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HYPOXIA-INDUCED 15-HETE ENHANCES THE CONSTRICTION OF INTERNAL CAROTID ARTERIES BY DOWN-REGULATING POTASSIUM CHANNELS

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Summary – Severe hypoxia induces the constriction of internal carotid arteries (ICA), which worsens ischemic stroke in the brain. A few metabolites are presumably involved in hypoxic vasoconstriction, however, less is known about how such molecules provoke this vasoconstriction. We have investigated the influence of 15-hydroxyeicosatetrienoic acid (15-HETE) produced by 15-lipoxygenase (15-LOX) on vasoconstriction during hypoxia. As showed in our results, 15-LOX level increases in ICA endothelia and smooth muscles. 15-HETE enhances the tension of ICA ring in a dose-dependent manner, as well as attenuates the activity and expression of voltage-gated potassium (Kv) channels. Therefore, the down-regulation of Kv channels by 15-HETE during hypoxia weakens the repolarization of action potentials and causes a dominant influx of calcium to enhance smooth muscle tension and ICA constriction.

The pathogenesis of cerebral ischemia includes thrombosis and hypoxia-induced vascular constriction, which lead to cell death [3]. The efforts to treat hypoxia-induced cerebral vasoconstriction in ischemia has been failed to achieve the primary goal. The reexamination of its underlying mechanisms is needed [10]. In addition to calcium influx, as hypoxia inhibits voltage-gated potassium (Kv) channels, the inhibition of Kv channels may be involved in hypoxic vasoconstriction through prolonging repolarization period for calcium entry [5, 8].

There are four subtypes of potassium channels in vascular smooth muscle cells, Kv, ATP-sensitive K⁺, inward rectification and large-conductance Ca²⁺-activated K⁺ [4]. Kv channels are encoded by genes of Kv1.0–Kv9.0, KvLQT and EAG, and some of them (Kv1.2, Kv1.5 and Kv2.1 [7]) are sensitive to hypoxia [11]. Which subtypes of Kv channels contribute to hypoxic cerebral vasoconstriction? In addition, several endothelial agents, e.g., endothelin, prostaglandin, leukotriene and cytochrome P450 metabolites, as well as 15-hydroxyeicosatetrienoic acid (15-HETE) induce hypoxic vasoconstriction [2, 12]. Hypoxia induces the expression of vascular 15-lipoxygenase (15-LOX) and increases the sensitivity of cerebral arteries to 15-HETE [14, 15]. Does 15-HETE serve as an essential mediator between hypoxia and Kv-channel inhibition for cerebral vasoconstriction?

To these questions, we examined the expression of 15-LOX by immunohistochemistry in vivo, the role of 15-HETE in hypoxic internal carotid arteries constriction by measuring its tension, the expressions of Kv2.1/Kv1.5 by western-blot and RT-PCR, as well as the activity of Kv channels by whole-cell recording in cerebral arterial smooth muscle cells (CASM) of rats. Our study reveals that 15-HETE induces hypoxic cerebral vasoconstriction via down-regulating Kv channels.

Methods

Wistar rats (225±25 g) were housed in the Animal Resource Center of Harbin Medical University. The procedures are approved by Institutional Animal Care and Use Committee. Rats were randomly divided into two groups. Group one was placed in normoxic environment as control, and another was maintained in a cage where fractional inspired oxygen (FiO₂) was reduced to 0.12 as hypoxic condition [14]. Living temperature was 22±2 °C, and relative humidity was 50±10 %. After nine days, rats were anesthetized by the intraperitoneal injection of 4 % sodium pentobarbital (40 mg/kg), and internal carotid arteries were surgically taken for subsequent experiments.

Internal carotid arteries rings were cut into 1.0–1.5 mm in length. Each fragment was mounted onto a tungsten wire, and immersed into an oxygenized KH solution (mM: NaCl, 118; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; NaHCO₃, 2.5; KH₂PO₄, 1.2; glucose, 6.0; pH 7.4) at 37 °C. After this equilibration for 40 min, the rings were loaded with a tension of 0.3 g. The relationships between vasoconstriction in internal carotid arteries rings and dose-response for 15-HETE were assessed in normoxic and hypoxic groups (n=8). 15-HETE (Cayman Chemical Company in USA) was added into KH solution from 10⁻⁸ M to 10⁻⁶ at 3 min intervals up to final concentrations.

The immunohistochemistry of 15-LOX: Rats were anesthetized (see above). 4 % paraformaldehyde in 0.1 M phosphate buffer solution (PBS) was perfused into left ventricle/aorta until their bodies were rigidity. The brains were quickly isolated and fixed in 4 % paraformaldehyde PBS for additional 24 hrs. Cortical tissues were sliced in cross section at 20 μm by a freezing microtome. Sections were washed by PBS for three times and stained by 15-LOX immunohistochemistry [14]. The distribution of 15-LOX (dark-brown in color) was observed under conventional optical microscope.

The culture of rat CASMs: internal carotid arteries rings were cut into small pieces, dispersed in cultural medium with 4 mg/ml papain [6] for 18 min at 37 °C, and transferred into the medium with collagenase (1 mg/ml, Invitrogen USA) [1] for 20 min at 37 °C. 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 % CO₂) for 3–5 days. The purity of CASMs 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 12h. Quiescent (growth-arrested) CASMCs were divided into three groups. Group one (normoxic control) was maintained in an incubator with a 5 % CO₂/95 % O₂. Hypoxia group were incubated in gas mixture composed of 3 % O₂, 5 % CO₂, and 92 % N₂. 1 mmol/L 15-HETE was added into the third group in an incubator containing 5 % CO₂ and 95 % O₂.

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 bandwidth 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 2M KOH). The osmolarity of pipette solution was 295–305 mOsmol, and the resistance of pipettes was 8–10 MW to have good access and prevent run-down in synaptic responses.

Western blotting: Primary-cultured CASMCs were gently washed thrice in cold PBS and placed in 200 μl lysis buffer (1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl-sulfate, 100 μg/ml phenylmethylsulfonyl fluoride and 30 μl/ml aprotinin) for 30 min on ice. The lysates were sonicated, and centrifuged at 12,000 rpm for 10 min at 4°C. Proteins in supernatant were quantified based on a method of Bradford, separated by sodium dodecyl-sulfate-PAGE and transferred to nitrocellulose membranes. The membranes were incubated in a blocking buffer (TBST solution with nonfat dry milk powder) for 1 hour at room temperature, and placed in affinity-purified rabbit polyclonal antibodies (1:500) specific to Kv1.5 and Kv2.1 (Santa Cruz USA) overnight at 4°C. Monoclonal antibody specific to smooth muscle β-actin was used for control. The membranes were washed and incubated with anti-rabbit horseradish peroxidase-conjugated IgG (1:5000) for 1 hour at room temperature. Bound antibodies were detected with an enhanced chemiluminescence-detection system (Amersham). The bands corresponding to the expected size were selected on a computerized scanner, and the pixel density within each band was determined by this computer after background correction for relative quantization.

RT-PCR: Sequences for rat Kv1.5 and Kv2.1 were obtained from GenBank™ database. Primers for rKv1.5a (M27158) are sense 5'-GGGCAAGATCGTGGGTT-3' and antisense 5'-GGCTTAAATACTCGGTGGTG-3' with 460 bp fragment. Primers for rKv2.1a (X16476) are sense 5'-CACCATCGCTCTGTCACTCA-3' and antisense 5'-GCAGGCCAGTTCTGTGTA-3' with fragment size 395 bp. Primers for β-actin (BC063166) 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 GelStar 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.

Statistical analysis: All values were presented as means and standard error, and calculated by using two-tailed analyses of variance (ANOVA) followed by Dunnett's test to examine the significance among experimental groups.

Results

15-HETE enhances the sensitivity of ICA to hypoxia-induced vasoconstriction

The expression of 15-LOX in internal carotid arteries was examined during hypoxia. Two groups of rats were placed under the conditions of hypoxia and control, respectively (see Methods). Nine days after the treatments, rats' brain tissues were isolated for immunohistochemistry. As showed in top panels of Fig. 1, the levels of 15-LOX in the layers of endothelia and smooth muscles are higher in hypoxia (1b) than control (1a), indicating that hypoxia elevates 15-LOX expression.

As 15-LOX produces 15-HETE, we tested the effect of 15-HETE on the sensitivity of internal carotid arteries tension to hypoxia. Figure 1c shows the different concentrations of 15-HETE vs. the tension of internal carotid arteries rings under the conditions of control (open symbols) and hypoxia (filled). Internal carotid arteries tension is higher under hypoxia than control in a dose-dependent manner, indicating that 15-HETE enhances the sensitivity of internal carotid arteries to hypoxia. Therefore, hypoxia may induce 15-LOX over-expression, and subsequently synthesized 15-HETE strengthens internal carotid arteries constriction.

In terms of mechanisms underlying hypoxic 15-HETE vasoconstriction, we studied the involvement of Kv channels, since Kv1.2, Kv1.5 and Kv2.1 were sensitive to hypoxia [4, 7]. If 15-HETE reduces Kv quantity and/or function, a delayed repolarization leads to dominant Ca²⁺ influx and enhances smooth muscle tension, i.e., hypoxic vasoconstriction.

15-HETE reduces the expression and function of Kv channels

We examined whether 15-HETE reduced the expressions of Kv channels in smooth muscles by using western-blot and RT-PCR, respectively, in which the levels of Kv proteins and mRNAs were read out by an enhanced chemiluminescence-detection system and a computerized scanner. Cerebral arterial smooth muscle cells (CASMC) were harvested and cultured under the conditions of hypoxia, 15-HETE and control (see Methods). Five days after the culture, CASMCs were collected in consistent quantities from three groups for the experiments.

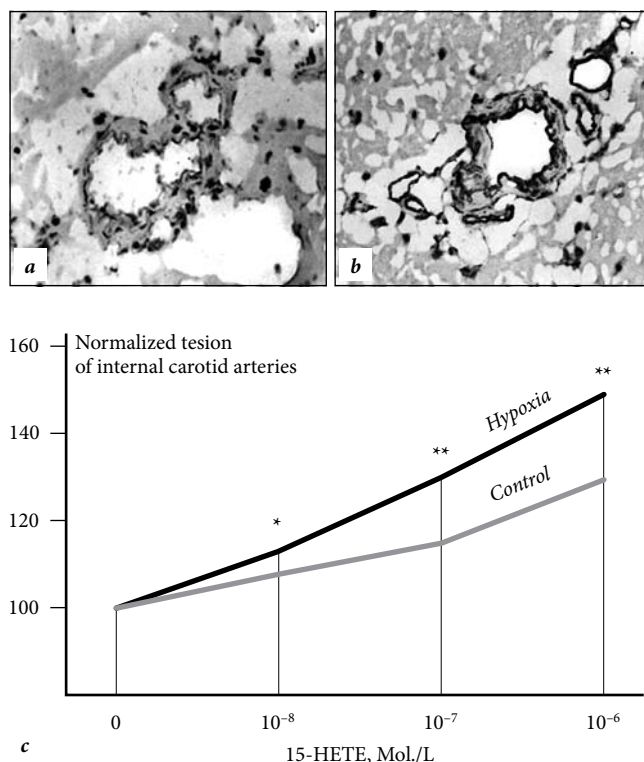


Figure 1. Hypoxia elevates the level of 15-LOX in endothelia and smooth muscles of internal carotid arteries, and enhances the sensitivity of internal carotid arteries tension to 15-HETE.

a, b – show the levels 15-LOX in the layers of internal carotid arteries endothelia and smooth muscles under the conditions of control (a) and hypoxia (b) by immunohistochemistry staining; c – 15-HETE induces tension of internal carotid arteries rings under control (open symbols) and hypoxia (filled) in dose-dependent manner ($n=8$; *, $p<0.05$; **, $p<0.01$).

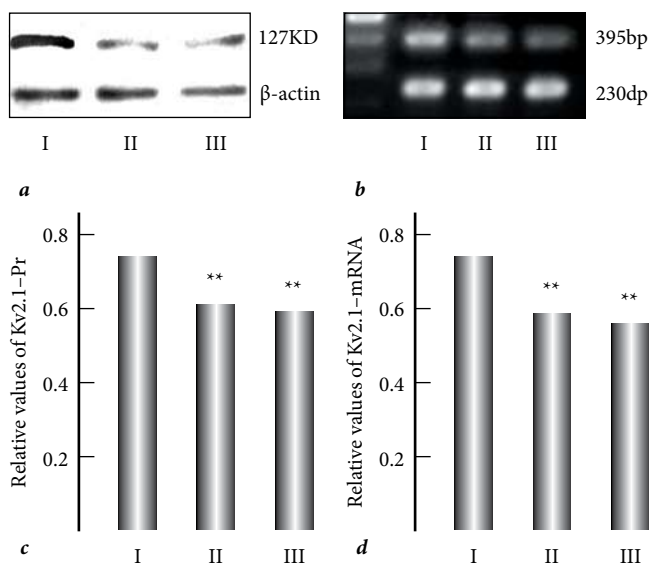


Figure 2. Hypoxia and 15-HETE attenuate the levels of Kv2.1 in epithelia and smooth muscles of internal carotid arteries.

a – shows the levels of Kv2.1 protein (Pr) detected by western-blot under the conditions of control (I), hypoxia (II) and 15-HETE (III); b – shows the statistic data of Kv2.1 proteins; c – shows the levels of Kv2.1 mRNA detected by RT-PCR under the conditions of control (I), hypoxia (II) and 15-HETE (III); d – shows the statistic data of Kv2.1 mRNAs. ** indicates $p<0.01$.

Figure 2 illustrates the effects of 15-HETE and hypoxia on Kv2.1 expression. In western-blot data of Kv2.1 proteins, the bands for Kv2.1 are lighter under hypoxia (II) and 15-HETE (III) than control (I). Relative values of Kv2.1 under

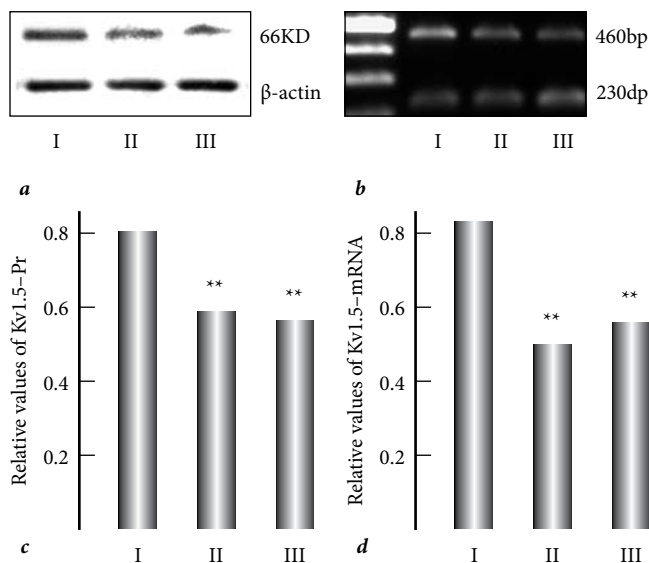


Figure 3. Hypoxia and 15-HETE down-regulate the levels of Kv1.5 in epithelia and smooth muscles of internal carotid arteries.

a – shows the levels of Kv1.5 protein (Pr) detected by western-blot under the conditions of control, hypoxia and 15-HETE; b – shows the statistic data of Kv1.5 proteins; c – shows the levels of Kv1.5 mRNA detected by RT-PCR under the conditions of control (I), hypoxia (II) and 15-HETE (III); d – shows the statistic data of Kv1.5 mRNAs. ** indicates $p<0.01$.

the conditions of control (I), hypoxia (II) and 15-HETE (III) are given in Figure 2b, in which the levels of Kv2.1 proteins are lower under hypoxia and 15-HETE significantly than control ($p<0.01$, $n=9$). In RT-PCR data of Kv2.1 mRNAs, the bands for Kv2.1 are lighter under hypoxia (II in Fig. 2c) and 15-HETE (III) than control (I). The relative values of Kv2.1 under the conditions of control (I), hypoxia (II) and 15-HETE (III) are showed in Figure 2d. The levels of Kv2.1 mRNA are lower significantly under hypoxia and 15-HETE than control ($p<0.01$, $n=10$). Therefore, 15-HETE during hypoxia attenuates Kv2.1 expression.

Figure 3 shows the effects of 15-HETE and hypoxia on Kv1.5 expression. In western-blot data of Kv1.5 proteins, bands for Kv1.5 are lighter under hypoxia (middle column) and 15-HETE (right) than control (left in Fig. 3a). Relative values of Kv1.5 proteins are lower significantly under hypoxia (middle bar) and 15-HETE (right) than control (left in Fig. 3b; $p<0.01$, $n=9$). In RT-PCR data of Kv1.5 mRNAs, the bands for Kv1.5 are lighter under hypoxia (middle column in Fig. 3c) and 15-HETE (right) than control (left). Relative values of Kv1.5 mRNA are lower significantly under hypoxia (middle bar) and 15-HETE (right) than control (left in Fig. 3D; $p<0.01$, $n=10$). Thus, 15-HETE during hypoxia attenuates Kv1.5 expression.

In addition to Kv2.1 and Kv1.5 expressions, we examined whether 15-HETE influenced the activity of Kv channels. Whole-cell Kv-currents on CSMCs were evoked by depolarization pulses and recorded by voltage-clamp, which were isolated by using TTX to block sodium channels and nimodipine to block calcium channels. Kv currents were recorded under the control (Fig. 4a) and 1 μ M 15-HETE in culture medium (Fig. 4b), in which 15-HETE completely blocked Kv currents. Figure 4c shows I-V curves for Kv channels under control (open symbols)

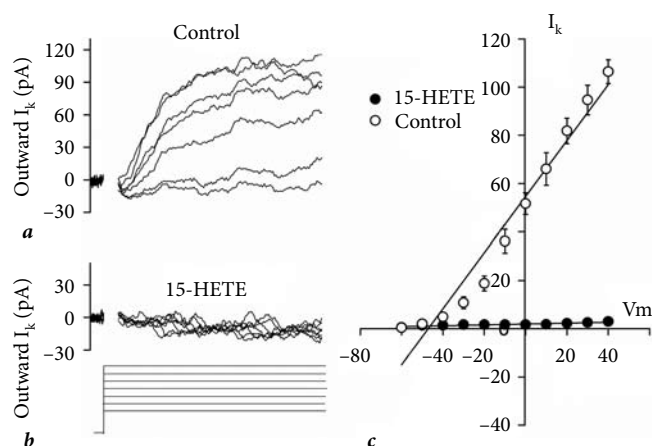


Figure 4. 15-HETE blocks whole-cell currents of voltage-gated potassium channels (VGPC) in smooth muscle cells of internal carotid arteries.

a, b – show the blockade of VGPCs by 15-HETE (control in “a” and 15-HETE application in “b”); c – shows the I–V curves of VGPCs under the conditions of control and 15-HETE application.

vs. 15-HETE addition (filled). This result indicates that 15-HETE produced during hypoxia blocks the activity of voltage-gated potassium channels.

Discussion

In studying the mechanisms underlying hypoxia-induced vasoconstriction, we found that the level of 15-LOX, which converts arachidonic acid into 15-HETE, in internal carotid arteries endothelia and smooth muscle is higher in hypoxia than control (Fig. 1a–b). 15-HETE enhances the sensitivity of internal carotid arteries rings to hypoxia (Fig. 1c), as well as down-regulates Kv2.1 and Kv1.5 channels' expressions (Fig. 2, 3) and activities (Fig. 4). Thus, hypoxia-induced 15-HETE enhances internal carotid arteries constriction via down-regulating Kv channels in smooth muscles. This is a novel mechanism to explain why hypoxia strengthens cerebral vasoconstriction, which worsens ischemia. Our study also suggests a therapeutic strategy to improve ischemic vascular occlusion by lowering 15-HETE level and preventing Kv channel down-regulation.

When ATP production reduces during hypoxia, cellular membrane may release numerous metabolites, including 15-HETE [2]. 15-HETE formation is attenuated by lipoxygenase inhibitors [13]. Hypoxia elevates 15-LOX expression (Fig. 1). Together these data, we suggest a chain reaction during hypoxia, in which the over-expressed 15-LOX converts arachidonic acid into 15-HETE, and 15-HETE enhances internal carotid arteries constriction (Fig. 1c) to intensify vascular occlusion during brain ischemia. Our study brings a new insight into pathology of cerebral ischemia.

Kv channels in type I of carotid body cells were inhibited during hypoxia [9]. Some Kv channels are sensitive to hypoxia. It is not clear about which subtypes of Kv channels on CSMCs are down-regulated during hypoxia, as well as which molecule is responsible for hypoxic inhibition of Kv channels that leads to the vasoconstriction. Kv1.2, Kv1.5, Kv1.2/Kv1.5 and Kv2.1 are the most spectacular subtypes of KV channel which are sensitive to hypoxia [7]. Kv 1.5 and

Kv2.1 transcript and protein are present in cerebral arterial smooth muscle. In addition, a substantial component of the voltage dependencies and kinetics of Kv currents in voltage-clamped cerebral arterial myocytes is consistent with Kv1 and Kv2 channels [7]. Thus in this test we examined the expression of Kv 1.5 and Kv 2.1. Our studies show that hypoxia and 15-HETE attenuate the expressions of Kv2.1 (Fig. 2) and Kv1.5 (Fig. 3), and that 15-HETE blocks the activity of Kv channels (Fig. 4). These findings suggest that Kv2.1 and Kv1.5 channels play a role in 15-HETE-induced internal carotid arteries vasoconstriction during hypoxia. There are indeed other functional components of Kv besides Kv 1.5 and Kv2.1, which need to be further studied in the future. We can use the proper blocker for each subtype of Kv, for instance, Dendrotoxin K for Kv1.1 and RTH-400 for Kv1.6, to examine whether other subtypes of potassium channels are inhibited by 15-HETE and involved in hypoxic constriction of internal carotid arteries.

Based on our study, 15-HETE produced during hypoxia down-regulates Kv1.5 and Kv2.1 expressions and functions (Fig. 2–4). An inhibition of Kv channels prolongs spikes' repolarization and lowers resting membrane potentials. The former allows a dominant Ca^{2+} influx on CSMCs during action potentials, and the latter reduces an energetic barrier to fire action potentials. Both scenarios may elevate cytoplasm Ca^{2+} and in turn induce cerebral vasoconstriction during hypoxia and ischemia. It remains to be studied how Ca^{2+} channels and Ca^{2+} influx are involved in hypoxic vasoconstriction, which we are doing now.

In summary, 15-HETE induces internal carotid arteries constriction by down-regulating Kv channels. 15-HETE is one of initiators for hypoxic internal carotid arteries constriction, and Kv2.1/1.5 channels are the targets of 15-HETE. This study is the first effort to elucidate the ion mechanisms of 15-HETE in hypoxic cerebral vasoconstriction.

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РОЛЬ 15-ГИДРОКСИЭЙКОЗАТЕТРАЕНОВОЙ КИСЛОТЫ В ИНАКТИВАЦИИ КАЛИЕВЫХ КАНАЛОВ И СПАЗМЕ ВНУТРЕННЕЙ СОННОЙ АРТЕРИИ ПРИ ГИПОКСИИ

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Тяжелая гипоксия вызывает спазм внутренней сонной артерии, который опасен ишемическим повреждением головного мозга. Некоторые метаболиты могут спровоцировать вазоконстрикцию, однако механизм их действия до конца не расшифрован. В эксперименте на крысах исследовали влияние 15-гидроксиэйкозатетраеновой кислоты (15-НЕТЕ), продуцируемой 15-липоксигеназой, на тонус сосудов при гипоксии. Обнаружено повышение уровня 15-липоксигеназы в эндотелии и гладкомышечных клетках внутренней сонной артерии. 15-НЕТЕ обуславливала дозозависимый спазм сосуда, а также снижение активности потенциалзависимых калиевых каналов. Таким образом, инактивация калиевых каналов 15-НЕТЕ при гипоксии приводит к ослаблению реполяризации активных потенциалов и притоку калия в клетки, что вызывает повышение тонуса гладкой мускулатуры сосудов и спазм внутренней сонной артерии.

Ключевые слова: потенциалзависимые калиевые каналы, гладкомышечные клетки, церебральный вазоспазм.

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THE INHIBITING EFFECT OF PHOTODYNAMIC THERAPY AND NOVEL RECOMBINANT HUMAN ENDOSTATIN ON THE *IN VIVO* GROWTH OF U251 HUMAN GLIOMA XENOGRAFTS

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Keywords: malignant tumors, endostar, hypoxia inducible factor, vascular endothelial growth factor.

Summary – Endostar, a novel recombinant human endostatin expressed in *Escherichia coli*, was approved by the State FDA in China. To investigate the effect of endostar and photodynamic therapy (PDT) on the *in vivo* growth of U251 glioma. Seven days after inoculation with U251 cells, nude mice with MRI-confirmed glioma were randomly assigned to 4 groups: PDT+endostar group; PDT group; endostar group and control group. In the PDT group, survival prolonged, accompanied by an increase in apoptosis, when compared with the control group. Furthermore, these changes were more pronounced in the PDT+endostar group. After PDT, hypoxia inducible factor-1 α (HIF-1 α) and vascular endothelial growth factor A (VEGF-A) expression was markedly increased and after endostar treatment, HIF-1 α and VEGF-A expression was significantly reduced. PDT, in combination with endostar, can significantly inhibit the growth of U251 glioma. This approach may represent a promising strategy in the treatment of malignant tumors.

Glioma is a common intracranial malignancy accounting for approximately 40–50 % of intracranial malignancies. The 5-year survival rate is approximately 30 % in patients with astrocytoma [14]. Photodynamic therapy (PDT) has been an effective auxiliary strategy in the treatment of glioma [11]. PDT treatment is based on the presence of

a drug with photosensitizing properties combined with visible or far red light and oxygen.

Following PDT, a state of hypoxia is induced within the tumor tissue as a result of rapid oxygen consumption [4]. Tissue hypoxia induces a plethora of molecular and physiological responses, including an adaptive response associated with gene activation. A primary step in hypoxia-mediated gene activation is the formation of the transcription factor complex (hypoxia inducible factor-1 – HIF-1). A number of HIF-1-responsive genes have been identified, including vascular endothelial growth factor (VEGF), erythropoietin, and glucose transporter-1. VEGF, also called vascular permeability factor, is an endothelial cell-specific mitogen involved in the induction and maintenance of the neovasculature in solid tumors [7].

A. Ferrario et al. [5] applied PDT in the treatment of mouse breast cancer. The results showed that HIF-1 α and VEGF expression was increased after PDT but was markedly decreased after treatment with angiogenesis inhibitors (IM862 and EMAP-2). R. Bhuvaneshwari et al. [2] revealed that subcurative PDT in an orthotopic model of prostate cancer increases not only VEGF secretion but also the fraction of animals with lymph node metastases. PDT followed