Original Article

Effect of β -sodium aescinate on hypoxia-inducible factor-1 α expression in rat brain cortex after cardiopulmonary resuscitation

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BACKGROUND: This study was undertaken to investigate the expression of hypoxia-inducible factor-1 α (HIF-1 α) in rat cerebral cortex and the effects of β -sodium aescinate (SA) administration after return of spontaneous circulation (ROSC).

METHODS: Sixty rats were divided into three groups: SA group, injected intraperitoneally with SA instantly after ROSC; control group, injected intraperitoneally with normal saline; and sham-operated group, without cardiac arrest or SA. The cardiac arrest model was established using asphyxiation and intravenous potassium chloride. Blood was sampled 1, 6, 12, and 24 hours after ROSC. Protein and mRNA levels of HIF-1 α , VEGF and EPO were detected in the cerebral cortex by immunohistochemistry and real-time RT-PCR; serum levels of NSE and S100 β were determined by enzyme-linked immunosorbent assays.

RESULTS: Serum S100 β and NSE were significantly increased in the control group versus the sham-operated group 1, 6, 12 and 24 hours after ROSC ($P < 0.05$). Protein and mRNA levels of HIF-1 α , VEGF and EPO were significantly increased in the control rats ($P < 0.05$). Serum NSE and S100 β were significantly decreased in the SA group versus the control group 1, 6, 12 and 24 hours after ROSC ($P < 0.05$). Protein and mRNA levels of HIF-1 α , VEGF and EPO were significantly increased in the SA group ($P < 0.05$).

CONCLUSIONS: The expression of HIF-1 α is increased in rat cerebral cortex after ROSC, and SA up-regulates the expression of HIF-1 α . The up-regulation of HIF-1 α improves the resistance of the cortex to ischemia and hypoxia and contributes to neuroprotection, possibly because of up-regulation of EPO and VEGF expression.

KEY WORDS: Cardiopulmonary resuscitation; HIF-1 α ; Erythropoietin; Vascular endothelial growth factor; β -sodium aescinate; Neuroprotection

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INTRODUCTION

Although an initial return of spontaneous circulation (ROSC) is achieved in 30%–40% of patients with cardiac arrest, only 10%–30% of these cases admitted to the hospital will be discharged with a good outcome.^[1] Most of the deaths and adverse neurologic outcomes after ROSC are due to the immediate and delayed effects of brain ischemia/reperfusion (I/R) injury.^[1,2] One reason

for these poor outcomes is a lack of neuroprotective medications capable of ameliorating I/R injury during the post-resuscitation period.^[3] A recent study^[4] found that hypoxia-inducible factor-1 α (HIF-1 α) is a key mediator of the cellular response to hypoxia and can regulate the transcription of many downstream genes, such as erythropoietin (EPO) and vascular endothelial growth factor (VEGF), which could exert neuroprotective

effects.

β -sodium aescinate (SA), extracted from the dried ripe fruits of the Chinese buckeye (*Aesculus chinensis*), has been used in clinical therapy because of its anti-apoptotic, anti-edematous, anti-inflammatory and antioxidant effects.^[5–7] However, few studies have examined the effect of SA on HIF-1 α expression in a brain cortex I/R injury after cardiopulmonary resuscitation. In the present study, we used a rat model of cardiac arrest to test the hypothesis that SA up-regulates the expression of HIF-1 α and its downstream targets EPO and VEGF at the mRNA and protein levels in rat cerebral cortex after ROSC.

METHODS

Animals and grouping

This study was carried out in strict accordance with the guidelines for animal care and use established by the Dalian Medical University Animal Care and Use Committee. The protocol was approved by the Committee on the Ethics of Animal Experiments of Dalian Medical University. All surgeries were performed under anesthesia and analgesia, and all efforts were made to minimize suffering.

Sixty adult male Sprague-Dawley rats (supplied by the experimental animal center of Dalian Medical University), weighing 300–350 g, were randomly divided into three groups ($n=20$): SA group, injected intraperitoneally with SA (5 mg/kg) instantly after the ROSC; control group, injected intraperitoneally with an equivalent volume of normal saline; and sham-operated group (sham), without cardiac arrest or SA administration.

Establishment of the rat cardiac arrest model

Before the operation, the rats were fasted overnight, but had free access to water. The rats were anesthetized with ketamine (100 mg/kg) by intraperitoneal injection. After anesthesia, the left femoral artery and vein were isolated. A catheter (Shanghai Puyi Medical Instruments Co., Ltd, Shanghai, China) was inserted into the left femoral vein and connected to a micro-infusion pump (Changsha Beyond Medical Devices Co., Ltd, Changsha, China) to administer normal saline (2 mL/h). To monitor arterial blood pressure and heart rate, an F24 catheter (Shanghai Puyi Medical Instruments Co., Ltd, Shanghai, China) was advanced from the left femoral artery into the thoracic aorta and connected to an animal ECG monitor (SurgiVet V3404, Friends Honesty Life Sciences Co.,

LTD, Hong Kong, China) through a transducer (ZH0144, Huaibei ZhengHua Company, Huaibei, China). After instrumentation, endotracheal intubation was performed and the rats were ventilated with a volume-controlled ventilator (Servo 900c, Siemens, Munich, Germany) with a tidal volume of 15 mL/kg, an FiO₂ of 0.21, and a ventilation rate of 12 to 20 breaths/min. End-tidal PCO₂ was monitored with in-line infrared capnograph (CO2SMOplus monitor, Respironics Inc., USA) placed in the airway. The ventilation rate and the tidal volume were adjusted to maintain normocapnia (35–45 mmHg). Arterial blood gases were analyzed to confirm adequate baseline ventilation (ABL80, Radiometer, Copenhagen, Denmark).

The rat cardiac arrest model was established using asphyxiation combined with intravenous potassium chloride solution.^[1] The criteria for cardiac arrest included the disappearance of spontaneous cardiac rhythm waveforms and a mean arterial pressure of less than 30 mmHg.

After 5 minutes of untreated cardiac arrest, CPR was started with chest compressions (160–200 beats/min) performed by the same investigator, an experienced CPR technician from our laboratory. Meanwhile, the rat was ventilated with a small animal ventilator (TKR-200C, Yatai KeLong Co., LTD, Beijing, China) with a respiratory rate of 80 beats/min, a tidal volume of 2.5 mL and 100% pure oxygen. Epinephrine (0.01 mg/kg) and atropine (0.01 mg/kg) were administered via the femoral vein. Additional doses of epinephrine were given, if needed, every 3 minutes until ROSC was achieved. ROSC was defined as an organized cardiac rhythm with a mean aortic pressure of more than 60 mmHg, continuously sustained for at least 10 minutes or more.

One, 6, 12 and 24 hours after ROSC ($n=5$), blood was drawn from the rat orbital venous plexus, and then the chest cavity was rapidly opened to expose the heart. The abdominal aorta and the inferior vena cava were separated and ligated. A syringe needle was inserted from the apex of the heart into the left ventricle and then to the aortic root. The puncture site of the needle was fixed with a hemostatic clamp. A small hole was cut in the auricula dextra. The heart was rapidly infused with normal saline until the lavage fluid became clear and the lobe was bright white, and then rapidly infused with 100 mL of 4% paraformaldehyde, followed by a slow infusion of 200 mL of 4% paraformaldehyde. The rats were sacrificed by decapitation, and the skull was opened to harvest brain tissues. The left hemisphere was fixed in

paraformaldehyde, and the right hemisphere was snap-frozen in liquid nitrogen and stored at -80°C . All of the parameters and records were in accordance with the Utstein-Style guidelines.^[8]

Enzyme-linked immunosorbent assay

NSE and S100 β proteins were detected using enzyme-linked immunosorbent assays (Zhongshan Golden Bridge Biotechnology Co., Ltd. Beijing, China), according to the manufacturer's instructions. Each sample was tested in triplicate.

Immunohistochemistry

EPO (1:150, Santa Cruz Biotechnology, Santa Cruz, CA, USA), HIF-1 α (1:200, Biosynthesis Biotechnology Co., LTD, Beijing, China) and VEGF (1:150, Santa Cruz Biotechnology) were detected by immunohistochemistry, according to the manufacturer's instructions. Image J software (National Institutes of Health, Bethesda, MD, USA) was used to determine the optical density of images. Five slices were selected in every group, and the average optical density of five high-power fields ($\times 400$) from each slice was measured. Each sample was tested in triplicate.

Real-time RT-PCR

Total RNA from frozen rat brain samples was isolated using a TRIzol kit (TaKaRa, Dalian, China) according to the manufacturer's instructions, followed by reverse transcription to generate first-strand cDNAs using the QuantiTect reverse transcription kit (Tiangen Biotech, Beijing, China), according to the manufacturer's instructions. The primer sequences for HIF-1 α were F-5'-ATTCTCCAAGCCTCCGA-3' and R-5'-TCATCCATTGACTGCCCC-3', the primer sequences for EPO were F-5'-GAATTGATGTCGCCTCCAGA-3' and R-5'-CCAAGCCGCTCCACTCCGAACA-3', the primer sequences for VEGF were F-5'-TTCAAGCCGTCCTGTGTGC-3' and R-5'-TCCAGGGCTTCATCATTGC-3', and the primer sequences for the endogenous reference β -actin were F-5'-GACCTGACTGACTACCTCAT-3' and R-5'-TCGTCATACTCCTGCTTGCT-3'. The primers were synthesized by Takara Biotechnology Co., LTD (Dalian, China). Real-time RT-PCR analysis was performed using a Quant script RT Kit (Tiangen Biotech, Beijing, China). The cycle threshold (Ct) values of the genes of interest were first normalized to the Ct value of β -actin from the same sample, and then the relative differences between the groups were calculated and expressed as relative

increases, setting the sham group as 1. Each sample was tested in triplicate.

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). The data were analyzed using Student's *t* test or one-way ANOVA, followed by the Bonferroni's correction for multiple comparisons. Pearson's product-moment correlation coefficient method was used for correlation analysis. Statistical significance was defined as $P < 0.05$. Statistical analysis was made using the SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA).

RESULTS

Serum S100 β and NSE

Compared with the sham group, the control group showed increased serum S100 β levels 1 hour after ROSC, peaked 6 hours after ROSC, and continued to be higher than those in the sham group 24 hours after ROSC. Serum NSE levels were increased in the control group versus the sham group 6 hours after ROSC, peaked 12 hours after ROSC, and continued to be higher than those in the sham group 24 hours after ROSC. Compared with the control group, the SA group showed reduced serum levels of S100 β and NSE at each time point (Table 1).

Expression of HIF-1 α , VEGF and EPO proteins in rat brain cortex

Compared with the sham group, the control group showed an increase in HIF-1 α , VEGF and EPO protein levels in the rat cerebral cortex 1, 6, 12 and 24 hours after ROSC. Compared with the control group, the SA group showed an increase in HIF-1 α , VEGF and EPO protein levels in the cortex at each time point (12 hours after ROSC shown in Figure 1).

Table 1. Serum S100 β and NSE levels (mean $\pm$ SD, $n=8$)

Variables	Time after ROSC			
	1 h	6 h	12 h	24 h
S100 β (ng/mL)				
Sham	0.002 $\pm$ 0.001	0.003 $\pm$ 0.001	0.002 $\pm$ 0.002	0.002 $\pm$ 0.001
Control	0.234 $\pm$ 0.108*	0.603 $\pm$ 0.210*	0.364 $\pm$ 0.137*	0.324 $\pm$ 0.126*
SA	0.132 $\pm$ 0.098 [#]	0.335 $\pm$ 0.127 [#]	0.211 $\pm$ 0.099 [#]	0.193 $\pm$ 0.107 [#]
NSE (ng/mL)				
Sham	2.113 $\pm$ 0.102	2.125 $\pm$ 0.904	2.103 $\pm$ 0.936	2.099 $\pm$ 0.101
Control	2.879 $\pm$ 0.859	4.483 $\pm$ 1.022*	5.691 $\pm$ 1.124*	8.834 $\pm$ 1.457*
SA	2.691 $\pm$ 0.121	3.276 $\pm$ 0.973 [#]	3.961 $\pm$ 1.003 [#]	5.074 $\pm$ 1.341 [#]

Compared with sham group, * $P < 0.05$; compared with control group, [#] $P < 0.05$; Sham: sham-operated group; SA: β -sodium aescinate group.

Figure 1. Expression of HIF-1 α , EPO and VEGF proteins in rat cerebral cortex. HIF-1 α in the sham (A), control (B) and SA (C) groups (original magnification $\times 400$); EPO in the sham (D), control (E) and SA (F) groups (original magnification $\times 400$); VEGF in the sham (G), control (H) and SA (I) groups (original magnification $\times 400$).

Table 2. HIF-1 α , EPO and VEGF mRNA expression in rat cerebral cortex (mean $\pm$ SD, $n=8$)

Variables	Time after ROSC			
	1 h	6 h	12 h	24 h
HIF-1α mRNA				
Sham	0.093 $\pm$ 0.025	0.098 $\pm$ 0.028	0.108 $\pm$ 0.027	0.096 $\pm$ 0.029
Control	0.094 $\pm$ 0.023	0.344 $\pm$ 0.063*	0.767 $\pm$ 0.057*	1.243 $\pm$ 0.056*
SA	0.120 $\pm$ 0.012	0.598 $\pm$ 0.091 [#]	1.205 $\pm$ 0.135 [#]	1.629 $\pm$ 0.164 [#]
EPO mRNA				
Sham	0.122 $\pm$ 0.018	0.114 $\pm$ 0.018	0.119 $\pm$ 0.017	0.117 $\pm$ 0.019
Control	0.115 $\pm$ 0.016	0.331 $\pm$ 0.093*	0.682 $\pm$ 0.248*	1.223 $\pm$ 0.065*
SA	0.115 $\pm$ 0.029	0.610 $\pm$ 0.091 [#]	1.211 $\pm$ 0.131 [#]	1.582 $\pm$ 0.189 [#]
VEGF mRNA				
Sham	0.033 $\pm$ 0.016	0.035 $\pm$ 0.013	0.036 $\pm$ 0.014	0.034 $\pm$ 0.012
Control	0.037 $\pm$ 0.017	0.046 $\pm$ 0.018*	0.096 $\pm$ 0.036*	0.117 $\pm$ 0.021*
SA	0.041 $\pm$ 0.015	0.640 $\pm$ 0.025 [#]	0.117 $\pm$ 0.018 [#]	0.153 $\pm$ 0.029 [#]

Compared with sham group, * $P<0.05$; compared with control group, [#] $P<0.05$; Sham: sham-operated group; SA: β -sodium aescinate group.

HIF-1 α , EPO and VEGF mRNA expression in rat cerebral cortex

Compared with the sham group, the control group showed increased mRNA expression of HIF-1 α , EPO

and VEGF 6 hours after ROSC, and continued to be higher than that in the sham group 24 hours after ROSC. Compared with the control group, the SA group showed an increased mRNA expression of HIF-1 α , EPO and VEGF at every time point (Table 2). Pearson's correlative analysis showed that the mRNA expression of HIF-1 α was positively correlated to the mRNA expression of EPO and VEGF in rat cerebral cortex (EPO: $r=0.952$, $P<0.05$; VEGF: $r=0.866$, $P<0.01$).

DISCUSSION

The present study demonstrates that the mRNA and protein expression of HIF-1 α , VEGF and EPO is significantly increased in rat cerebral cortex after ROSC, and up-regulated by SA. Also, rat serum S100 β and neuron specific enolase (NSE), markers of neuronal injury, are significantly decreased by SA. Thus, we speculate that SA-induced neuroprotection might be associated with the up-regulated expression of HIF-1 α and its downstream target genes VEGF and EPO at the

mRNA and protein levels.

HIF-1 is a nuclear protein that activates gene transcription, including that of EPO, VEGF, iNOS and heme oxygenase-1, in response to cellular hypoxia.^[4,9]

HIF-1 is a heterodimer composed of HIF-1 α and HIF-1 β subunits. Both subunits are induced by hypoxia and rapidly decay upon return to normoxia. Thus, HIF-1 α can induce adaptive pathophysiological responses to hypoxia and ischemia.^[9]

VEGF is a very important target gene of HIF-1 α , and a major regulator of angiogenesis.^[4] After hypoxia-ischemia, the over-expression of HIF-1 α induces an increase in VEGF expression.^[4,10] VEGF can promote angiogenesis and microcirculation reconstruction, and thus increase the blood and oxygen supply to ischemic tissues and contribute to the recovery from brain injury caused by ischemia and hypoxia.^[4] Over-expression of VEGF can also exert protective effects on brain ischemia-reperfusion injury by protecting endothelial cells, reducing brain edema, and scavenging oxygen free radicals. Furthermore, VEGF has a direct protective effect on neurons by stimulating the growth of axons, promoting division, migration and survival of glial cells, and contributing to nerve regeneration.^[11] Inhibition of HIF-1 α expression down-regulates VEGF expression and leads to vascular defects.^[12]

EPO is expressed in brain tissues, and contributes to neuroprotection.^[13] Hypoxia can rapidly activate HIF-1 α and thus promote the expression and release of EPO.^[14] EPO can increase the number of red blood cells and the oxygen content in blood.^[14] EPO protects the brain from ischemic-reperfusion injury through several mechanisms: anti-apoptotic effects, protection of vascular endothelial cell integrity, angiogenesis, antioxidant effects by up-regulating the expression of antioxidant, inhibition of pro-inflammatory cytokine release and inflammatory cell infiltration, improvement of synaptic transmission, and enhanced resistance to ischemia.^[15-17]

The current study has some limitations. First, our study focused on the cerebral cortex, as it is known to play a vital role in the recovery of function after cardiac arrest. However, the hippocampus is the brain region most vulnerable to global ischemic injury and may therefore show greater effects. Second, we established a rat cardiac arrest model using asphyxiation combined with intravenous potassium chloride solution, but ventricular fibrillation-induced cardiac arrest is more commonly found in patients.

In conclusion, SA up-regulates the expression of HIF-1 α and its downstream target genes EPO and VEGF

at the mRNA and protein levels after ROSC, which may explain its neuroprotective effects.

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Ethical approval: The present study was approved the Dalian Medical University Animal Care and Use Committee, Dalian, China.

Conflicts of interest: The authors declare that there is no conflict of interest.

Contributors: Kang J conceived and designed the experiments. Gong P and Ding QL performed the experiments. Ren YB analyzed the data. Gao DN contributed reagents, materials and analysis tools. Ding QL wrote the paper. All authors contributed to editing the final manuscript for content and style.

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