Tanshinone IIA Prevented Brain Iron Dyshomeostasis in Cerebral Ischemic Rats

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Key Words

Tanshinone IIA • Cerebral ischemia • Iron • Divalent metal transporter 1 • Transferrin receptor • Ferroportin 1 • Hephastin

Abstract

Background: Tanshinone IIA is a lipid-soluble compound extracted from Chinese herb Danshen which was commonly used in the treatment of cerebrovascular diseases. Brain iron homeostasis is very essential for normal physiological functions of neurons, and brain iron accumulation contributes to many neurological disorders. The present study was aimed to determine whether Tanshinone IIA protects against cerebral ischemic injury via maintaining brain iron homeostasis. **Methods:** Wistar Rats were orally administered with Tanshinone IIA (4, 20 and 100 mg/kg/d) for one week, and then subjected to cerebral ischemia by middle cerebral artery occlusion (MCAO) for 2 h. *In vitro*, cultured neurons were pretreated with Tanshinone IIA (2, 10 and 50 μ M) for one week, and then exposed to hypoxia for 24 h. Brain iron content was determined by Flame Atomic Absorption Spectrophotometer and intracellular free iron level was measured by laser scanning Confocal

microscopy. Expression levels of iron transport proteins including DMT1, TfR, Fpn1 and Heph were assessed by Western blot technique. **Results:** Tanshinone IIA pretreatment resulted in a significant reduction of cerebral infarct volume in MCAO rats. Compared with control rats, cerebral ischemia considerably augmented total iron content in the brain of MCAO rats, which was also effectively restricted by Tanshinone IIA pretreatment. MCAO rats exhibited the increased expression of iron influx proteins DMT1 and TfR, and the decreased expression of iron efflux proteins Fpn1 and Heph compared with control rats, which was responsible for elevated iron content in the ischemic brain. Tanshinone IIA pretreatment prevented the dysregulation of these four iron transport proteins and maintained brain iron homeostasis. *In vitro* studies also confirmed that Tanshinone IIA alleviated the hypoxia-induced decline of cell viability and the overload of intracellular free iron level in neurons through downregulating the expression of DMT1 and TfR, and upregulating the expression of Fpn1 and Heph. **Conclusion:** Tanshinone IIA protected brain tissues against ischemic and hypoxic damage *in vivo* and *in vitro* by mediating brain iron homeostasis which was associated with the downregulation of DMT1 and TfR, and the upregulation of Fpn1 and Heph. These

results provide new insights into molecular mechanisms of ischemia-induced brain iron abnormalities and suggest maintaining brain iron homeostasis may be a novel therapeutic strategy for ischemic cerebrovascular diseases.

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Introduction

Iron is an essential metal element for supporting human body health. It widely exists in the brain, and plays an important role in the oxidative metabolism, the activation of enzymes, the synthesis of dopamine and serotonin, etc [1-3]. A large body of evidence has shown that the maintenance of brain iron homeostasis is essential for normal biological structure and functions of neurons, which mainly depends on the balance of iron uptakes and releases from cells in the mammalian brain [1-5]. The deficiency or excess of brain iron content as a consequence of the imbalance of brain iron metabolism may cause severe clinical symptoms. A lack of iron is responsible for anemia and excessive iron accumulation in human body is an important cause for neurodegenerative diseases, coronary diseases or even cancers [3]. Cerebral ischemia was shown to lead to brain iron accumulation and neurons iron deposition [4-5]. Brain iron overload-induced neurotoxicity contributed to an increased infarct volume in MCAO animals [6]. Level of free iron was also observed elevated in serum and cerebrospinal of patients with cerebral infarction [7]. The dysregulation between iron influx proteins DMT1 and TfR, and iron efflux proteins Fpn1 and Heph has been proposed as a major trigger for the imbalance of cellular iron homeostasis in ischemic brains [3, 6]. Excessive iron accumulation in the brain promotes the production of free radical, aggravates iron-induced neuronal damage, and causes neuronal degenerative diseases [8-9]. So, the alleviation of brain metal dyshomeostasis is taken as novel therapeutic avenues to prevent ischemic damage in the brain.

Tanshinone IIA is a lipid-soluble compound purified from the Chinese herb Danshen (the rhizome of *Salvia miltiorrhiza*), a well-known traditional Chinese medicine used for the treatment of cerebrovascular and cardiovascular diseases such as stroke [10-11]. Some studies have showed that Tanshinone IIA could protect the brain against ischemic injury, and its mechanisms were associated with the reduction of oxidative products and the suppression of redox-induced inflammatory responses [11-

13]. For instance, Lam et al reported administration of Tanshinone IIA via intraperitoneal injection significantly reduced the brain infarct volume by about 30% in mice subjected to transient focal cerebral ischemia and enhanced the neurological scores [14]. Tanshinone IIA also could reduce the production of NO and inhibit the expression of iNOS and translocation of NF- κ B, and thus inhibit cell apoptosis in the brain [15].

However, whether brain iron metabolism is involved in the protective effects of Tanshinone IIA on cerebral ischemia remained unknown. Therefore, we hypothesize that Tanshinone IIA may prevent cerebral ischemia-induced brain iron imbalance via regulating iron transport proteins. Here, we examined the total brain iron and the expression of DMT1, TfR, Fpn1 and Heph proteins in rats subjected to MCAO and Tanshinone IIA pretreatment. Our results suggest that Tanshinone IIA plays a neuroprotective role in cerebral ischemia-induced brain damage via modulating iron transporters to maintain the balance of brain iron metabolism.

Materials and Methods

Drugs and Animals

Tanshinone IIA (purity 99%) was purchased from Xian Guanyu Bio-tech Co. Ltd, China, and was dissolved in dimethylsulphoxide (DMSO). The final concentration of DMSO was not more than 0.01% in all assays of this study. Male Wistar adult (220~250 g) and neonatal rats (1-3 days) were obtained from the Animal Center of Affiliated Second Hospital of Harbin Medical University. Wistar Rats were housed in a climate-controlled environment at an ambient temperature of 20°C with a 12/12 h light/dark cycle. The use of animals was in accordance with the requirements of the Ethics Committee of Harbin Medical University.

Cerebral Ischemia Induced by Middle Cerebral Artery Occlusion

Male Wistar rats were randomly divided into five groups: Control group, Cerebral infarction group (MCAO), Tanshinone IIA 4 mg/kg/d group, Tanshinone IIA 20 mg/kg/d group, Tanshinone IIA 100 mg/kg/d group. The rats were anesthetized with chloral hydrate (0.3 ml/kg). The procedure for middle cerebral artery occlusion (MCAO) using an intraluminal suture was described in detail previously [16]. In brief, the right common carotid artery, internal carotid artery, and external carotid artery were surgically exposed. A 4-0 monofilament nylon suture (Beijing Sunbio Biotech Co Ltd) with a rounded tip was aseptically introduced into the right carotid artery, in an antegrade fashion towards the carotid bifurcation. It was then directed distally up the right internal carotid artery to a distance of 17.5 ± 0.5 mm from the carotid bifurcation to occlude the origin of middle cerebral artery for 2 h. All surgical proce-

dures were performed under sterile conditions. After surgery, survival rats were housed at ambient temperature of 22°C. Tanshinone IIA (4, 20 and 100 mg/kg/d) were orally administered daily one week before operation. In control rats, the thread was immediately removed as soon as the origin of the middle cerebral artery was reached.

Measurement of Cerebral Infarction Volume

The brain was removed carefully and dissected into coronal 2-mm sections, and sequentially immersed into a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in PBS at 37°C for 30 min. The samples were fixed in 4% paraformaldehyde solution at 4°C as previously described [17]. The volume of infarction in the cerebral cortex of the right middle cerebral artery territory of each brain slice was measured by an image analyzer, Image-Pro Plus Data Analysis Program (Media Cybernetics, Silver Spring, Md, USA).

Measurement of Brain Iron Content

Brain iron content was determined by Flame Atomic Absorption Spectrophotometer (Shimadzu AA-6800, Japan) [18]. Tissue samples (0.1~0.2 g) from brain cortex were digested with a mixture of HNO₃ and HClO₄ (4:1) for 10 h, then heated to boiling. The final clear residue was adjusted to a final volume of 10.0 ml with 2 % HNO₃.

Primary Neuron Culture

Neonatal rat neurons were isolated and cultured as described previously [19]. Briefly, 1-3 days old Wistar rats were decapitated and their brains were aseptically removed. Brain tissues from the cortex were dissected, minced and trypsinized with 0.125 % trypsin at 37°C for 25~30 min. The cells were suspended in DMEM containing 10 % FBS, centrifuged and pooled. Neuronal cells were plated onto dishes at a density of 10⁵ cells/cm² and incubated at 37 °C in a humidified air with 5% CO₂ for 72 h, and then 4 ug/ml Cytarabine was added into the medium to deplete gliocytes.

Hypoxic Culture in Vitro

After cultured for another 4 days, neurons were treated with or without Tanshinone IIA. In order to mimics cerebral ischemia *in vivo*, cultured neurons were then exposed to hypoxia for 24 h by using a hypoxia chamber (temperature: 37°C, 1% O₂, 94% N₂ and 5% CO₂). Tanshinone IIA (2, 10 and 50 uM) was given for one week previous hypoxic insult.

MTT Assay for Cell Viability

Cell Proliferation Kit I [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT); Roche Molecular Biochemicals, Laval, PQ, Canada] was used to quantify survival of neural cells suffering from hypoxic injury according to the manufacturer's protocol.

Measurement of Neuronal Fe²⁺ Concentration ([Fe²⁺])

Intracellular Fe²⁺ concentration ([Fe²⁺]_i) was assessed using Calcein AM. Briefly, cells were washed with PBS, added final concentration Calcein AM (0.125 μmol/L) in the medium. Pluronic F-127 (1 mg/ml), a mild detergent, was used to load

Fig. 1. Effect of Tanshinone IIA on the volume of cerebral infarction and the level of brain iron in rats subjected to middle cerebral artery occlusion (MCAO). (A) Pretreatment with Tanshinone IIA (4, 20 and 100 mg/kg/d, orally, 7 days) significantly reduced the infarct volume of rat brains subjected to MCAO. (B) Tanshinone IIA (4, 20 and 100 mg/kg/d, orally, 7 days) also attenuated the accumulation of brain iron level in MCAO rats. Data are expressed as mean $\pm$ SEM. ###P < 0.001 vs. Control; *P < 0.05 vs. MCAO; ***P < 0.001 vs. MCAO, n = 10.

Calcein AM. After loading beyond light at 37°C for 30 min, fluorescent changes of Calcein AM -loaded cells were detected using laser scanning Confocal microscope (Olympus FV-300) with 488 nm for excitation from an argon ion laser and 530 nm for emission. 5~7 cells were randomly selected in each vision and analyzed statistically.

Western Blot

For western blot analysis, the procedures were described as previously [17]. In brief, protein samples (100 ug) were fractionated on a 10% SDS-polyacrylamide gel. After electrophoretically transferred moist to a Pure Nitrocellulose Blotting membrane (Pall Life Science), membranes were blocked by 5% nonfat dry milk in PBS and incubated 2 h at room temperature, then the blots were incubated overnight at 4°C with DMT1, Fpn1 and Heph polyclonal antibodies (Alpha Diagnostic, San Antonio, TX), and TfR monoclonal antibodies (Zymed, San Francisco, CA), with GAPDH (Santa Cruz Biotechnology, USA) as an internal control. The immunoreactivity was detected using odyssey software (Infrared Imaging

Fig. 2. Effects of Tanshinone IIA on the expression of iron transport proteins including DMT1, TfR, Fpn1 and Heph in MCAO rats. (A, B, C, D) Western blotting was performed to examine the expression of DMT1, TfR, Fpn1 and Heph proteins in the brain tissues. (A, B) Tanshinone IIA pretreatment (20 mg/kg/d, orally, 7 days) significantly inhibited the expression of DMT1 and TfR proteins, while significantly enhanced Fpn1 and Heph levels in the ischemic brain tissues (C, D). Values are given normalized to band intensity of GAPDH used as internal control. All values are expressed as mean $\pm$ SEM. #P < 0.05 vs. Control; ###P < 0.001 vs. Control; ***P < 0.001 vs. MCAO, n = 10.

System LI-COR, Biosciences, Lincoln, NE, USA). Western blot bands were quantified using Odyssey v1.2 software by measuring the band intensity (area $\times$ OD) in each group and normalizing to the internal control.

Statistics

Data are expressed as mean $\pm$ SEM and analyzed using SPSS13.0 software. One-way ANOVA followed by Bonferroni's post-hoc test was used to test the significance in different groups. A two-tailed P < 0.05 was considered a significant difference.

Results

Tanshinone IIA reduced the infarct volume and attenuated brain iron overload in cerebral ischemic rats

Infarct volume induced by cerebral ischemia in the rats administered, or not, with Tanshinone IIA 4, 20 and 100 mg/kg/d were shown in Fig. 1A. Cerebral infarct volume in MCAO rats was significantly attenuated by Tanshinone IIA in a dose-dependent manner. Tanshinone IIA 20 mg/kg/d pretreatment considerably decreased the

average infarct volume from 163.2 ± 7.9 mm³ in MCAO rats to 99.5 ± 8.3 mm³ (P < 0.001, n = 10). It indicates that Tanshinone IIA exerts a neuroprotective role in ischemia-induced brain injury.

To further determine whether brain iron metabolism is involved in neuroprotective actions caused by Tanshinone IIA, we observed the total brain iron content in different groups. As displayed in Fig. 1B, total iron content in the brain was much higher in MCAO rats (541 ± 34.7 mg/kg, n = 10) compared with control rats (230 ± 31.7 mg/kg, P < 0.001, n = 10). Nevertheless, Tanshinone IIA 20 mg/kg/d pretreatment significantly inhibited brain iron overload induced by cerebral ischemia (258 ± 31.6 mg/kg, P < 0.001 vs. MCAO, n = 10) (Fig. 1).

Effects of Tanshinone IIA on the expression of DMT1, TfR, Fpn1 and Heph proteins

Western blotting analysis was performed to determine the relative levels of DMT1, TfR, Fpn1 and Heph proteins in the brain tissues from different groups. The expression of iron import proteins DMT1 and TfR was significantly increased in the brains of MCAO rats

Fig. 3. Effects of Tanshinone IIA on the cell viability and intracellular iron level of neurons after 24 h hypoxia. (A) Cell viability was determined by MTT assay, expressed as relative level of surviving cells. Hypoxia induce a significantly reduction in the cell viability. Pretreatment with Tanshinone IIA (2, 10 and 50 μ M) markedly increased the survival rate of neurons after 24 h hypoxia insult. (B) Intracellular free iron level was assayed by laser scanning Confocal with the Calcein AM (0.125 μ mol/L). (C) The relative $[\text{Fe}^{2+}]_i / (F_{\text{max}}/F_0)$ was detected in neurons exposed to hypoxia and Tanshinone IIA (2, 10 and 50 μ M). The results showed that Tanshinone IIA (2, 10 and 50 μ M) pretreatment significantly inhibited intracellular free iron accumulation induced by 24 h hypoxia. ### $P < 0.001$ vs. Control; * $P < 0.05$ vs. Hypoxia; ** $P < 0.01$ vs. Hypoxia; *** $P < 0.001$ vs. Hypoxia, $n = 8$.

compared with control rats ($P < 0.001$, $n = 10$, Fig. 2A, 2B). Meanwhile, the expression of iron export proteins Fpn1 and Heph in the brains of MCAO rats was obviously decreased compared with control rats ($P < 0.05$, $n = 10$, Fig. 2C, 2D). Tanshinone IIA 20 mg/kg/d pretreatment remarkably attenuated the downregulation of TfR and DMT1 proteins, as well as the elevation of Fpn1 and Heph expressions in cerebral ischemic rats. It implies that Tanshinone IIA ameliorates ischemia-induced brain iron overload through regulating iron transport proteins.

Tanshinone IIA prevented hypoxic injury and intracellular iron overload in cultured neurons

In order to further confirm the protective effect of Tanshinone IIA on cerebral ischemic injury, cultured neurons were pretreated with Tanshinone IIA 2, 10 and 50 μ M for one week and then exposed to hypoxia for 24 h to mimics cerebral ischemia *in vivo*. The cells viability of neurons was significantly decreased after 24 h hypoxia ($P < 0.05$, Fig. 3A). Pretreatment with Tanshinone IIA 2, 10 and 50 μ M significantly enhanced the neuronal survival after 24 h hypoxia in a concentration-dependent manner. This suggests that Tanshinone IIA plays a strong protective role in hypoxia-induced neuronal injury.

We further observed the protective effects of Tanshinone IIA on hypoxia-induced iron imbalance in cultured neurons. Exposure of neurons to hypoxia for

24 h caused a significant accumulation of intracellular free iron concentration (Fig. 3B). Tanshinone IIA 2, 10 and 50 μ M pretreatment markedly inhibited the overload of intracellular free iron in cultured neurons exposed to hypoxia ($P < 0.05$, Fig. 3C). This suggests that the alteration of intracellular iron levels is involved in the neuroprotective actions of Tanshinone IIA.

Tanshinone IIA regulated brain iron transports in cultured neurons

In vitro, exposure of cultured neurons to hypoxia also induced an obvious increase of DMT1 and TfR proteins expression, as well as the downregulation of Fpn1 and Heph proteins ($P < 0.05$, Fig. 4), which indicates that hypoxia causes intracellular iron overload via disrupting brain iron import and export proteins. Tanshinone IIA 50 μ M pretreatment was shown to effectively reverse hypoxia-induced the upregulation of DMT1 and TfR (Fig. 4A and 4B), and the downregulation of Fpn1 and Heph (Fig. 4C and 4D) ($P < 0.001$, $n = 8$).

Discussion

The main finding of this study is that Tanshinone IIA alleviated brain iron disturbance via reducing the expression of DMT1 and TfR proteins, and increasing the expression of Fpn1 and Heph proteins in ischemic brains,

Fig. 4. Effects of Tanshinone IIA on brain iron transport proteins in cultured neurons. Hypoxia induced a significant elevation of DMT1 (A) and TfR (B) expression in cultured neurons. Tanshinone IIA 50 μ M pretreatment prevented these alteration induced by hypoxic insult. Tanshinone IIA 50 μ M attenuated hypoxia-induced the decrease of Fpn1 (C) and Heph (D) expression in cultured neurons. The values were normalized to GAPDH. ##P < 0.01 vs. Control; ###P < 0.001 vs. Control; ***P < 0.001 vs. Hypoxia, n = 8.

which contributes to its neuroprotective role in ischemia-induced brain injury.

Ischemic cerebrovascular diseases such as stroke remain a major factor causing death all over the world, with a gradual consistent increase in mortality and morbidity on population level. A large body of evidence suggests that cerebral ischemia is likely to lead to the excessive brain iron accumulation [1-2]. Iron deposition in the neurons accords for the generation of free radical and the promotion of membrane lipid peroxides, which, in turn, causes and aggravates neuronal damage [8-9]. Accordingly, restoring or alleviating iron dyshomeostasis in the brain has emerged as a new effective strategy to treat ischemic brain diseases.

Brain iron homeostasis is mainly controlled by the balance between brain iron uptake and iron release via regulating iron transport proteins [1, 3]. Besides the well-known transferrin receptor (TfR) pathway, some new discovered iron transport proteins such as DMT1, Fpn1 and Heph participate in the modulation of brain iron metabolism as well. The neuronal iron uptake is mediated by TfR followed by DMT1 to transport iron into the cytosol, where the iron is directed to incorporation into enzymes or storage in ferritin [3]. Pharmacological blockage of TfR/DMT1-mediated iron uptake is an effective

method to treat cerebral ischemic injury. Ferroportin 1 (Fpn1) is a membrane-bound protein that mediates ferrous iron export through the cell membrane. Fpn1 widely exists in the brain and blood-brain barrier, suggesting that Fpn1 plays an important role in iron output in neurons and iron transport in the blood-brain barrier [20]. The downregulation of Fpn1 protein in ischemic brains reduces the export of neurons iron, and at the same time brain iron intake is commonly increased, which will cause the accumulation of brain iron in neurons. Hephaestin functions to transform ferrous into ferric iron, and helps transporters 1 to release iron in cells. Hephaestin also may regulate the DMT1 and affect iron transports. Song et al. showed that the overexpression of Fpn1 and/or Heph might directly contribute to iron efflux process from neurons, and thus prevent cellular iron accumulation and eventually protect cells from iron-induced oxidative stress [21].

Our results showed that oral administration of Tanshinone IIA markedly reduced the brain infarct volume compared with that in MCAO rats, and increased the cell viability of neurons after 24 h hypoxic insult. These results suggest that Tanshinone IIA protects rats against cerebral ischemic injury, which is consistent with the previous observations [11]. More importantly, Tanshinone IIA was found to inhibit cerebral ischemia-induced brain

iron overload by promoting iron efflux and decreasing iron influx, which mainly resulted from the reduction of DMT1 and TfR expression, and the increase of Fpn1 and Heph expression.

Reactive oxygen species (ROS) is a very important reason for the pathogenesis of stroke. Abundant oxygen free radicals are generated in ischemic brain tissues, which contribute to the loss of mitochondrial potential and neuronal death after an episode of ischemia [8-9]. Although it is widely recognized that generation of ROS is responsible for cerebral ischemic damage, the sources and mechanisms of ROS generation during ischemia are not clearly understood. As we know, free iron ion plays an essential role in the oxidative metabolism and respiratory chain, especially in case of the overload of iron storage. Excessive iron accumulation in the brain can lead to the production of oxygen free radical, potentiate stress-induced neurotoxicity, and result in neurological disorders. In the present series of experiments, total iron content in the brain was significantly augmented in MCAO rats *in vivo*, and intracellular iron level was also elevated in cultured neurons exposed to hypoxia *in vitro*, accompanied by reduced infarct size and improved cell viability, respectively. These results implicate brain iron overload as an important cause of ischemia or hypoxia-induced oxidant stress and cellular injury.

Tanshinone IIA has presented antioxidant activities in a variety of cells such as cardiomyocytes [13, 22]. Several studies also demonstrated that Tanshinone IIA exerted a protective action on mitochondrial membrane potential and decreased cell death secondary to oxidative stress [11, 15, 23]. However, there is little information about whether brain iron metabolism is involved in

neuroprotection induced by Tanshinone IIA in ischemic brains. Our results showed that Tanshinone IIA attenuated the decrease of cell viability in neurons exposed to hypoxia, and prevented the augment of brain iron level induced by ischemia *in vivo* and hypoxia *in vitro*. It suggests that maintaining brain iron balance contributes to the attenuation of oxidant stress-induced neuronal damage by Tanshinone IIA. It was further revealed that the expression of Fpn1 and Heph was upregulated, but the expression of DMT1 and TfR was downregulated in rats pretreated by Tanshinone IIA. It suggests that Tanshinone IIA exhibits an inhibitory effect on brain iron overload by modulating iron transport proteins.

Taken together, our data uncovered that Tanshinone IIA restored brain iron homeostasis through upregulating iron export proteins and downregulating iron import proteins, which contributed to its neuroprotective effects on ischemic and hypoxic damage. It provides new insights into the mechanisms of ischemia-induced aberrant iron metabolism and may be one novel therapeutic target for ischemic cerebrovascular diseases.

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