2-(2-Benzofuranyl)-2-Imidazoline Mediates Neuroprotection by Regulating the Neurovascular Unit Integrity in a Rat Model of Focal Cerebral Ischemia

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Background: We showed previously that 2-(2-benzofuranyl)-2-imidazoline (2-BFI), a ligand to type 2 imidazoline receptor (I2R) exerts neuroprotective effects in ischemia stroke via an unknown mechanism. The present study was to investigate whether 2-BFI can protect the neurovascular unit (NVU) using a rat model of 90 min focal cerebral ischemia. *Methods:* Rats were randomly divided into three groups: thesham-operated group; the vehicle control group and the 2-BFI group which received 2-BFI (3 mg/kg) immediately after the start of middle cerebralartery occlusion (MCAO). Neurological deficit score, infarct size, apoptosis level, brain water content and Evans Blue extravasation were assessed at 24 h after stroke. Expressions of occludin and zonula occludens 1 (ZO-1), collagen IV, aquaporin-4 (AQP-4), matrix metalloproteinase-9 (MMP-9) and MMP-2 were assessed by Western blotting. *Results:* 2-BFI treatment was associated with significant improvement of neurological performance and decreased infarct volume at 24 h after stroke. Apoptosis level reduced significantly by 2-BFI compared to the vehicle group ($34.3 \pm 5.4\%$ vs $56.1 \pm 7.9\%$, $p < 0.05$). Significant decreased of brain water content ($79.5 \pm 2.6\%$ vs $84.62 \pm 2\%$, $p < 0.05$) and Evans Blue extravasation (1.2 ± 0.5 vs 2.5 ± 0.41 μg/g, $p < 0.05$) of ipsilateral hemisphere was observed in 2-BFI group compared to vehicle group. Expressions of occludin, ZO-1 and collagen IV were significantly higher while MMP-9 level significantly lower in 2-BFI group. AQP-4 and MMP-2 showed no difference between 2-BFI and the vehicle groups. *Conclusions:* These results suggest that the neuroprotective effects of 2-BFI in acute ischemic brain damage are at least partly due to the drug's ability to improve the functions of NVU.
Key Words: Focal ischemia—2-(2-benzofuranyl)-2-imidazoline—neurovascular unit—blood-brain barrier—neuroprotection.

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Introduction

Ischemic stroke is a major cause of death and long-term disability worldwide. Thrombolysis with recombinant tissue plasminogen activator is so far the only proven and effective treatment for acute ischemic stroke, but it is accessible to only a fraction of stroke patients because of strict selection criteria. For this reason, researchers have been trying for decades to develop neuroprotective strategies to mitigate or eliminate different aspects of ischemia injury.[1]

One of the drivers of ischemia-induced damage is excitotoxicity, which caused by glutamate binding to N-methyl-D-aspartate receptors (NMDARs), leading to excessive Ca^{2+} influx, which triggers a plethora of downstream signaling pathways and ultimately programmed neuronal death. Pharmacological inhibition of NMDAR can ameliorate excitotoxicity-mediated neuronal death and protect the brain after cerebral ischemia. However, in the clinic, NMDAR antagonists have failed to yield the expected benefits in stroke patients.[2,3] This is largely because of side effects arising from blocking glutamate, which plays far-reaching, essential roles in neural development, excitatory synaptic transmission, and plasticity.[4] These failures have led to the hypothesis that drugs that block NMDAR transiently and reversibly may ameliorate excitotoxicity-evoked brain damage and may be well tolerated.[5] One such candidate may be 2-(2-benzofuranyl)-2-imidazoline (2-BFI), a ligand of the type 2 imidazoline receptor (I_2R), which our previous work has shown can protect the brain from transient ischemia injury in a rat model.[6,7] 2-BFI blocks NMDAR reversibly and noncompetitively, similarly to the noncompetitive NMDAR antagonist memantine.[8] Our previous work suggests that the neuroprotective effects of 2-BFI are due in part to its ability to protect endothelial cells besides protecting neurons from apoptosis,[9] but the detailed mechanisms by which 2-BFI protects the brain from ischemia remain unknown.

During the last decade, our understanding of stroke pathophysiology has substantially improved. Instead of focusing strictly on neurons, studies of stroke injury have begun to consider the wider environment of neurons and their interaction with neighboring glial cells and the endothelium, which together make up the neurovascular unit (NVU).[10] Memantine protects the NVU by suppressing activation of matrix metalloproteinase (MMP)-9, which is a collagenase that degrades the extracellular matrix.[11]

We hypothesized that the neuroprotective effects of 2-BFI in ischemic brain are due to its ability to preserve NVU integrity. Using a rat model of transient ischemia, we examined the effects of 2-BFI on apoptosis in neurons in the penumbra area, as well as on blood brain barrier (BBB) integrity. We assessed BBB integrity in terms of water content, extravasation of Evans blue dye, and expression of proteins related to BBB permeability, including occludin, zonula occludens 1 (ZO-1), aquaporin-4 (AQP-4), and collagen IV, as well as the enzymes MMP-9 and MMP-2.[12]

Materials and Methods

Middle Cerebral Artery Occlusion (MCAO) in Rats

Animal experiments were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University and were performed in accordance with the principles outlined in the U.S. National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

Temporary focal ischemia was induced in adult male Sprague–Dawley rats weighing 250-280 g using the intraluminal vascular occlusion method as previously described,[13] with minor modifications. After 90 minutes of MCAO, the suture was removed to restore blood flow. Sham-operated rats were manipulated in the same way, but the middle cerebral artery was not occluded. All surgical procedures were performed under an operating stereomicroscope, and rectal temperature was monitored and maintained at 37.5°C using a thermostatically controlled heating pad. Rat brains were removed at 24 hours after surgery.

Rats were randomly divided into 3 groups: a sham-operated group; a saline control group, in which saline (3 mL/kg) was given immediately after the start of MCAO; and a 2-BFI group, which received 2-BFI (3 mg/kg; Tocris, Bristol, United Kingdom) immediately after the start of MCA occlusion. Our previous studies showed this dose of 2-BFI to be optimal (data were not shown). Both saline and 2-BFI were administered through the femoral vein. Researchers performing procedures were blinded to whether they were administering saline or 2-BFI.

Neurological Deficit Testing

At 24 hours after surgery, the animals were blindly evaluated using 2 motor testing paradigms: the Longa test[13] and the beam balance performance test.[14] Higher scores on these tests indicate more serious deficits. Rats were trained for the beam balance test 3 days before the trial. Results from neurological testing were analyzed blindly.

Infarct Volume

At 24 hours after surgery, the rats were euthanized with 10% chloral hydrate (500 mg/kg, intraperitoneal). After euthanasia, coronal brain sections 2 mm thick were removed and stained with a 4% solution of 2,3,4-triphenytetrazolium-chloride (Sigma, St. Louis, MO) at 37°C for 20 minutes. The infarct area of each section was measured using ImageJ software (http://www.uhnresearch.ca/facilities/wcif/download.php). To minimize edema-induced error, the infarct volume measured