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Research Report

Cilostazol reduces ischemic brain damage partly by inducing metallothionein-1 and -2

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ABSTRACT

The neuroprotective effect of cilostazol, an antiplatelet drug, was examined after 24 h permanent middle cerebral artery (MCA) occlusion in mice, and explored the possible underlying mechanism by examining metallothionein (MT)-1 and -2 induction in vivo. Cilostazol (30 mg/kg) was intraperitoneally administered at 12 h before, 1 h before, and just after MCA occlusion. Mice were euthanized at 24 h after the occlusion, and the neuronal damage was evaluated using 2,3,5-triphenyltetrazolium chloride (TTC) staining. Cilostazol significantly reduced the infarct area and volume, especially in the cortex. Real-time RT-PCR revealed increased mRNA expressions for MT-1 and -2 in the cortex of normal brains at 6 h after cilostazol treatment without MCA occlusion. MT-1 and -2 immunoreactivity was also increased in the cortex of such mice, and this immunoreactivity was observed in the ischemic hemisphere at 24 h after MCA occlusion (without cilostazol treatment). The strongest MT-1 and -2 immunoreactivity was detected in MCA-occlused mice treated with cilostazol [in the peri-infarct zone of the cortex (penumbral zone)]. These findings indicate that cilostazol has neuroprotective effects in vivo against permanent focal cerebral ischemia, especially in the penumbral zone in the cortex, and that MT-1 and -2 may be partly responsible for these neuroprotective effects.

1. Introduction

The metallothioneins (MTs) are a family of four low molecular weight, metal-binding proteins with a high cystein content, known as MT-1, MT-2, MT-3, and MT-4 (Hamer, 1986). In adult mice, MT-1 and MT-2 are found in all organs, MT-3 is expressed mainly in the brain (Palmiter et al., 1992), and MT-4 is most abundant in certain stratified squamous epithelial tissues (Quaife et al., 1994). Previous studies suggest that MT-1 and -2 serve as important regulators of metal homeostasis, and also as a source of zinc for incorporation into proteins, including transcription factors (Zeng et al., 1991; Palmiter, 1998). MT-1 and -2 are able to prevent zinc deficiency and toxicity in vivo (Dalton et al., 1996; Kelly et al., 1996; Lee et al., 1996), and have also been proposed to function as detoxifiers of other reactive metals and free radicals (Thornalley and Vasak, 1985; Liu et al., 1991, 1995). Interestingly, MT-1 isoform-overexpressing transgenic mice apparently possess a degree

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of protection against neuronal damage in a cerebral ischemia reperfusion model (Van Lookeren Campagne et al., 1999), and MT-1,2-knock-out mice have larger infarcts than wild-type mice after 2 h transient focal ischemia (Trendelenburg et al., 2002).

Cilostazol, an antiplatelet drug used to treat intermittent claudication, has been reported to increase the intracellular level of cyclic AMP by inhibiting its hydrolysis by type III phosphodiesterase. Its principal actions include inhibition of platelet aggregation (Kimura et al., 1985; Kohda et al., 1999), antithrombosis in feline cerebral ischemia, and vasodilation via mediation of increased cyclic AMP level (Tanaka et al., 1989). Recently, Lee et al. (2005) found that cilostazol has a neuroprotective effect against cerebral infarcts in rat brains subjected to middle cerebral artery (MCA) occlusion followed by 24 h reperfusion, and that this effect is exerted via antioxidant and antiapoptotic actions. Since Michael and Robert (1989) noted that dibutyryl cyclic AMP increased significantly liver MT-1 and -2, it seemed possible that cilostazol might reduce ischemic brain damage at least partly by inducing MT-1 and -2.

The purposes of this study, on mice, were therefore to examine (1) whether cilostazol induces MT-1 and -2 in the brain and (2) whether cilostazol reduces, via an MT-related mechanism, the brain damage occurring after permanent MCA occlusion.

Table 1 – Physiological variables before and after ischemia in vehicle-treated mice and cilostazol-treated mice

Parameters	Vehicle	Cilostazol
Mean blood pressure (mm Hg)		
Before ischemia[a]	73.8±2.5	72.6±1.7
After ischemia[b]	79.6±2.8	77.7±3.9
pH		
Before ischemia[a]	7.34±0.05	7.32±0.03
After ischemia[b]	7.31±0.06	7.26±0.03
$PaCO_2$ (mm Hg)		
Before ischemia[a]	36.5±5.7	35.5±2.1
After ischemia[b]	38.3±5.2	43.8±3.0
PaO_2 (mm Hg)		
Before ischemia[a]	164.0±18.9	163.0±5.8
After ischemia[b]	147.8±14.3	141.8±8.1
Regional CBF (%)	20.2±1.0	23.1±0.6

Blood pressure was monitored via the femoral artery (using PowerLab/8sp data acquisition equipped with a transducer amplifier). pH, $PaCO_2$, and PaO_2 were obtained from femoral arterial blood samples [50 μl samples being withdrawn twice (before ischemia and 30 min after MCA occlusion)]. There were no significant differences in the above parameters between vehicle-treated and cilostazol-treated mice. Regional CBF (rCBF) was measured before and 30 min after MCA occlusion (MCAO) in vehicle-treated and cilostazol-treated mice. Animals were operated initially under isoflurane anesthesia. rCBF was determined by laser-Doppler flowmetry, the tip of the probe being placed on the intact skull over the ischemic cortex (2 mm posterior and 6 mm lateral to bregma). There was no significant difference in rCBF between vehicle-treated and cilostazol-treated mice. Data are expressed as the mean±SEM ($n=4$).

[a] Immediately before MCA occlusion.

[b] 30 min after MCA occlusion.

2. Results

2.1. Physiological variables

There was no significant difference between the vehicle and cilostazol groups in the following physiological variables: mean arterial blood pressure, blood pH, partial pressures of carbon dioxide ($PaCO_2$) and oxygen (PaO_2), and regional cerebral blood flow (rCBF) in the core area whether they were measured before or after 30 min ischemia (Table 1).

2.2. Effect of cilostazol on infarct size and volume

Animals injected i.p. with cilostazol at 30 mg/kg, at 12 h before, 1 h before, and just after the induction of focal ischemia showed no behavioral changes, except for the neurologic deficits induced by the ischemia. Twenty-four hours after MCA occlusion, an ischemic zone was consistently identified in the cortex and striatum of the left cerebral hemisphere. The infarct area was smaller in the cilostazol-treated group than in the vehicle-treated (control) group (Figs. 1A–C). Infarct volume was also smaller in the cilostazol-treated group (Fig. 1F). A cilostazol-induced reduction in infarct area could be seen in total area, and especially, in the cortex (Figs. 1D, E, G, and H). In the subcortex, infarct area and volume tended to be smaller after cilostazol, but statistical significance was not reached for infarct area ($p=0.168$) or infarct volume ($p=0.082$).

2.3. Real-time RT-PCR and immunohistochemistry

We examined whether cilostazol might induce MT-1 and -2 mRNAs in the brain using real-time RT-PCR. Cilostazol (30 mg/kg, i.p.) was administered three times (at 12 h before, 1 h before, and just after focal ischemia), as mentioned in Experimental procedures. Six hours after the last cilostazol injection, the mice were decapitated. In 5-mm-thick coronal block slices of hemisphere at the striatal level, real-time RT-PCR revealed increases in the mRNA expressions for both MT-1 and -2 in cilostazol-treated mice without MCA occlusion (vs. the vehicle-treated group) (Fig. 2).

We also determined whether cilostazol treatment and/or MCA occlusion might induce MT-1 and -2 in the brain by examining MT-1 and -2 immunoreactivity (Fig. 3). At 24 h after the start of the ischemia, MT-1 and -2 immunoreactivity was detected in the ipsilateral hemisphere. MT-1 and -2 immunoreactivity was also increased in the cortex of cilostazol-treated mice (without ischemia). The strongest MT-1 and -2 expression was detected in cilostazol-treated, MCA-occluded mice in the peri-infarct area at 24 h after the occlusion. On the other hand, no MT-1 or -2 expression was observed in control (no ischemia) mice.

3. Discussion

We examined the neuroprotective effects of cilostazol after 24 h permanent MCA occlusion in mice, and we explored the possible underlying mechanism by examining MT-1 and -2 induction in vivo. Cilostazol-treated mice showed significantly