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Research report

Protective effects of 2-(2-benzonfuranyl)-2-imidazoline combined with tissue plasminogen activator after embolic stroke in rats

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HIGHLIGHTS

- 2-BFI (0.5 h) combined with rt-PA (6 h) can reduce infarct damages in eMCAO rats.
- 2-BFI (0.5 h) combined with rt-PA (6 h) can decrease apoptosis cells in eMCAO rats.
- 2-BFI (0.5 h) combined with rt-PA (6 h) can inhibit BAX/BCL-2 levels in eMCAO rats.

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ABSTRACT

Stroke is the third leading cause of death and disability in developing countries. The effective therapy for acute ischemic stroke is thrombolysis with recombinant tissue plasminogen activator (rt-PA) within 4.5 h of stroke onset. An effective post-ischemic neuroprotectant would extend the advantages of rt-PA, and protect against complications of thrombolysis. We previously reported that 2-(2-benzofuranyl)-2-imidazoline (2-BFI), a newly discovered ligand for high-affinity type 2 imidazoline receptor (I2R), provides neuroprotection against ischemic stroke in rats. Here we investigated the protective effects of 2-BFI in combination with delayed intravenous rt-PA after stroke induced by embolic middle cerebral artery occlusion (eMCAO) in rats. Infarct size was determined using 2,3,5-triphenyltrazolium chloride staining, while neurological deficit was assessed based on neurological score. Numbers of apoptotic cells *in vivo* were estimated using TUNEL stain, and expression of the pro-apoptotic protein BAX and anti-apoptotic protein BCL-2 were quantified by Western blotting. The results showed that 2-BFI (3 mg/kg) administered at 0.5 h after embolic MCAO combined with rt-PA (10 mg/kg) administered at 6 h reduced brain infarct size, mitigated neurological deficit, decreased the number of TUNEL-positive cells, down-regulated BAX expression, and up-regulated BCL-2 expression. These findings suggest that 2-BFI may extend the therapeutic window of rt-PA to 6 h after embolic stroke onset in rats.

1. Introduction

Stroke is a major public health problem in developing countries, especially China. It is often associated with death and long-term disability, and it places an enormous economic burden on families and societies (Abilleira et al., 2011; Feigin, 2005; Mukherjee and Patil, 2011; Towfighi and Saver, 2011). Therefore, it is essential to develop effective strategies to reduce stroke-related mortality and improve recovery.

Current therapies for ischemic stroke focus on thrombectomy, thrombolysis, and neuroprotection (Berkhemer et al., 2015a). Recombinant tissue plasminogen activator (rt-PA) has proven effective in several large clinical trials as a thrombolysis treatment after cerebral infarction (Berkhemer et al., 2015b; Goyal et al., 2015), and it was approved by the US Food and Drug Administration in 1996 (Lapchak, 2013). At most medical centers, the first choice for thrombolytic therapy to improve recovery from ischemic stroke is to administer rt-PA within 4.5 h of the event (Hacke et al., 2008; Liao et al., 2013). Therapy with rt-PA is effective only when administered within a short window after the event, and its use is associated with various complications including intracerebral hemorrhage, brain edema and disruption of the blood brain barrier (BBB) (Blinzler et al., 2011; Delgado et al., 2010;

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Niego and Medcalf, 2014). Risk of these adverse events has led researchers to explore neuroprotective strategies to mitigate or eliminate ischemia injury and extend the effective therapeutic window for rt-PA.

Neuroprotection to mitigate effects of ischemic stroke can be achieved using 2-(2-benzofuranyl)-2-imidazoline (2-BFI), which binds to one type of imidazoline receptor (IR). First reported in 1984 (Bousquet et al., 1984), three types of IR have been defined (Head and Mayorov, 2006). I_1R, which can be labeled specifically using clonidine, is expressed mainly in the rostral ventrolateral medulla and lowers blood pressure. I_2R, which can be labeled using idazoxan, is expressed widely in the cardiovascular and nervous systems, as well as in the kidney, and it offers an allosteric binding site for monoamine oxidase. I_3R can regulate insulin secretion of pancreatic beta cells (Dong et al., 2008; Head and Mayorov, 2006; Jiang et al., 2010; Olmos et al., 1996).

2-BFI is a newly discovered high-affinity I_2R ligand that protects against glutamate toxicity *in vitro* (Jiang et al., 2010). It inhibits excessive influx of Ca^{2+}, mitigating excitotoxicity-mediated neuronal death and cerebral ischemia *in vivo* (Han et al., 2009). Our previous study demonstrated that 2-BFI blocks the *N*-methyl-D-aspartate receptor (NMDAR) reversibly and noncompetitively, similarly to the non-competitive NMDAR antagonist memantine (Han et al., 2013). In this way, 2-BFI regulates neurovascular unit integrity in a rat model of cerebral ischemia (Zhang et al., 2018). In fact, the neuroprotective effects of 2-BFI appear not to be limited to ischemic stroke: in a rat model of autoimmune encephalomyelitis, 2-BFI decreases the expression of neuronal injury markers such as inflammatory cytokines, apoptosis-inducing factor (AIF), and amyloid beta (Zhu et al., 2015). 2-BFI also reduces expression of the pro-apoptotic protein BAX and increases expression of the anti-apoptotic protein BCL-2 following cerebral ischemia (Han et al., 2010). BAX, a pore-forming cytoplasmic protein of the BCL-2 family, translocates to the outer mitochondrial membrane and affects permeability between the intermembrane space and cytosol, contributing to cell death (Yu et al., 2011). Anti-apoptotic BCL-2, also of the BCL-2 family, lies in the cytoplasmic side of the outer mitochondrial membrane, nuclear envelope, and endoplasmic reticulum. It regulates the release of apoptogenic cytochrome *c* and blocks translocation of AIF from the mitochondria to the nucleus (Chiou et al., 2006). These results suggest that 2-BFI may protect against brain injury arising from ischemic stroke.

Therefore we tested here whether combining the neuroprotective effects of 2-BFI with the well-established thrombolytic drug rt-PA could improve outcomes in rats subjected to embolic middle cerebral artery occlusion (eMCAO). We also examined whether this combination would be effective over a longer therapeutic window than rt-PA alone. Few studies have examined the combination of 2-BFI and rt-PA, which deserves greater consideration because of the urgent need to improve stroke treatments.

2. Results

2.1. 2-BFI combined with rt-PA protects the brain against eMCAO

eMCAO produced large infarction areas on the ipsilateral side of the brain that were not observed in sham-operated animals. The smallest infarction areas were observed in rats treated with 2-BFI (0.5 h) combined with rt-PA (6 h) (Fig. 1A). 2-BFI (0.5 h) led to smaller infarct area than most other treatments, whereas rt-PA (6 h) led to greater infarct area than most other treatments. 2-BFI (0.5 h) with rt-PA (6 h) led to significantly smaller infarct volumes than 2-BFI alone, rt-PA alone or the combination of 2-BFI (0.5 h) and rt-PA (8 h). Timing of rt-PA administration was key: 2-BFI (0.5 h) combined with rt-PA (8 h) led to infarct areas similar to those in animals treated with 2-BFI (0.5 h) alone (Fig. 1B).

Consistent with these results suggesting less severe brain injury with 2-BFI than with rt-PA, eMCAO rats treated with 2-BFI (0.5 h) combined with rt-PA (6 h) showed the best recovery of neurological function. Neurological scores were similar between animals treated with 2-BFI (0.5 h) alone or combined with rt-PA (8 h).

These results suggest that of the various treatments we tested, 2-BFI (0.5 h) combined with rt-PA (6 h) can optimally protect the brain against eMCAO damage in rats.

2.2. 2-BFI combined with rt-PA reduces brain cell apoptosis induced by eMCAO

In order to determine whether the observed neuroprotective effects of 2-BFI combined with rt-PA reflect reduced apoptosis, brain tissues from the different treatment groups were TUNEL-stained. Rats subjected to eMCAO showed a large number of apoptotic cells on the ipsilateral side of the cortex, which was not observed in sham-operated rats. 2-BFI (0.5 h) with rt-PA (6 h) markedly decreased cell apoptosis after eMCAO (Fig. 2A). On its own, 2-BFI (0.5 h) effectively reduced the number of TUNEL-positive cells on the ischemic side of the brain, but rt-PA (6 h) appeared to increase the number of TUNEL-positive cells. Significantly fewer positive cells were observed in animals treated with the combination of 2-BFI (0.5 h) and rt-PA (6 h) than in those treated with 2-BFI alone, rt-PA alone or the combination of 2-BFI (0.5 h) and rt-PA (8 h). Similar numbers of positive cells were observed in animals treated with 2-BFI (0.5 h) alone or with rt-PA (8 h) (Fig. 2B). These results strongly suggest that 2-BFI (0.5 h) combined with rt-PA (6 h) can optimally inhibit eMCAO-induced apoptosis in rat brain.

2.3. 2-BFI combined with rt-PA down-regulates BAX expression in ischemic brain

To begin to explore how 2-BFI combined with rt-PA may inhibit apoptosis in eMCAO brains, brain lysates from the different treatment groups were assayed for the pro-apoptotic protein BAX using Western blotting. eMCAO up-regulated BAX expression, which the combination of 2-BFI (0.5 h) and rt-PA (6 h) dramatically inhibited (Fig. 3A). While 2-BFI (0.5 h) reduced BAX expression on the ischemic side of the brain, rt-PA (6 h) increased BAX levels. BAX levels were significantly lower in animals treated with the combination of 2-BFI (0.5 h) and rt-PA (6 h) than in those treated with 2-BFI alone, rt-PA alone or the combination of 2-BFI (0.5 h) and rt-PA (8 h). BAX expression was higher in animals treated with the combination of 2-BFI (0.5 h) and rt-PA (8 h) than in those treated with 2-BFI (0.5 h) alone (Fig. 3B). These results suggest that the neuroprotective effects of the combination of 2-BFI and rt-PA are due at least in part to inhibition of eMCAO-induced apoptosis, and that the optimal treatment in our experiments was 2-BFI (0.5 h) combined with rt-PA (6 h).

2.4. 2-BFI combined with rt-PA up-regulates BCL-2 expression in ischemic brain

To further explore how 2-BFI combined with rt-PA may inhibit apoptosis in eMCAO brains, brain lysates from the different treatment groups were assayed for the anti-apoptotic protein BCL-2 using Western blotting. eMCAO obviously decreased BCL-2 expression in the ischemic area, and this effect was reversed by the combination of 2-BFI (0.5 h) and rt-PA (6 h) (Fig. 4A). While 2-BFI (0.5 h) inhibited BCL-2 down-regulation in ischemic areas of the brain, rt-PA (6 h) further down-regulated BCL-2. BCL-2 expression was significantly higher in animals treated with the combination of 2-BFI (0.5 h) and rt-PA (6 h) than in those treated with 2-BFI alone, rt-PA alone or the combination of 2-BFI (0.5 h) and rt-PA (8 h). BCL-2 expression was similar in animals treated with 2-BFI (0.5 h) alone or combined with rt-PA (8 h) (Fig. 3B). These results further support that the combination of 2-BFI and rt-PA exerts neuroprotective effects at least in part by inhibiting eMCAO-induced apoptosis, and that the optimal treatment in our experiments was 2-BFI (0.5 h) combined with rt-PA (6 h).