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Neurovascular protection conferred by 2-BFI treatment during rat cerebral ischemia

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ABSTRACT

Stroke is caused by vascular dysfunction and currently there are no effective therapeutics to stroke induced brain damage. In contrast to an intense emphasis on neuroprotection, relatively few studies have addressed means of vascular protection in cerebral ischemia. Here we discovered that the ligand to immidazolin receptor, 2-BFI, not only provided potent neuroprotection during middle cerebral artery occlusion in rat, which confirmed our previous reports, but also protected the integrity of the cerebral vasculature. Treatment with 2-BFI twice daily after the occlusion of the middle cerebral artery for 14 d significantly improved the neurological deficits, reduced brain infarction, and importantly, protected the cerebral vasculature as evidenced by the increased expression of an endothelial marker, von Willebrand factor, and better preservation of the cerebral vasculature, as viewed under a confocal microscope on rat brain perfused with FITC-labeled dextran. These results indicated that 2-BFI contributes to protection of neurovasculature. Understanding the molecular mechanisms could eventually lead to development of more effective therapies for stroke.

1. Introduction

Stroke is the third leading cause of death and disability in the developed world and is rapidly becoming the No. 1 killer in developing countries such as China [8,9,13,17]. However, stroke therapeutics still represent the largest unmet medical need. So far, apart from thrombolytic drugs, there are no effective treatment for stroke-induced brain damage in spite of tremendous progress made in the understanding of the fundamental mechanism of neuronal death caused by stroke [17].

Our recent studies showed that ligands to the type 2 immidazoline receptor (I_2R), such as 2-(2-benzofu-ranyl)-2-imidazoline (2-BFI) and Idazoxan, are potently neuroprotective against hypoxia–ischemia damage both *in vitro* and *in vivo* [4,12,20]. Possible mechanisms of neuroprotection by I_2R ligands are believed to be through ameliorating excitotoxicity by directly modulating NMDA receptor-mediated calcium influx in neurons [12]. I_2R ligands directly bind to NMDA receptors [18] and may block NMDA receptor-gated calcium channels [3,15,16]. Amongst I_2R ligands, 2-BFI was found to be the most effective in neuroprotection against glutamate toxicity *in vitro* [12] and cerebral ischemia *in vivo* [4,20]. Our recent studies demonstrated that 2-BFI not only transiently and reversibly blocked calcium influx in cultured cortical neurons in response to glutamate-mediated excitotoxicity, but also provided a much longer lasting neuroprotection against glutamate toxicity in comparison with other I_2R ligands, such as idazoxan [12].

There are three types of imidazoline receptors based on imidazoline binding sites: I_1 site is labeled by clonidine and the I_2 site is labeled by idazoxan and other selective molecules such as 2-BFI. A putative I_3 site has also been described [6]. I_1R, found in the rostral ventrolateral medulla, mediates the sympathoinhibitory actions of imidazolines to lower blood pressure; I_2R represents an allosteric binding site of monoamine oxidase, while I_3R is known to regulate insulin secretion from pancreatic beta cells. So far only ligands to I_2R, such as 2-BFI and idazoxan, have been found to prevent ischemia-hypoxia induced brain damage both *in vitro* and *in vivo* [3,12,15,18].

The neurovascular unit comprises of a framework that integrates responses in all cell types, such as neurons and vascular cells [2]. It has come to the realization that therapies must not only protect neurons, but also the neurovascular unit as a whole in order to preserve the proper function of neurons [17]. In contrast to intense emphasis on neuroprotection, relatively few studies have

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addressed the means of vascular protection in cerebral ischemia. In this study, we tested the possibility of whether 2-BFI has a therapeutic protective effect not only on neurons, but also the vasculature. 2-BFI was administered only after the onset of stroke. We found that 2-BFI was not only potently neuroprotective, but also preserved the integrity of the cerebral vasculature. 2-BFI and its derivatives are promising candidates for further development as a therapeutic drug for stroke.

2. Materials and methods

2.1. Experimental reagents

2-BFI was purchased from Tocris (Bristol, Avon, UK) and 2,3,5-triphenyltetrazolium chloride (TTC) was from Sigma Co. (St Louise, MA, USA). A primary polyclonal antibody against von Willebrand factor was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and FITC-labeled dextran was purchased from Sigma Co. (USA). An immunohistochemistry kit was purchased from Bio Co., Ltd. (Beijing, China).

2.2. Experimental animals

All animal procedures were conducted following an institutionally approved protocol in accordance with guidelines set by the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize suffering and to reduce the number of animals used. Healthy male Sprague–Dawley rats (3–4 months old at 280–300 g body weight) were obtained from the Experimental Animal Center of Wenzhou Medical College. All animals were fed with food and water *ad librum* and housed under a 12 h per day light–dark cycle. Rats were separated into a sham-operated group ($n = 18$), a MCAO group ($n = 36$) which was further randomly separated into a MCAO only group ($n = 18$) and 2-BFI-treated group ($n = 18$).

2.3. Middle cerebral artery occlusion (MCAO) in rat

Procedures for transient occlusion of the middle cerebral artery were as previously described [10,14]. Briefly, rats were anesthetized with 10% chloral hydrate. After a midline incision in the neck, the right external carotid artery was carefully exposed and dissected. A monofilament silicon-coated nylon suture was inserted from the external carotid artery to the right internal carotid artery (18 ± 0.5 mm) to occlude the origin of the right middle cerebral artery. After 120 min of occlusion, the thread was removed to allow for reperfusion. The external carotid artery was ligated and the wound was closed. Sham-operated rats ($n = 18$) underwent an identical surgery except that the suture was not inserted. The body temperature of rat was maintained at 37.0 ± 0.5 °C using a heating pad.

Immediately following MCAO, rats were randomly divided into a MCAO only group ($n = 18$), which received saline injection i.p. and a 2-BFI treatment group ($n = 18$), which were injected with 2-BFI i.p. at 1.5 mg/kg twice per day for seven days. At the end of the treatment, rats were anesthetized and perfused through the heart with 4% paraformaldehyde in phosphate buffered saline (PBS). The brains were then removed for brain infarction quantification and tissue fixation for histology.

2.4. Measurement of the neurological dysfunction

A neurological examination was performed at 0 h after MCAO to determine a similar loss of neurological function before randomly separating them into different groups. All MCAO rats showed turning towards the right side and an inability to put forward the right forepaw. The neurological deficits were subsequently measured on days 1, 3, 7 and 14 after reperfusion. The neurological deficits were scored on a five-point scale modified from those previously described by Longa et al. [14]: 0: no deficit; 1: failure to extend left forepaw fully; 2: circling to the left; 3: falling to the left; 4: no spontaneous walking with a depressed level of consciousness.

2.5. Measurement of infarct volume

At the desired reperfusion time point, rats were anesthetized and killed. Brains were removed and cut into 2 mm thick coronal slices. Sections were immersed into a 2% TTC solution and incubated with TTC for 30 min at 37 °C before fixation with 4% paraformaldehyde. The brain slices were evaluated by an investigator blinded to treatment. Infarcted areas were visualized as regions lacking the typical brick-red staining of normal brain tissue. These areas were quantified with a computer-assisted program, Image-Pro Plus (V6.0). The uncorrected infarct area and the total areas of both hemispheres were calculated for each coronal slice. The corrected infarct area in slices was calculated to compensate for brain edema. Corrected infarct volumes were calculated by multiplying the corrected area by the slice thickness and summing the volume [7,11].

2.6. Immunohistochemistry

Immunostaining was performed on brain sections using a streptavidin–peroxidase Histostain-Plus Kit (Zymed Laboratories, South San Francisco, CA, USA) according to the manufacturer's instructions [7,11]. In brief, endogenous peroxidase activity was blocked by a 30 min incubation at room temperature in 1% hydrogen peroxide. After several washes with phosphate buffered saline, sections were incubated in a blocking solution (2% goat serum, 0.1% Triton X-100, 1% bovine serum albumin in PBS) for 1 h at room temperature. The sections were then incubated with rabbit anti-von Willebrand factor (vWF) at a 1:200 dilution at 37 °C for 3 h. Sections were then washed with PBS and incubated with a biotinylated goat anti-rabbit secondary antibody (1:1000) for 1 h at room temperature. An avidin–biotin complex and diaminobenzidine (DAB) were used to obtain a visible reaction product. Sections were dehydrated and sealed under a coverslip. A Leica microscope equipped with a digital camera was used for examination and photography of the slides, respectively.

2.7. Visualization of cerebral microvessels using confocal microscopy

After 14 d of reperfusion, rats from each treatment group ($n = 6$ from each group) were anesthetized and injected through the tail vein 1 ml of 5% FITC-labeled dextran. Rats were killed after 10 min and brains were fixed in 4% freshly prepared paraformaldehyde for 48 h. Coronal brain sections at 60 μm were cut and examined under a confocal microscope. Images were taken under a 200× magnification with high resolution (1340 × 1340 dpi) and processed using Image Pro plus (V6.0). The fluorescent area from the area of interests was measured and calculated to reflect the regional cerebral vessel integrity.

2.8. Statistical analysis

All values were recorded using Microsoft Excel and graphs were generated using Prism 5.0. Data is presented as the mean ± standard errors (SEM). A comparison between two groups was performed using the Student's *t*-test and a comparison among multiple groups was performed using one-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* test. $p < 0.05$ is considered statistically significant.

3. Results

3.1. 2-BFI protects the brain against MCAO

Rats were subjected to 2 h MCAO followed by injection of 2-BFI twice per day at 2 h, 24 h (1 d), 36 h (3 d), 84 h (7 d) and 168 h (14 d) after reperfusion. Significant improvement in neurological deficits occurred in the 2-BFI treated group just after 24 h of reperfusion when compared with the MCAO only group (Fig. 1A; $p < 0.05$, $n = 8$). Remarkably, rats treated with 2-BFI at all time points, including 1 d, 3 d, 7 d, and 14 d, showed significant improvement in neurological deficit when compared with the MCAO only group. Fig. 1B showed TTC staining of rat brains after 3 d reperfusion, highlighting reduced infarct size in the brain. The MCAO only group after 3 d reperfusion had an average infarct size of 45.4% ± 4.5 compared with 29.6% ± 7.3 in the 2-BFI treated group ($n = 6$, $p < 0.05$), as shown in Fig. 1C. An histological examination was also performed using hematoxylin and eosin (H and E) staining (Fig. 1D) showing reduced brain damage in the 2-BFI treated group (arrows indicate healthy neurons).

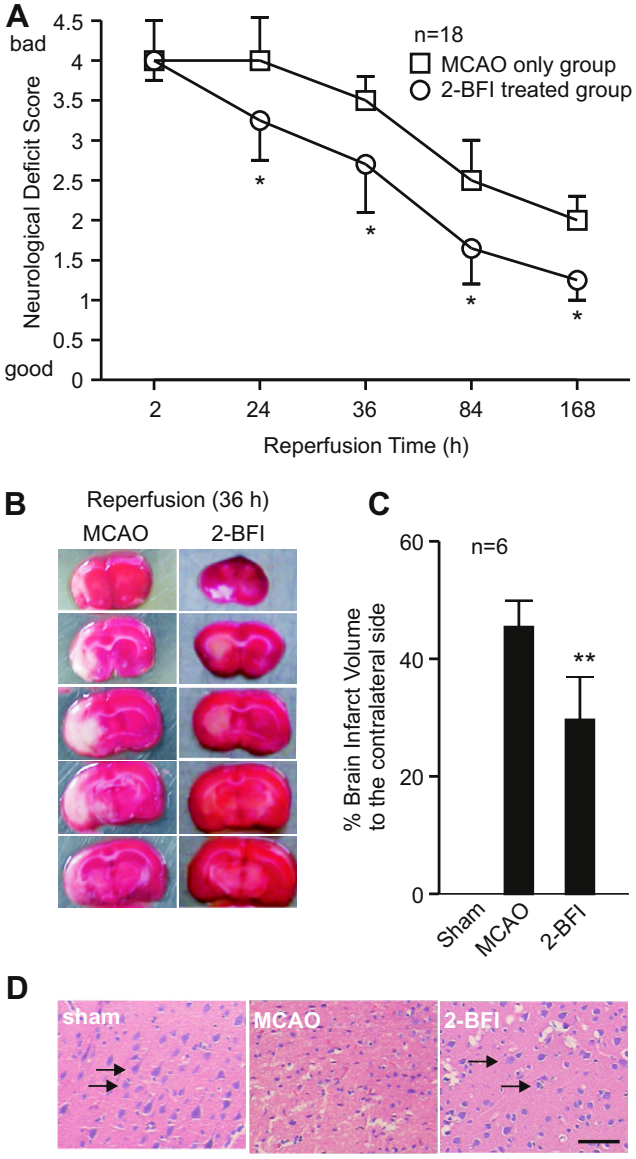

Fig. 1. Improvement in neurological function and reduction in brain infarction size following MCAO in rats treated with 2-BFI. After 2 h reperfusion, MCAO-induced neurological deficits in rats were evaluated as described in the Section 2 (A). Rats showed a clear sign of turning and an inability to put forward the opposite side of the forepaw. These rats were grouped and either treated with 2-BFI (2-BFI-treated group) or left untreated (MCAO only group). 2-BFI was given twice per day for 7 d at 1.5 mg/kg. A significant improvement in neurological function occurred in the 2-BFI treated rats at all the time points examined. TTC staining was performed on rat brains treated with or without 2-BFI after 36 h reperfusion (B). The infarct volume was measured and plotted in panel C. Brains were also embedded in paraffin and sectioned for H and E staining as shown in D. Arrows indicate healthy neurons in the brain. Scale bar = 50 μm. Data represents the mean ± SEM. * in (A) and ** in (D) indicates a statistical significant difference with $p < 0.05$ and $p < 0.01$, respectively, by Student's *t*-test.

3.2. Treatment with 2-BFI enhanced von Willebrand factor (vWF) expression

vWF is a large multimeric glycoprotein present in blood plasma and produced constitutively in endothelium. Staining of this factor reflects the integrity of the microvessel system in the brain. As shown in Fig. 2A (and inset A′), sham-operated rat brain expressed vWF along the vessel walls and in very small vessels. Following MCAO (7 d post reperfusion), a significant reduction in the number of vWF positive cells occurred (Fig. 2B and D). In contrast to the MCAO only brain, 2-BFI treatment significantly preserved the number of vWF positive cells (Fig. 2C and D), indicating 2-BFI protected the cerebrovasculature. The number of vWF positive cells in the brain were counted in a 20× microscopic field and presented in Fig. 2D which demonstrated significant reduction of vWF positive cells in the MCAO only group ($n = 4$, $p < 0.05$).

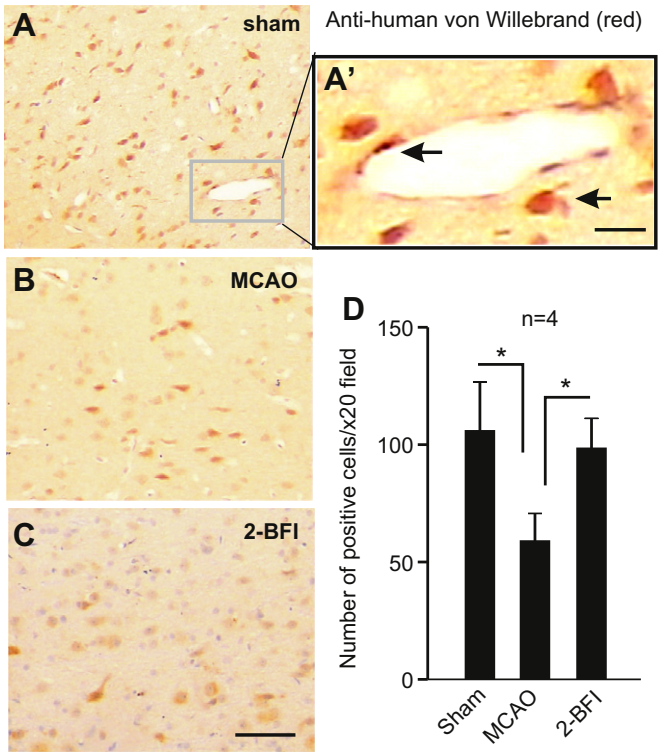

Fig. 2. Immunohistochemical evaluation of von Willebrand factor expression in the MCAO brain. At the end of 7 d treatment with 2-BFI, rats were killed and the brains were removed for paraffin embedding and tissue sectioning as described in the Section 2. Immunohistochemical staining using a primary antibody to vWF showed positive staining of endothelial cells in the sham-operated rat brains (A and A′; arrows indicate positive cells). The number of cells expressing vWF was significantly reduced in the MCAO only rat brain (B and D). In contrast, 2-BFI treatment preserved vWF expression in the rat brain (C and D). Scale bar = 50 μm. The number of vWF positive cells were counted from randomly selected 20× microscopic fields and plotted in D. Data represents the mean ± S.E.M. * indicates a statistical significant difference with $p < 0.05$ by one-way ANOVA and a Tukey's *post hoc* test for significant groups.

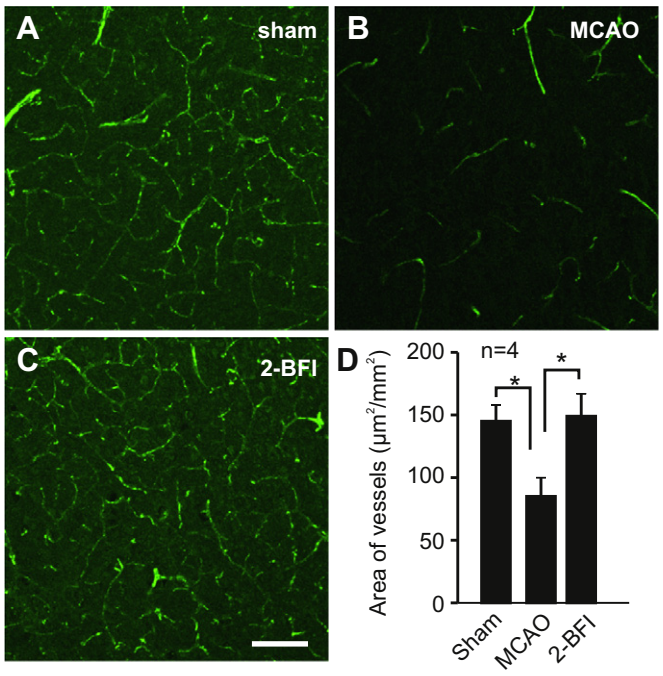

Fig. 3. 2-BFI treatment preserved functional cerebral vasculature after MCAO in rats. After 14 d reperfusion, MCAO rats were injected with FITC-dextran and killed for brain collection. Thick brain coronal sections (60 μm) were cut and examined under a confocal microscope to determine microvessel density and integrity (A–C). This method allowed for the determination of functional microvessels. MCAO brain clearly showed a significant reduction in microvessel density (B and D), while treatment with 2-BFI preserved the functional microvessels. Scale bar = 100 μm. Data represents the mean ± SEM * indicates a statistical significant difference with $p < 0.05$ by one-way ANOVA and a Tukey's *post hoc* test.

3.3. Preservation of functional microvessels in 2-BFI treated brain

In order to further demonstrate that treatment with 2-BFI protected microvessels from MCAO induced damage, FITC-dextran was infused in the cerebral circulation. Brain slices were examined under a confocal microscope. As shown in Fig. 3A, sham-operated rat brain has dense FITC-dextran filled microvessels. In contrast, MCAO at 14 d reperfusion showed a clear reduction in the density of functional microvessels (Fig. 3B). Interestingly, treatment with 2-BFI clearly preserved the integrity and density of functional microvessels (Fig. 3C). The density of the microvessels was measured as described in the Methods section and shown in Fig. 3D, which illustrated a significant reduction in the density of FITC-dextran positive vessels in the MCAO only group in comparison with the sham-operated and 2-BFI treated groups. The group treated with 2-BFI showed a density of 154.4 ± 10.7 $\mu m^2/mm^2$ in comparison to MCAO only group which had a density of 92 ± 6.2 $\mu m^2/mm^2$ ($p < 0.05$).

Collectively, these data demonstrated that 2-BFI given after MCAO was effective in protecting the brain from MCAO-induced damage, possibly through protecting the integrity of the cerebral vasculature.

4. Discussion

In the present study, we investigated the protective effect of 2-BFI on the cerebral vasculature. We found that administering 2-BFI after cerebral ischemia significantly protected both neurons and cerebral vasculature. The results clearly indicted that giving 2-BFI during different reperfusion times was effective in providing long-term protection of the brain. The protective mechanism may not only be through a direct protection against neuronal death as we have previously reported [4,5], but importantly, through an indirect way by protecting the cerebral vasculature.

Our previous study has shown that 2-BFI at 1.5 mg/kg represents the optimal dose for neuroprotection against MCAO-induced neuronal damage [5]. The present study confirmed that this dosage was effective in brain protection, but also extended this observation to show that giving this dosage of 2-BFI for 7 d after MCAO was also effective in brain protection. The possible molecular mechanism of neuroprotection conferred by 2-BFI may be through a direct blockade of NMDA receptors, similar to what we have shown that 2-BFI can transiently reduce glutamate-induced calcium entry through NMDA receptors in a relatively fast on-and-off fashion [12]. However, blocking glutamate excitotoxicity could not account, in an obvious way, for the death of non-neuronal cells, such as astrocytes, vascular cells, or microglia in ischemic tissue. The glutamate hypothesis may have oversimplified the complexity of the cell death process and underestimated the diversity of expressing cell types as well as the heterogeneity of glutamate responses following receptor over-stimulation in stroke [1,17].

To explore other possible ways that 2-BFI protected the brain during long-term reperfusion, we examined the expression of vWF and the functionality of cerebral microvessels using a FITC-dextran perfusion test. The results showed clear preservation of microvasculature integrity in 2-BFI treated rats. Although it is not possible at this time to determine whether 2-BFI has a direct target on vascular endothelial cells, it is a plausible idea that 2-BFI protected neurons through reducing vascular damage and promoting vascular genesis after stroke. These data indicated that 2-BFI may have multiple targets on multiple cell types. Indeed, we have shown recently that 2-BFI was effective in suppression of inflammation-induced brain and spinal cord injury through suppression of microglia activation, mitochondrial changes and immune system response [19–21]. Collectively, these studies strongly suggest that 2-BFI is an excellent candidate for development as a therapeutic drug for stroke.

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