(Fetalvero et al., 2006). Cilostazol increased the intracellular cAMP level by inhibiting phosphodiesterase type III and might contribute to maintaining the physiological contractile phenotype, thereby attenuating the pathological remodeling, including perivascular collagen deposition, of small vessels.

Other pathways might also be involved in the mechanisms of cilostazol-mediated alleviation of WM lesions. First, cilostazol might have alleviated the WM lesions independent of the small-vessel pathology because cilostazol directly reduces the apoptotic cell death of oligodendroglia in chronic, cerebral hypoperfusion (Lee et al., 2006) through the cAMP-responsive element-binding protein phosphorylation pathway (Watanabe et al., 2006). Second, cilostazol might have improved the microcirculation by suppressing both the endothelial expression of adhesion molecules (Park et al., 2006; Hashimoto et al., 2006) and the endothelium-independent vasodilation (Nakamura et al., 2006). Third, suppression of the microglial activation might reduce the level of cytotoxic substances, including inflammatory cytokines such as TNF-α (Wakita et al., 2003).

In conclusion, the findings reported herein indicate that long-term treatment with cilostazol attenuates the phenotypic modulation of SMCs, cerebral small-vessel pathology associated with WM lesions in SHRSP. These results signify that cilostazol is a promising candidate for the treatment of SVD.

4. Experimental procedures

4.1. *Animals and treatments*

Male SHRSP (8 weeks old; $n=18$) and their normotensive control strain, male WKY rats (8 weeks old; $n=9$), were obtained from Japan SLC, Inc. (Hamamatsu, Japan). They were maintained on a 12-hour light–dark cycle with free access to laboratory chow and water. The rats were divided into three groups: 1) cilostazol-treated SHRSP, which were fed with a chow containing 0.1% cilostazol (a generous gift from Otsuka Pharmaceutical, Tokyo, Japan); 2) vehicle-treated SHRSP, fed with a standard rat chow; and 3) WKY rats, fed with a standard rat chow ($n=9$ each group). Body weight and systolic blood pressure were measured in all animals once a week throughout the study (20 weeks of treatment). Systolic blood pressure was measured in the warmed, conscious, and restrained state using the tail-cuff method (TK-370A, Unicom Inc, Tokyo, Japan).

4.2. *Histopathology and immunohistochemistry*

Animals were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.) at 28 weeks of age and perfused transcardially at 20 mL/min with 0.01 mol/L phosphate-buffered saline (PBS) followed by 4% paraformaldehyde and 0.2% picric acid in 0.1 mol/L PBS, pH 7.4. The brains were removed, and the coronal brain blocks were postfixed for 24 h in 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4). Fixed coronal brain blocks including the caudoputamen were dehydrated and embedded in paraffin. The remaining coronal blocks were stored in 20% sucrose in 0.1 mol/L PBS (pH 7.4) until use for cryosectioning. Paraffin-embedded tissue was sectioned at 4-μm thickness. The sections were stained with hematoxylin and eosin for examination of the overall morphology; elastica van Gieson's stain for examining the perivascular collagen deposition and Klüver–Barrera staining for examining the WM lesions. For immunohistochemistry, the paraffin sections were incubated overnight with a mouse monoclonal antibody directed against human smooth-muscle actin (hSMA; diluted 1:100; DAKO). Cryosections were taken on a cryostat (20-μm thickness) and incubated overnight at 4 °C with the following primary antibodies: OX6 (diluted 1:100; Sera Lab), against major histocompatibility complex (MHC) class II antigen as a marker for activated microglia, and embryonic smooth-muscle myosin heavy chain (SMemb; diluted 1:3000; Yamasa) as a marker for synthetic SMCs. These sections were subsequently incubated with biotinylated antimouse IgG (diluted 1:200; Vector Laboratories). Immunoreactivity was visualized using the avidin–biotin complex method (Vector Laboratories).

4.3. *Morphometric analysis*

In all animals, 10 to 15 lenticulostriate arteries with an external diameter between 30 and 75 μm in the caudate putamen, and hypothalamus were analyzed. Vessel sections with a long-axis/short-axis ratio <1.3 were chosen as suitable cross-sections. Medial and luminal cross-sectional area was quantitatively analyzed in the sections immunostained for hSMA. The ratio of the cross-sectional medial area to the luminal area (wall area/lumen area ratio) was assessed as an index of wall thickening. Perivascular fibrosis was assessed by the ratio of the collagen area to the total vessel area with elastica van Gieson's stain (collagen area/total vessel area ratio), in which the total vessel area was defined as medial plus luminal area (Akishita et al., 2000). Each field was analyzed using the Image J freeware obtained from the National Institutes of Health. The pathological changes in the WM were quantified in terms of the numerical density of MHC class II-immunopositive microglia and the degree of WM lesions. The MHC class II-immunopositive cells were counted in a predefined area (per 0.25 mm^2) of the corpus callosum. Severity of the WM lesions was graded as reported previously (Wakita et al., 1994): normal (grade 0); disarrangement of the nerve fibers (grade 1), formation of marked vacuoles (grade 2), and the disappearance of myelinated fibers (grade 3).

4.4. *Statistical analysis*

Data were expressed as mean±SEM. Systolic blood pressure was compared between the groups by repeated-measure ANOVA. Differences in the grading score for WM lesion were determined by the nonparametric Kruskal–Wallis test with subsequent group comparisons using Mann–Whitney *U* test. Other statistical analyses were carried out by one-way ANOVA followed by the Bonferroni correction. A value of $p<0.05$ was considered statistically significant.

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