Research Report

Protective effects of propofol against whole cerebral ischemia/reperfusion injury in rats through the inhibition of the apoptosis-inducing factor pathway

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Abstract

Cerebral ischemia/reperfusion (I/R) injury could cause neural apoptosis that involved the signaling cascades. Cytochrome c release from the mitochondria and the followed activation of caspase 9 and caspase 3 are the important steps. Now, a new mitochondrial protein, apoptosis-inducing factor (AIF), has been shown to have relationship with the caspase-independent apoptotic pathway. In this study, we investigated the protective effects of propofol through inhibiting AIF-mediated apoptosis induced by whole cerebral I/R injury in rats. 120 Wistar rats that obtained the permission of the animal care committee of Harbin Medical University were randomly divided into three groups: sham group (S group), cerebral ischemia/reperfusion injury group (I/R group), and propofol treatment group (P group). Propofol (1.0 mg/kg/min) was administered intravenously for 1 h before the induction of ischemia in P group. The apoptotic rate in three groups was detected by flow cytometry after 24 h of reperfusion. The mitochondrial membrane potential (MMP) changes were detected via microplate reader. The expressions of B-cell leukemia-2 (Bcl-2), Bcl-2 associated X protein (Bax) and AIF were evaluated using Western blot after 24 h, 24 h and 48 h of reperfusion. The results of our study showed that apoptotic level was lower in P group compared with I/R group and propofol could protect MMP. The ratio of Bcl-2/Bax was significantly higher in P group compared with I/R group. The translocation of AIF from mitochondrial to nucleus was lower in P group than that in I/R group. Our findings suggested that the protective effects of propofol on cerebral I/R injury might be associated with inhibiting translocation of AIF from mitochondrial to the nucleus in hippocampal neurons.

1. Introduction

Apoptosis has been tested play a critical role in lots of neurologic diseases. Apoptosis included activation of caspase-dependent and caspase-independent signaling pathways. While caspase-dependent pathway via release of cytochrome c from mitochondria, and then followed by apoptosome formation and activation of caspase3, caspase6, have been studied in detail. By now, little is known about the contribution of caspase-independent signaling after global cerebral ischemia (Harukuni and Bhardwaj, 2006).

Apoptosis inducing factor (AIF) is expressed in central nervous system and appears to play an important role in neuronal apoptosis induced by glutamate toxicity or oxidative stress (Yu et al., 2002; Zhang et al., 2002). AIF is a mitochondrial flavor protein which could mediate caspase-independent apoptotic cell death through its nuclear degrading activities (Daugas et al., 2000a, 2000b; Dumont et al., 2000; Ferri and Kroemer, 2000; Susin et al., 1999; Vieira et al., 2000). AIF translocated from mitochondria to nuclei where it induces caspase-independent DNA fragmentation. AIF is synthesized as a cytoplasmic ~67-kDa precursor which gives rise to a mature ~62-kDa protein that located in the mitochondrial inter-membrane space. When pathological permeabilization of the mitochondria, AIF is then processed to a ~57-kDa protein and followed released and translocated to the nucleus. The translocation to nucleus of AIF is associated with chromatin condensation and caspase-independent large-scale DNA fragmentation (Cheung
Propofol is an intravenous anesthetic that is commonly used in induce and maintain anesthesia. Many experimental evidences suggested that propofol had neuroprotective effect against cerebral ischemia injury (Ergun et al., 2002; Ito et al., 1999; Yano et al., 2000; Zheng et al., 2008). Although many studies have evaluated the mechanisms of propofol, it is still not totally clear. The brain protective mechanisms of propofol are complex. Our prior study had shown that propofol could inhibit the caspase-dependent apoptosis pathway. It is unknown, however, whether the brain protective effect of propofol is due to the caspase-independent pathway. Accordingly, the authors speculated that AIF signaling pathway is a candidate for contributing to the caspase-independent apoptotic process after cerebral ischemic injury. To test this hypothesis, we studied if apoptosis inducing factor (AIF), a major representative of caspase-independent apoptotic signaling is released from mitochondria and translated to nucleus following whole cerebral I/R injury and whether propofol could inhibit the process.

2. Results
2.1. Physiological parameters

The physiological variables of each group of rats were not statistically significant in terms of weight, mean arterial blood pressure (MAP), or arterial blood gas tension analysis, as shown in (Table 1). The levels of arterial oxygen pressure (PaO₂) and arterial carbon dioxide pressure (PaCO₂), as well as the blood pH, were kept within the normal range. The MAP was kept within predetermined limits (40 ± 5 mm Hg) during the ischemic period in the I/R group and propofol group of rats.

2.2. Apoptosis assay assessed by flow cytometry (FCM)

Neuronal apoptosis in the hippocampal from different groups was detected by FCM at 24 h after reperfusion. The distribution of apoptotic neurons was observed in each group. Few apoptotic neurons were found in the sham group. However, many apoptotic neurons were induced in the I/R group. Compared with the I/R group, the level of apoptosis was significantly decreased in propofol group. The percentage of apoptotic neurons was also calculated in the three groups. The percentage of apoptotic neurons in the I/R group (50.9 ± 3.2%) was higher (n=5, P < 0.05 vs. sham), compared with the sham group (6.4 ± 0.8%); the percentage of apoptotic neurons in the propofol treatment group (24.7 ± 1.2%) was lower compared with the I/R group, (n=5, P < 0.05 vs. I/R, see detail in Fig. 1).

2.3. Mitochondrial membrane potential analysis

Mitochondrial membrane potential (MMP) changes were detected at 6 h, 24 h, and 48 h after reperfusion and were determined via microplate reader. A lower light absorption photometric value of mitochondria reflected a lesser opening of the mitochondrial permeability transition pore, and the functionality of the mitochondria was taken as an approximation of normal neuron. The light absorption photometric values were 0.28 ± 0.08, 2.41 ± 0.4, and 1.2 ± 0.27 at sham group, I/R group and propofol group at 6 h after reperfusion, respectively; 0.28 ± 0.08, 2.56 ± 0.26, and 1.48 ± 0.19 at sham group, I/R group and propofol group at 24 h after reperfusion, respectively; 0.28 ± 0.08, 2.64 ± 0.16, and 1.68 ± 0.21 at sham group, I/R group and propofol group at 48 h after reperfusion, respectively.

The light absorption in I/R group and propofol group are higher than that in the sham group (P < 0.05 at each time point, respectively). The light absorption in propofol group was lower than that in the I/R group (as shown in Fig. 2, P < 0.05 at each time point, respectively).

2.4. Western blot analysis of Bcl-2 and Bax

The expression of Bcl-2 and Bax was evaluated by Western blot analysis. The results showed that the ratio of Bcl-2/Bax in P group (2.2 ± 0.63) was significantly higher than that in I/R group (1.1 ± 0.31) at 24 h after reperfusion (P < 0.05 vs I/R, Fig. 3).

2.5. Translocation of AIF

The expressions of AIF protein in different part of neuron were examined using Western blot in sham group, I/R group and propofol group at 6 h, 24 h and 48 h after reperfusion. The results showed that the total AIF protein level was not significantly changed after ischemia, and clear translocation of AIF from mitochondria to the nucleus was observed in 6 h, 24 h and 48 h after reperfusion. The AIF relative levels of mitochondrial fraction in I/R group and propofol group were lower than that in sham group (n=5, P < 0.05 vs sham). The AIF relative level of mitochondrial fraction in I/R group was lower than that in propofol group (n=5, P < 0.05 vs I/R). The AIF relative levels of nuclear fraction in I/R group and propofol group were higher than that in sham group (n=5, P < 0.05 vs sham). The AIF relative level of nuclear fraction in I/R group was higher than that in propofol group (n=5, P < 0.05 vs I/R, see detail in Fig. 4).

3. Discussion

Our study showed that propofol (1.0 mg/kg/min) significantly reduced the neural apoptosis level, ameliorated the opening of the mitochondrial permeability transition pore, increased the expression of apoptosis related gene Bcl-2, decreased the expression of Bax and decreased the translocation of AIF from mitochondria to the nucleus after cerebral ischemia/reperfusion injury.

In this study, we choose the clinical dose 1.0 mg/kg/min propofol as the test dose, and according to our past study, this dose could provide better cerebral protective effect and less circulation depress. The current study performed that AIF is a caspase-independent signaling pathways and important mediator of neuronal apoptosis and propofol treatment could decrease the translocation of AIF from mitochondria to nucleus after whole cerebral I/R injury. To our knowledge, this is the first report to implicate AIF in neuronal apoptosis about propofol treatment in whole cerebral I/R injury.
AIF, a pro-apoptotic protein that is located inside the mitochondrial membrane space, is released from mitochondria to the nucleus. AIF is associated with nuclear condensation and caspase-independent neuronal apoptosis following stretch injury or traumatic brain injury (Cao et al., 2003; Cheung et al., 2005; Zhu et al., 2003). Studies had shown that injury could cause AIF to be released from mitochondria into the cytoplasm and then into the nucleus, where it can induce apoptosis (Loetscher et al., 2001; MacManus et al., 1997; Sugawara et al., 2002; Yao et al., 2001; Zhan et al., 2001). Previous reports have tested that toxic concentrations of glutamate, oxygen-glucose deprivation and exposure to free radicals could result in nuclear AIF translocation to neurons (Cao et al., 2003; Culmsee et al., 2005; Plesnila et al., 2004; Wang et al., 2004; Zhang et al., 2002). In mitochondria, AIF is involved in oxido-reduction and is considered to be a reactive

**Fig. 1.** The results of apoptotic neurons in rat hippocampus in sham group, I/R group and propofol group at 24 h after reperfusion. The level of apoptotic neurons was measured by flow cytometry. A-C showed the results of apoptotic neurons in sham, I/R, and propofol group at 24 h after brain I/R injury, respectively. D, The percentage of apoptotic cells of different groups at 24 h after reperfusion (n=5, *P < 0.05 vs sham; #P < 0.05 vs I/R).

**Fig. 2.** Showed the light absorption photometric value of mitochondria in the three groups at 6 h, 24 h and 48 h after reperfusion, respectively (n=5, *P < 0.05 vs sham; #P < 0.05 vs I/R).

**Fig. 3.** Western blot showed Bcl-2 and Bax protein expression in the hippocampus in sham group, I/R group and propofol group at 24 h after reperfusion. A showed the immune-reactivity of Bcl-2 and Bax. B showed relative changes of Bcl-2 protein in sham group, I/R group and propofol group at 24 h after I/R reperfusion (n=5, *P < 0.05 vs sham; #P < 0.05 vs I/R).
AIF associated with less nuclear pyknosis and translocation of AIF that CypA knock-out mice had significant neuroprotective ability and large-scale DNA fragmentation (Daugas et al., 2000a, 2000b). Upon pathological permeabilization of the mitochondria, AIF is released and translocated to the nucleus, and then causes nuclear shrinkage and condensation and large-scale DNA fragmentation (Daugas et al., 2000a, 2000b).

The mechanisms of AIF cause nuclear pyknosis are interest and still unclear. Study showed that the translocation of AIF to isolated nuclei induced pyknosis and large-scale (50 kDa) DNA fragmentation after 30 s (Susin et al., 1999). Pyknosis is a hallmark of neuronal cell death, and AIF had been found in pyknotic nuclei following global cerebral ischemia, focal cerebral ischemia, and TBI. These findings suggested that AIF maybe responsible for this process. AIF did not have DNase activity, indicated that AIF may need to associate with other factors to induce the pyknosis (Braun et al., 2001; Cregan et al., 2002; Susin et al., 1999, 2000). Recently, studies suggested that AIF could induce DNA degradation only when it binded and translocated to the nucleus together with cyclophilin A (CypA), a cytoplasmic protein. Zhu et al. had shown that CypA knock-out mice had significant neuroprotective ability associated with less of nuclear pyknosis and translocation of AIF from mitochondria to nucleus (Zhu et al., 2003, 2007). So, we speculated that CypA may play an important role in AIF mediated nuclear Pyknosis after cerebral I/R injury. So, next step, we should explore the CypA mechanism of mitochondrial AIF release in further.

Our study showed that AIF translocated from mitochondria to the nucleus after 6 h following I/R injury. The process was accompanied by the opening of mitochondrial permeability transition pore that caused the loss of mitochondrial membrane integrity. The results supported the notion that the loss of mitochondrial integrity may play an important role in mediating ischemic neuronal apoptosis by triggering a diversity of death pathways involving both caspase-dependent and caspase-independent signaling pathways.

The upstream mechanisms involved in AIF release have been explored. Studies have shown that the Bcl-2 family members Bcl-2 and Bax are necessary for AIF release in neurons (Cregan et al., 2002) and are major mediators of cell apoptosis after ischemia injury. Furthermore, overexpression of Bcl-2 could block AIF release after focal ischemia (Murphy et al., 2000; Zhao et al., 2003). Inhibition of AIF translocation is a potential target for I/R injury treatment, and the current findings suggested that Bcl-2 overexpression maybe a possible way to accomplish this. Bcl-2 proteins reside on the outer mitochondrial membrane oriented toward the cytosol, and govern ion transportation and protect against breaches in the membrane. Bcl-2 also has various roles in normal cells, including regulating proton flux (Shimizu et al., 1998), buffering calcium mobilization (Foyouzi-Youssefi et al., 2000; Murphy et al., 2001; Pinton et al., 2002), antioxidant effects (Albrecht et al., 1994; Esposti et al., 1999), blocking cytochrome c (Green and Reed, 1998; Kowalski et al., 2000) and regulating AIF release (Daugas et al., 2000a, 2000b; Tsujimoto and Shimizu, 2000), and inhibiting caspase activity (Srinivasan et al., 1996; Zhang et al., 1999). Studies demonstrated previously that overexpression of Bcl-2 blocks neuronal death in vitro and in vivo. Therefore, our results showed that propofol treatment could increase the expression of Bcl-2 and decrease the expression of Bax. The results reinforce the notion that Bcl-2 may be the important step for AIF translocation from mitochondria and redistributed to the nucleus in neurons after I/R injury.

In conclusion, our results showed that AIF, a caspase-independent apoptotic regulated molecule, is released from the mitochondria in neurons after whole cerebral I/R injury and propofol could inhibit the process. Furthermore, the mitochondrial release and nuclear translocation of AIF after I/R injury maybe have relationship with the regulation of anti-apoptotic protein Bcl-2 and the opening of mitochondrial permeability transition pore. Taken together, AIF mediates caspase-independent cell death following brain I/R injury. Hence, inhibition of AIF-mediated cell apoptosis signaling pathway may be a therapeutic option for brain injured patients.

4. Materials and Methods

4.1. Animal preparation and experimental groups

The study was allowed by the animal care committee of Harbin Medical University, Heilongjiang, China and was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. Male Wistar rats (n = 120, provided by the animal research center of the second Affiliated Hospital of Harbin Medical University), weighing from 250 to 300 g, were randomly divided into three study groups (sham group, I/R group, and propofol group), and then into subgroups based on the reperfusion time (6 h, 24 h and 48 h after reperfusion). The rats in
sulting supernatant was centrifuged for 10 min at 8000 g. The homogenate was centrifuged in isolated mitochondria were performed using the MMP colorimetric method kit with a microplate reader at a wavelength of 450 nm. Mitochondrial membrane potential changes in three groups were detected by assessing changes in the light absorption photometric value at 6 h, 24 h, and 48 h after reperfusion.

4.6. Western blot analysis of Bcl-2 and Bax

After decapitation, hippocampal samples were solubilized by sonication for 5 seconds on ice in 0.5 ml of lysis buffer (Tris–Cl 50 mM, pH 7.5, EDTA 2 mM, NaCl 100 mM, 1% Nonidet P-40, supplemented with protease inhibitor cocktail purchased from Sigma). The solubilized tissues were then centrifuged at 12,000 rpm for 5 min at 4 °C, and supernatants containing protein were collected for immune blot analysis. An equivalent amount of total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) on 10% polyacrylamide gels, and then transferred to poly-vinylidene membranes. Membranes were blocked with 5% dry milk in PBS/0.1% (v/v) Tween 20 (PBST). Membranes were then incubated with primary antibodies (anti-Bcl-2, anti-BAX, and anti-actin, all from Santa Cruz) diluted in 2% BSA in PBST overnight at 4 °C. The membranes were then washed three times with PBST (10 min each time), incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 45 min at room temperature, and then washed three times with PBST. Immuno-reactive bands were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences) using a standard X-ray film.

4.7. Western blotting analysis

For the detection of AIF release and nuclear translocation, mitochondrial and nuclear protein extracts were prepared from the hippocampus in the three groups at 6 h, 24 h and 48 h after reperfusion (n = 5). After centrifugation at 13,500 × g for 15 min at 4 °C, cell lysates were collected. Protein concentration was assessed using the bicinchoninic acid (BCA) protein assay. The aliquots were then mixed with Laemmli sample buffer and boiled at 100 °C for 5 min. The samples (100 μg protein) were resolved on 10% SDS–PAGE gels, followed by transfer to nitrocellulose membranes. For visualization, blots were probed with antibodies against AIF (1:500 dilution) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000 dilution) at room temperature (21–23 °C) for 1 h, and detected using horseradish peroxidase-conjugated secondary antibodies (1:10000, Cell Signaling). The obtained digital images of the Western blots were used for densitometry measurements (Gel-Pro analyzer 4.0, Media Cybernetics). Western blot bands were quantified using Odyssey v3.0 software by measuring the band intensity for each group, and normalizing to GAPDH band as an internal control.

4.8. Data analysis

Quantitative data in this study are expressed as the mean ± the standard deviation. The data were analyzed using one-way analysis of variance (ANOVA) followed by the post hoc Fisher protected least squares difference (PLSD). P values < 0.05 were deemed to be statistically significant.

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