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Brain Research

Research Report

Neuroprotective effects of cilostazol are mediated by multiple mechanisms in a mouse model of permanent focal ischemia

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ARTICLE INFO

Article history:
Accepted 12 January 2015
Available online 21 January 2015

Keywords:
Permanent focal ischemia
Cilostazol
Pleiotropic effect
Oxidative stress
NADPH oxidase 2

ABSTRACT

The phosphodiesterase (PDE) 3 inhibitor cilostazol, used as an anti-platelet drug, reportedly can also ameliorate ischemic brain injury. Here, we investigated the effects of cilostazol in a permanent focal ischemia mice model. Male Balb/c mice were subjected to permanent middle cerebral artery occlusion. Mice were then treated with either cilostazol (10 or 20 mg/kg) or vehicle administered at 30 min and 24 h post-ischemia, and infarct volumes were assessed at 48 h post-ischemia. Mice treated with 20 mg/kg of cilostazol or vehicle were sacrificed at 6 h or 24 h post-ischemia and immunohistochemistry was used for brain sections. Treatment with 20 mg/kg of cilostazol significantly reduced infarct volumes to 70.1% of those with vehicle treatment. Immunohistochemistry results for 8-hydroxydeoxyguanosine (OHdG) expression showed that some neurons underwent oxidative stress around the ischemic boundary zone at 6 h post-ischemia. Cilostazol treatment significantly reduced the percentage of 8-OHdG-positive neurons (65.8±33.5% with vehicle and 21.3±9.9% with cilostazol). Moreover, NADPH oxidase (NOX) 2-positive neurons were significantly reduced with cilostazol treatment. In contrast, immunohistochemistry results for phosphorylated cyclic-AMP response element binding protein (pCREB) showed that there were significantly more pCREB-positive neurons around the ischemic boundary zone of cilostazol-treated mice than in those of vehicle-treated mice at 24 h post-ischemia. These results suggested that cilostazol might have multiple mechanisms of action to ameliorate ischemic tissue damage, by attenuating oxidative stress mediated by suppressing NOX2 expression by ischemic neurons and an anti-apoptotic effect mediated through the pCREB pathway.

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http://dx.doi.org/10.1016/j.brainres.2015.01.022
0006-8993/

1. Introduction

Stroke remains a leading cause of death and disability; nevertheless, despite intensive research, few treatment options are available. Numerous neuroprotective drugs have shown varying degrees of effectiveness in animal stroke models, but none of these have been found to improve the outcomes of patients with ischemic stroke (Savitz and Fisher, 2007). In Japan, only edaravone is routinely used in clinical settings. Because ischemic stroke triggers a number of molecular mechanisms that results in neuron death, a single neuroprotective agent that affects only one aspect of the ischemic cascade is unlikely to have any substantial clinical benefits. Thus, it is reasonable to hypothesize that a combination therapy or a single agent that acts on multiple pathways of the ischemic cascade might more successfully prevent against or ameliorate ischemic injury in acute stroke (Savitz and Fisher, 2007).

Cilostazol, a phosphodiesterase (PDE) 3 inhibitor and an anti-platelet drug, is commonly prescribed for ischemic stroke during the chronic phase. Recently, the Cilostazol Stroke Prevention Study (CSPS) II reported that cilostazol was superior to aspirin for further reducing vascular events (Shinohara et al., 2010). Additionally, Choi et al. reported that treatment with cilostazol could reduce the infarct volumes in a cerebral ischemia model (Choi et al., 2002); subsequently, numerous researchers have focused on its pleiotropic neuroprotective effects (Table 1).

Although some of its therapeutic mechanisms have been suggested, the molecular mechanisms involved with cilostazol actions have not been completely determined. Thus, in the present study, we investigated what effects cilostazol had in a mouse model of permanent focal ischemia.

2. Results

2.1. *Cilostazol reduces infarct volumes in a dose-dependent manner*

Using 2,3,5-triphenyltetrazolium chloride (TTC) staining, infarct volumes were evaluated at 48 h after permanent MCAo was established. The representative photomicrographs shown in Fig. 1A indicated that the infarct areas were located in the ipsilateral neocortex and that treatment with cilostazol dose-dependently salvaged the tissue damage of the ischemic boundary zones. A quantitative analysis of infarct volumes indicated that the mean percentage of infarct volumes was 20.1±3.8% in the hemispheres of vehicle-treated mice. When mice were treated with 10 or 20 mg/kg of cilostazol, mean infarct volumes decreased to 16.3±6.2% (10 mg/kg) or 14.1±4.9% (20 mg/kg), respectively. Thus, treatment with 20 mg/kg of cilostazol significantly reduced the infarct volumes to 70.1% of those in control mice ($P<0.05$; Fig. 1B). We did not find any hematoma in the ischemic lesion of both cilostazol-treated and vehicle-treated animals.

2.2. *Cilostazol reduces neuron oxidative stress around the ischemic boundary zone*

8-hydroxydeoxyguanosine (OHdG) is a marker of oxidative stress, and an immunoreaction against 8-OHdG reflects DNA damage due to reactive oxygen species (ROS). Double immunostaining for 8-OHdG and NeuN, a neuron marker, showed that some neurons underwent oxidative stress in around the ischemic boundary zone at 6 h post-ischemia (Fig. 2 A). Treatment with 20 mg/kg of cilostazol reduced the numbers of 8-OHdG-positive neurons, and this effect to relieve oxidative

Table 1 – Previous reports about neuroprotective effects of Cilostazol.

Authours	Year	Model	Administration	Effects and mechanisms
Choi et al.	2002	tMCAo(2 h)	Iv, 10 mg/kg ×2	Infarct↓, apoptosis↓, scavenging ROS
Lee et al.	2003	tMCAo(2 h)	Oral, 30 mg/kg ×2	Infarct↓
Lee et al.	2004	tMCAo(2 h)	Oral, 20–50 mg/kg ×2	Infarct↓, apoptosis↓, pCREB↑
Honda et al.	2006	pMCAo	Oral, 50 mg/kg ×2	Infarct↓, axonal damage↓, CBF↑
Wakida et al.	2006	pMCAo	Ip, 30 mg/kg ×3	Infarct↓, MT-1 & MT-2↑
Watanabe et al.	2006	chCHP	Oral, 50 mg/kg/day	Cognitive function↑, axonal damage↓, pCREB↑
Lee et al.	2006	chCHP	Oral, 60 mg/kg/day	Axonal damage↓, inflammation↓, TNF-α↓
Ye et al.	2007	tMCAo(30 min)	Ip, 10 mg/kg	Infarct↓, neurological dysfunctions↓
Yuzawa et al.	2008	tMCAo(2 h)	Oral, 30 mg/kg	Infarct↓, CBF↑
Nonaka et al.	2009	tMCAo(2 h)+NBO	Ip, 3 mg/kg	Infarct↓, CBF↑, apoptosis↓, eNOS↑
Nonaka et al.	2009	tMCAo(2 h)	Ip, 10 mg/kg	Infarct↓, edema↓, hemorrhagic transformation↓
Lee et al.	2009	Transient global ischemia	Oral, 60 mg/kg/day	CA1 damage↓, neurogenesis↑, pCREB↑
Ishiguro et al.	2010	tMCAo(6 h)+tPA	Ip, 10 mg/kg	Hemorrhagic transformation↓, BBB protection
Tanaka et al.	2010	tMCAo(45 min)	Ip, 10 mg/kg/day	Infarct↓, neurogenesis↑, pCREB↑
Ito et al.	2010	Photothrombotic occlusion	Oral, 100 mg/kg	Infarct↓, NO↑
Omote et al.	2012	SHR-SP	Ip, 100 mg/kg/day	Infarct↓, oxidative stress↓
Omote et al.	2014	SHR-SP	Ip, 100 mg/kg/day	NVU protection, MMP-9↓
Toda et al.	2014	tMCAo(90 min)	Oral, 50 mg/kg/day	Infarct↓, oxidative stress↓

Abbreviations; tMCAo: transient middle cerebral artery occlusion, pMCAo: permanent middle cerebral artery occlusion, chCHP: chronic cerebral hypoperfusion, NBO: normobaric hyperoxia, SHR-SP: stroke-prone spontaneous hypertensive rat, pCREB: phosphorylated cyclic-AMP response element binding protein, CBF: cerebral blood flow, MT: metallothionein, TNF: tumor necrosis factor, eNOS: endothelial nitric oxide synthase, BBB: blood-brain barrier, NVU: neuro-vascular unit, MMP: matrix metalloproteinase.