Cerebrospinal fluid from rats given hypoxic preconditioning protects neurons from oxygen-glucose deprivation-induced injury

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Abstract

Hypoxic preconditioning activates endogenous mechanisms that protect against cerebral ischemic and hypoxic injury. To better understand these protective mechanisms, adult rats were housed in a hypoxic environment (8% O₂/92% N₂) for 3 hours, and then in a normal oxygen environment for 12 hours. Their cerebrospinal fluid was obtained to culture cortical neurons from newborn rats for 1 day, and then the neurons were exposed to oxygen-glucose deprivation for 1.5 hours. The cerebrospinal fluid from rats subjected to hypoxic preconditioning reduced oxygen-glucose deprivation-induced injury, increased survival rate, upregulated Bcl-2 expression and downregulated Bax expression in the cultured cortical neurons, compared with control. These results indicate that cerebrospinal fluid from rats given hypoxic preconditioning protects against oxygen-glucose deprivation-induced injury by affecting apoptosis-related protein expression in neurons from newborn rats.

Key Words: nerve regeneration; hypoxic preconditioning; cerebrospinal fluid; cerebral cortex; oxygen-glucose deprivation; neurons; apoptosis; Bcl-2/Bax; neural regeneration

Introduction

Ischemic cerebrovascular disease-induced vascular dementia substantially affects the health and quality of life of patients. Therefore, it is necessary to develop neuroprotective strategies that relieve cortical neuronal injury after ischemic cerebrovascular events and improve the prognosis of vascular dementia (Meguro et al., 2013; Kwon et al., 2014; Liu et al., 2014; Sakr et al., 2014; Sun et al., 2014). Hypoxic preconditioning (HPC) activates a complex series of endogenous protective mechanisms that reduce hypoxic injury (Benitez et al., 2014; Monson et al., 2014; Sheldon et al., 2014; Suryana and Jones, 2014; Liu et al., 2015). HPC has protective effects against ischemic/hypoxic brain injury and ischemic cerebrovascular disease (Gidday, 2006; Benitez et al., 2014; Monson et al., 2014; Sheldon et al., 2014; Suryana and Jones, 2014; Liu et al., 2015). HPC has been demonstrated to protect against ischemic brain injury induced by acute cerebral infarction as evaluated by measuring infarct volume, neurological function assessment and apoptosis assay (Niu et al., 2009). However, it is difficult to implement HPC in the clinical setting. Nonetheless, HPC is currently a hot topic in translational medicine research.

Cerebrospinal fluid (CSF) is primarily secreted by the choroid plexus of the lateral ventricles, and contains a variety of electrolytes, proteins, glucose and growth factors (Cui et al., 2001; Lehtinen et al., 2011; Niu et al., 2011). CSF has been shown to induce the differentiation of neural stem cells into neurons and astrocytes (Lehtinen et al., 2011; Niu et al., 2011). CSF has also been shown to be neuroprotective for neurons and PC12 cells (Niu et al., 2011). In the present study, we investigate the potential neuroprotective effects of CSF obtained from rats given HPC on cortical neurons exposed to hypoxia, and we examine the underlying mechanisms of action.

Materials and Methods

Experimental animals

A total of 345 healthy adult Wistar rats weighing 200–250
et al., 2011). Neurons were cultured with 10% CSF from rats subjected to HPC for 1 day, and then exposed to OGD for 1.5 hours. In the HPC CSF group, neurons were cultured with normal rat CSF and high-glucose Dulbecco's modified Eagle's medium (DMEM) at 1:9 for 1 day, and then exposed to OGD for 1.5 hours. In the normal CSF group, neurons were cultured with PBS, neurons were incubated with goat anti-rabbit IgG-FITC (1:100; Boster, Wuhan, China) at 37°C for 30 minutes. Neurons were then washed, mounted with glycerol (4.5.3; Bio-Rad) (Yang et al., 2009). Flow cytometry Neurons were washed with 0.125% trypsin for approximately 4 minutes. Cells were collected and placed in centrifuge tubes and made into a single-cell suspension. Thereafter, 1 mL of cells at 1 × 10^6/mL was centrifuged at 1,000 r/min for 10 minutes. The supernatant was discarded, and the cells were resuspended in 200 µL binding buffer, and 10 µL annexin V-FITC and 5 µL propidium iodide were added in the dark at room temperature for 20 minutes. For the control, 400 µL of PBS was added instead of dye. Samples were filtered through a 200-mesh sieve and detected immediately with a flow cytometer (FACSCalibur; Bio-Rad) (Yang et al., 2009). Immunofluorescence assay After removal of the medium, neurons were washed twice with 0.01 M PBS (pH 7.4) at 37°C, fixed with 4% paraformaldehyde for 30 minutes, washed twice with PBS, and air-dried. Neurons were stained according to instructions provided by the Neuron Specific Enolase kit (Sigma), and observed under the fluorescence microscope (Radiance 2100; Bio-Rad, Hercules, CA, USA) (Yang et al., 2007a, 2008). The cortices were removed from neonatal Wistar rats under sterile conditions, sliced into 1-mm³ blocks in cold D-Hanks buffer, digested with 0.125% trypsin at 37°C in a 5% CO₂ incubator for 10 minutes, and filtered with a 200-mesh sieve. Cells at 5 × 10^6/mL were incubated in a 0.01% poly-L-lysine (Sigma, St. Louis, MO, USA)-coated plate. Then, 24 hours later, the medium was completely replaced with fresh medium. From then on, half of the medium was replaced every 3 days. At 8 days, primary cortical neurons were identified as follows: cells were washed twice with 0.1 M PBS, fixed with 4% paraformaldehyde for 30 minutes, washed twice with PBS, and air-dried. Neurons were stained according to instructions provided by the Neuron Specific Enolase kit (Sigma), and observed under the fluorescence microscope (Radiance 2100; Bio-Rad, Hercules, CA, USA) (Yang et al., 2009; Niu et al., 2011). Preparation of rat CSF In accordance with a previous method (Vannucci et al., 1998), adult rats were placed in a low-oxygen chamber (self-made, 8% O₂, 92% N₂, 37°C) for 3 hours for HPC. Then, after housing under normal oxygen for 12 hours, rats were intraperitoneally anesthetized with 10% chloral hydrate (3–4 mg/kg), and surgically operated. The atlantooccipital membrane was punctured, and the cerebrospinal fluid was expressed using a 0.22-µm microporous membrane, sterilized, and stored at −80°C for subsequent use. Culture and identification of neurons Primary neurons, cultured for 8 days, were randomly assigned to normal control, oxygen-glucose deprivation (OGD), normal CSF and HPC CSF groups. In the normal control group, neurons were cultured with normal high-glucose medium (Life Technologies, San Francisco, CA, USA). In the OGD group, neurons were exposed to OGD for 1.5 hours. In the normal CSF group, neurons were cultured with normal rat CSF and high-glucose Dulbecco's modified Eagle's medium (DMEM) at 1:9 for 1 day, and then exposed to OGD for 1.5 hours. In the HPC CSF group, neurons were cultured with 10% CSF from rats subjected to HPC for 1 day, and then exposed to OGD for 1.5 hours. Each group consisted of six wells. OGD culture was as follows: neurons were washed three times with glucose-free Earle's solution, and then incubated with glucose-free Earle's solution containing 1 mM Na₂SO₄ at 37°C, 5% CO₂ and saturated humidity (Niu et al., 2011).
Results
CSF from rats given HPC reduced apoptosis in cortical neurons exposed to OGD
In the normal control group, weak red (propidium iodide) and green (annexin V) fluorescence, a few stained cells, and a few early apoptotic neurons were visible. In the OGD group, there was abundant dark green and red fluorescence, and many bare red nuclei, middle and late apoptotic neurons and dead neurons were visible. In the normal CSF group, many cells had a green membrane and a red nucleus. Most were middle and late apoptotic neurons, although a few were early apoptotic neurons. Green and red fluorescence intensity was weaker in the normal CSF group than in the OGD group (P < 0.01). In the HPC CSF group, there were a few fluorescent cells and many cells with green membranes, characteristic of cells undergoing early apoptosis. Green and red fluorescence intensity was significantly weaker in the HPC CSF group than in the OGD and normal CSF groups (P < 0.01; Figure 1, Table 1).

CSF from rats given HPC increased the survival rate of cortical neurons exposed to OGD
Flow cytometry revealed that the survival rate was highest in the normal control group, and lowest in the OGD group. Compared with the OGD group, cell survival rate was significantly higher in the normal CSF group (P < 0.01). Furthermore, the survival rate was significantly greater in the HPC CSF group than in the OGD and normal CSF groups (P < 0.01; Figure 2, Table 1).

CSF from rats given HPC increased Bcl-2 expression and decreased Bax expression in cortical neurons exposed to OGD
The number of Bcl-2-positive cells was low in the normal control and OGD groups. The intensity of Bcl-2 immunofluorescence was significantly greater in the normal CSF group than in the normal control and OGD groups (P < 0.05). No significant difference in the number of Bcl-2-positive cells was detectable between the normal CSF and OGD groups. Bcl-2 immunofluorescence intensity and the number of Bcl-2-positive cells were significantly higher in the HPC CSF group than in the OGD and normal CSF groups (P < 0.01, P < 0.05; Figure 3, Table 2).

For Bax, in the normal control group, only minor stellate fluorescence was visible, the fluorescence intensity was low, and only a few Bax-positive cells were detectable. The fluorescence intensity of Bax immunolabeling and the number of Bax-positive cells were significantly lower in the HPC CSF and normal CSF groups than in the OGD group (P < 0.05 or P < 0.01). Bax immunofluorescence intensity and the number of Bax-positive cells were significantly lower in the HPC CSF group than in the normal CSF group (P < 0.01; Figure 3, Table 2).

The Bcl-2/Bax ratio was significantly higher in the normal CSF and HPC CSF groups than in the OGD group (P < 0.01). The Bcl-2/Bax ratio was significantly greater in the HPC CSF group than in the normal CSF group (P < 0.01; Table 2).

Discussion
Following HPC (i.e., exposure to a short period of nonfatal hypoxia), cells acquire tolerance to subsequent longer-term ischemic/hypoxic injury (Vannucci et al., 1998; Cui et al., 2001; Lu et al., 2004; Lu et al., 2005; Gidday, 2006; Zhang et al., 2007a, b, 2008); however, the underlying mechanisms are complex and unclear (Vannucci et al., 1998; Cui et al., 2001; Lu et al., 2004; Lu et al., 2005; Gidday, 2006; Zhang et al., 2007a, b, 2008). HPC induces numerous changes, including changes in behavior and metabolism, cellular morphology, as well as neurochemical and molecular biological alterations (Vannucci et al., 1998; Cui et al., 2001; Lu et al., 2004; Lu et al., 2005; Gidday, 2006; Zhang et al., 2007a, b, 2008).

At present, HPC cannot be directly applied in the clinical setting. However, cerebrospinal fluid components can easily traverse the blood-brain barrier. A better understanding of the effects of CSF from animals given HPC may help identify factors that protect against hypoxic/ischemic injury.

In the present study, we found high levels of neuronal apoptosis after 1.5 hours of OGD. Consistent results were obtained using both flow cytometry and confocal laser scanning microscopy. Both normal CSF and CSF from animals given HPC had protective effects on neurons subjected to OGD. The number of apoptotic neurons was reduced in the normal CSF and HPC CSF groups compared with the OGD group. HPC CSF displayed a better neuroprotective effect than normal CSF.

We also sought to clarify the mechanisms mediating the neuroprotective effects of HPC CSF. We examined whether HPC CSF inhibits apoptosis, thereby enhancing cell survival after OGD. Bcl-2 and Bax are critical apoptotic proteins. Bcl-2 suppresses the opening of the mitochondrial permeability transition pore and the start of the apoptotic cascade by regulating caspase-activating factors, reducing intracellular oxidative stress, inhibiting intracellular calcium-activated DNA cleavage, blocking pro-apoptotic pathways, and by maintaining mitochondrial structure and function. In contrast, Bax, which is downstream of p53, is activated by apoptotic stimuli and binds to Bcl-2 on the mitochondrial membrane, forming a heterodimer. Bax counters the anti-apoptotic effects of Bcl-2, and Bax homodimers can directly initiate the apoptotic cascade (Ramalingam and Kim, 2014; Sharifi et al., 2014; Wang et al., 2014a, b; Wu et al., 2014; Yang et al., 2014; Zhao and He, 2015). The Bcl-2/Bax protein ratio determines the ultimate fate of cells. Apoptosis occurs when Bax is dominant, and cell survival is observed when Bcl-2 is dominant (Ramalingam and Kim, 2014; Sharifi et al., 2014; Wang et al., 2014a; Wang et al., 2014b; Wu et al., 2014; Yang et al., 2014; Zhao and He, 2015). We observed that Bcl-2 expression and the Bcl-2/Bax ratio were significantly higher in the HPC CSF group than in the OGD and normal CSF groups. Bax expression was significantly lower in the HPC CSF group than in the OGD and normal CSF groups.
**Table 1** Effects of HPC CSF on cultured cortical neuron apoptosis and survival

| Group            | Annexin-V-FITC (fluorescence intensity) | Propidium iodide (fluorescence intensity) | Survival rate (%) |
|------------------|----------------------------------------|------------------------------------------|-------------------|
| Normal control   | 0.06±0.01                              | 0.03±0.01                                | 74.34±3.52        |
| OGD              | 2.62±0.165                             | 2.98±0.16                                | 47.26±2.68        |
| Normal CSF       | 1.61±0.17**                            | 2.08±0.02**                               | 53.64±1.82**      |
| HPC CSF          | 0.68±0.08**##                          | 0.24±0.03**##                            | 62.28±2.74**      |

The data are expressed as the mean ± SD, and were analyzed with one-way analysis of variance and Dunnett’s two-tailed t-test. **P < 0.01, vs. OGD group; ##P < 0.01, vs. normal CSF group. OGD: Oxygen glucose deprivation; CSF: cerebrospinal fluid; HPC CSF: CSF from rats given hypoxic preconditioning. Experiments were performed six times.

**Table 2** Effect of HPC CSF on Bcl-2 and Bax expression in cultured cortical neurons

| Group            | Fluorescence intensity | Number of positive cells (/40-fold field) |
|------------------|------------------------|------------------------------------------|
|                  | Bcl-2                  | Bax                                      | Bcl-2/Bax         |
| Normal control   | 0.03±0.01              | 0.37±0.02                                | 4.45±0.34         |
| OGD              | 0.04±0.01              | 1.66±0.07                                | 8.26±2.58         |
| Normal CSF       | 0.64±0.04*             | 0.87±0.32†                               | 12.08±2.69        |
| HPC CSF          | 1.85±0.07**##          | 0.49±0.04**##                            | 16.48±4.68**      |

The data are expressed as the mean ± SD, and were analyzed with one-way analysis of variance and Dunnett’s two-tailed t-test. *P < 0.05, **P < 0.01, vs. OGD group; #P < 0.05, ##P < 0.01, vs. normal CSF group. OGD: Oxygen glucose deprivation; CSF: cerebrospinal fluid; HPC CSF: CSF from rats given hypoxic preconditioning. Experiments were performed six times.
Therefore, HPC CSF maintains mitochondrial structure and function, improves hypoxic tolerance, and inhibits apoptosis by upregulating Bcl-2 expression and downregulating Bax expression. HPC CSF may mediate neuroprotection via other mechanisms as well; however, further study is required to identify the neuroprotective factors and their mechanisms of action.

In this study, normal rat CSF was also found to have neuroprotective effects, possibly because it contains factors that enhance neuronal survival, such as nerve growth factor (Cui et al., 2001; Lehtinen et al., 2011; Niu et al., 2011). CSF likely contains numerous neuroprotective factors that easily traverse the blood-brain barrier. CSF also has minimal adverse effects, and protects against ischemic injury. CSF can be used to produce novel drugs that may have therapeutic potential for ischemic brain injury.

In summary, CSF from rats given HPC reduced injury to OGD-exposed cortical neurons and exhibited neuroprotective effects. The neuroprotective mechanism appears to involve the upregulation of Bcl-2 expression, the downregulation of Bax expression, and an increase in the Bcl-2/Bax ratio.

Author contributions: YBZ, ZDG, MYL, JZN, and MFY conceived, designed and performed the experiments. SJL, XMJ and GWL analyzed the data and wrote the paper. All authors conceived and designed the experiments, analyzed data, wrote the paper and approved the final version of the paper.

Conflicts of interest: None declared.

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