release numerous metabolites, including 15-HETE [12, 13]. 15-HETE formation is attenuated by lipoxygenase inhibitors [22]. Hypoxia elevates 15-LOX expression and over-expression 15-LOX increased 15-HETE (**Figure 2**).

In our research, cerebral arterial smooth muscle cells (CASMCs) were divided into five groups and placed under the condition of hypoxia or normoxia respectively. Kv2.1 and Kv1.5 channels' expression (**Figure 3**) and activity (**Figure 4**) were inhibited in hypoxia and 15-LOX over-expression cells compared to cells placed under normoxic condition. However, no suppression was observed under hypoxic condition when 15-LOX gene was knockout or inhibited by NDGA. Taking all the results, we suggested a chain reaction during hypoxia, in which the over expressed 15-LOX converts arachidonic acid into 15-HETE, and 15-HETE via down-regulating Kv channels enhances cerebral vasoconstriction to intensify vascular occlusion during brain ischemia. The arachidonic acid/15-LOX/15-HETE/Kv channels pathway could be involved in hypoxia-induced vasoconstriction [16, 22]. In studying the mechanisms underlying hypoxia induced vasoconstriction. We found that the level of 15-LOX, which converts arachidonic acid into 15-HETE, in ICA endothelia and smooth muscles higher in hypoxia than control (**Figure 2**). 15-HETE enhances the sensitivity of ICA rings to hypoxia (**Figure 1**), as well as down-regulates Kv2.1 and Kv1.5 channels' expressions (**Figure 3**) and activities (**Figure 4**). Thus, hypoxia-induced 15-HETE enhances ICA vasoconstriction via down-regulating Kv channels in smooth muscles. This is a novel mechanism to explain why hypoxia strengthens cerebral vasoconstriction, which worsens ischemia.

In summary, AA/15-LOX/15-HETE induces vasoconstriction by down-regulating Kv channels, and Kv2.1/1.5 channels are the targets. This study is the first effort to elucidate the ion mechanisms of AA/15-LOX/15-HETE in hypoxic cerebral vasoconstriction. Our study also suggests a therapeutic strategy to improve ischemic vascular occlusion by lowering 15-HETE level and preventing Kv channel down-regulation, which made 15-LOX as a new target for the treatment of cerebral hypoxia.

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Disclosure of conflict of interest

None.

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