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Research article

Cilostazol alleviates white matter degeneration caused by chronic cerebral hypoperfusion in mice: Implication of its mechanism from gene expression analysis

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ABSTRACT

Cilostazol is known to alleviate white matter demyelination due to chronic cerebral hypoperfusion in rodent models, although their pharmacological mechanisms remain unclear. In this study, we investigated the protective effect of cilostazol in relation to gene expression profile. Bilateral common carotid artery stenosis (BCAS) mice were treated with oral administration of cilostazol or placebo starting from a week after the surgery. Demyelination of the cingulum was compared between the 2 groups 2, 6, and 10 weeks after initial drug administration. Also, to examine temporal gene expression change during demyelination, DNA microarray analysis was conducted using samples from the corpus callosum of 2nd and 6th week BCAS mice. For genes that showed more than 2-fold up-regulation, their increase was validated by qPCR. Finally, to determine the effect of cilostazol towards those genes, their expression in the corpus callosum of 6-week placebo-treated and cilostazol-treated BCAS mice was compared by qPCR.

Amelioration of myelin loss was observed in cilostazol-treated group, showing significant difference with those observed in placebo group after 10-week treatment. Gene ontology analysis of the 17 up-regulated (FDR $<$ 0.01) genes showed that majority of the genes were related to cell development processes. Among the validated genes, expression of *Btg2* was significantly promoted in the corpus callosum of BCAS mice by administration of cilostazol. Results of this study suggest that activation of Btg2 may be one of the key pharmacological effects of cilostazol towards the white matter during chronic ischemia.

1. Introduction

Subcortical ischemic vascular dementia (SIVD) is the most common subtype of vascular cognitive impairment syndrome (VCI), clinically defined as cognitive decline under the evidence of subcortical brain infarction [1,2]. Patients with SIVD suffer from vast white matter degeneration due to chronic cerebral hypoperfusion caused by fibrohyalinosis of the medullary artery, which is often slowly progressive with poor neurological outcome [3,4]. Although number of patients with SIVD is predicted to increase in accordance with the advancement of aging society, there is no established treatment for this pathological condition to date, and therapeutic breakthrough is long awaited.

For profound understanding of SIVD, several animal models have been developed to this day [5–8]. Among them, bilateral carotid artery occlusion (BCAO) rats and bilateral carotid artery stenosis (BCAS) mice are considered to be the most reliable models that replicate pathophysiology of SIVD. Ever since their development, various pharmacological studies for the treatment of SIVD had been carried out, and some have shown potential efficacy of drugs that are currently used for other medical purposes [9–14]. Cilostazol, a phosphodiesterase 3 inhibitor, is one of them, and it has been proposed that the drug would ameliorate ischemic white matter degeneration in SIVD models by directly interacting with white matter components such as blood-brain barrier and oligodendrocyte progenitor cells, aside from its native antithrombotic and vasodilatory function [15,16]. However, pharmacological mechanisms of these interactions still remain obscure.

In this study, we aimed for comprehensive observation of the protective effect of cilostazol towards SIVD in relation to gene expression profile. Following pathological confirmation of the potency of the drug to white matter degeneration, we utilized DNA microarray analysis to embrace temporal gene expression change in the corpus callosum of BCAS mice, to identify key genes, and to detect alteration in multiple

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genes within major functional pathways. Upon identifying genes with significant up-regulation during chronic cerebral ischemia, we investigated how the expressions of those genes were modified by administration of cilostazol in BCAS mice.

2. Material and methods

2.1. Mice and surgical procedure

Male 9–10 weeks old C57BL/6JJcl mice (22–25 g) were purchased from Japan Clea (Tokyo, Japan) and housed in specific pathogen-free conditioned 12-h light/dark cycle room with free access to food and water throughout the experiment. After a week of habituation, mice were randomly selected to receive BCAS or sham surgery. BCAS surgery was performed as previously described [8]. Anesthesia was induced with 4% isoflurane (Abbott Japan, Japan) and maintained by 1.5% during operation. Following midline incision, bilateral common carotid arteries were carefully isolated from vagal nerves. To keep body temperature at 37.0 ± 0.5 °C, heating pad (Nissinrika, Japan) was used when necessary. After application of 0.18 mm diameter microcoils (Samini, Japan), we sutured the wound and discontinued the anesthesia and waited for recovery before returning mice to their cage. No inter-/post-surgical complications were observed.

2.2. Laser-doppler flowmetry

Skin overlying the right skull of sham-operated and BCAS mice (n = 4 each) was removed under anesthesia with isoflurane (4% for induction, 1.5% for maintenance). Then, plastic guide cannula (outer diameter 5 mm, inner diameter 3 mm, length 4 mm) was perpendicularly attached to the skull 2 mm posterior and 2 mm lateral to the bregma with dental cements (Sunmedical, Japan). Cerebral blood flow (CBF) was detected by inserting 3 mm probe (Omegawave, Japan) into the cannula, and analyzed with computer-based laser blood flowmeter (OMEGAFLO-Lab, Omegawave). CBF was recorded shortly before and after the operation, and 1, 7, 14, 35, 50 days after the surgery.

2.3. Cilostazol preparation and administration protocol

Cilostazol (Wako, Japan) was dissolved by 0.5% carboxymethyl cellulose sodium salt (CMC; Wako, Japan) to the concentration of 10 mg/mL for oral administration. Each mouse was weighed daily to determine the dose of the drug. For gavage tool, a soft disposable feeding tube made from polytetrafluoroethylene (Fuchigami, Japan) was used to avoid esophageal perforation. As described in Fig. 1, 40 BCAS mice were randomly separated into cilostazol-treated group (n = 16), and placebo-treated group (n = 24). The drugs were started 1 week after the BCAS surgery. The former group received cilostazol (100 mg/kg/day) for a maximum of 10 weeks, while the other received 0.5% CMC (10 mL/kg/day) for the same period. Dose of cilostazol was determined in reference to previous studies [17,18]. Mice were sacrificed after 2, 6, and 10 weeks of drug administration in cilostazol group (n = 4 for 2 and 10, n = 8 for 6), and 2, 4, 6, 8, and10 weeks in placebo group (n = 4 for 2, 4, 8, and10, n = 8 for 6) for sample collection.

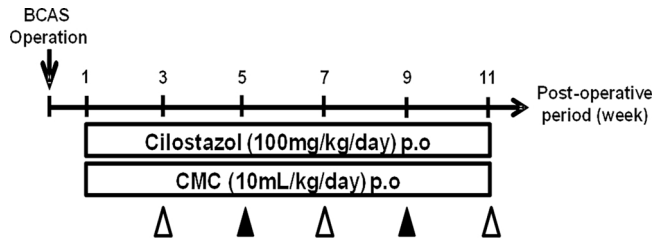

Fig. 1. Protocol for cilostazol administration.
BCAS mice were treated either by cilostazol (n = 16) or CMC (placebo; n = 24) starting from 1 week up to 10 weeks after the operation. White arrowhead stands for point of sacrifice for both groups (week 3 & 11: n = 4 for each group/week, week 7: n = 8 for each group/week). Black arrowhead stands for point of sacrifice for CMC group only (n = 4/week).

2.4. Immunohistochemistry

2.4.1. Preparation of sections

After gentle cervical translocation, mice were transcardially perfused with 50 mL 0.15 M sodium chloride and 50 mL 4% paraformaldehyde. Promptly removed brain *en bloc* was post-fixed with 4% paraformaldehyde overnight at 4 °C. Samples were then either processed as frozen sections or paraffin sections. For frozen sections, post-fixed samples were cryoprotected using 20% sucrose in phosphate-buffered saline (PBS). Next, we cut out area between anterior 1.5 mm and posterior 2.5 mm of bregma which included majority of the corpus callosum with mouse brain matrix (Muromachi, Japan) and frosted with liquid nitrogen. We then prepared consecutive 10 μm-thick coronal frozen sections by cryostat (Microm HM525, Thermo Fisher Scientific, USA). Sections were kept at −30 °C until use after dehydration. For paraffin sections, cut brains were post-fixed in 4% paraformaldehyde for 48 h and embedded with paraffin using HistoStar (Thermo Fisher Scientific). Paraffin blocks were then sliced to 6 μm-thick coronal sections by Microm HM400 (Thermo Fisher Scientific), and kept at room temperature until use.

2.4.2. Kluver-Barrera (KB) staining

Paraffin-embedded sections were deparaffinized and hydrated to 95% ethanol. Then, they were left in 60 °C oven overnight in Luxol Fast Blue Solution (Muto Chemical, Japan). After rinsing excess stain with 95% ethanol and distilled water followed by differentiation with 0.1% lithium carbonate, they were counterstained with 0.1% Cresyl Violet Acetate (Muto Chemical) containing few drops of 10% acetic acid. Samples were then rinsed with 95% alcohol and dehydrated before mounted with resinous medium (Muto Chemical). Samples were examined with Axiophot2/Axioplan2 microscope (Zeiss, Germany), and pictures were taken with AxioCam HRc (Zeiss).

2.4.3. Myelin proteolipid protein (PLP) staining

Frozen sections were permeabilized with Tris-buffered saline (pH 7.4) containing 0.1% Triton-X, followed by 25-min heat-induced antigen retrieval in 95 °C pH 7.4 sodium citrate buffer. After cooling them down to room temperature, sections were incubated with 5% normal goat serum for 1 h at room temperature. Next, they were incubated overnight with anti-proteolipid protein (PLP; a largest component of myelin) antibody (1:100, Abcam, UK) at 4 °C. After washing with PBS for 10 min 3 times, sections were incubated with secondary antibody (Alexa Flour 594 conjugated rabbit IgG antibody, 1:500, Thermo Fisher Scientific) for 1 h at room temperature. Then, they were washed with PBS again for 10 min 3 times and covered with ProLong Diamond antifade mountant (Thermo Fisher Scientific). After blinding the samples, immunostaining was examined with Axiophot2/Axioplan2 fluorescence microscope, and all the pictures were taken under the exposure time of 150 ms with AxioCam HRc. Picture data (zvi file, RGB color) were converted to 16 bit gray scale with Image J (imagej.nih.gov/ij/) by using its plug-in tool Bio-Formats (LOCI, USA). The cingulum: a bundle of white matter fibers that project from cingulate gyrus interrelating components of the limbic system, was chosen as a region of interest (ROI) as it showed most notable rarefaction within the white matter during chronic hypoperfusion (Fig. 2). We then measured mean fluorescence intensity of the corresponding area. After blinding the stained samples, Intensity of 3 different area of cingulum per mouse (n = 4 for each week) was measured to calculate average and standard error of the mean within each group. Relative intensity towards the average of 2-week CMC-treated mice was measured for comparison.