Clinical and Pathological Improvement in Stroke-Prone Spontaneous Hypertensive Rats Related to Pleiotropic Effect of Cilostazol

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Abstract

Background and Purpose: Cerebral infarction is a major cause of death or decreasing activities of daily living (ADL). This study aimed to investigate the efficacy of commonly used anti-platelet drugs on stroke, and motor and cognitive functions in relation to oxidative stress markers and insulin-like growth factor 1 receptor (IGF-1R).

Methods: Stroke-prone spontaneously hypertensive rats (SHR-SP) were treated with vehicle, aspirin, clopidogrel and cilostazol from 8 to 10 weeks of age. Physiological parameters, regional cerebral blood flow (rCBF) and serum lipids were examined. Motor and cognitive functions were evaluated weekly by rotarod and water maze task. Spontaneous infarct volume, oxidative stress markers for lipid, protein and DNA at the ischemic boundary zone of spontaneous infarction and the IGF-1R positive cell ratio in the hippocampus were immunohistochemically examined in brain sections. IGF-1Rβ expression in the hippocampus was assessed by western blotting.

Results: The anti-platelet drugs, cilostazol and clopidogrel, reduced the spontaneous infarct volume more than aspirin. Only cilostazol improved motor and cognitive functions with a significant increase ($p<0.05$) in the memory-related IGF-1R positive ratio and IGF-1Rβ expression in the hippocampus. Cilostazol reduced the 4 oxidative stress markers in affected neurons in SHR-SP regardless of blood pressure, rCBF or

serum lipid levels.

Conclusion: The present results suggest that a possible pleiotropic effect of cilostazol resulted in the reduction of spontaneous infarct volume and preservation of motor and spatial cognitive functions. The increase of IGF-1R positive cells in the hippocampal CA1 region could partly explain the preservation of spatial cognitive function in SHR-SP.

Introduction

Cerebral infarction is a major cause of death and decreasing the activities of daily living (ADL), and prevention of stoke is an important problem that needs to be solved. Anti-platelet drugs such as aspirin, clopidogrel and cilostazol are the most powerful and commonly used drugs in daily clinical settings for preventing stroke. Hypertension, dyslipidemia, diabetes mellitus and obesity are well known vascular risk factors (VRF) to cause stroke, vascular dementia and Alzheimer's disease. Recent studies have reported that improvement of such VRF reduces risks for both stroke and dementia.[1]

Stroke-prone spontaneously hypertensive rats (SHR-SP) are a good model for spontaneous stroke in relation to hypertension and dyslipidemia by loading salt and high fat and cholesterol (HFC).[2, 3] SHR-SP is also known as cerebral small-vessel disease[4]

and vascular dementia[5] models, characterized by multiple lacunar infarctions and white-matter lesions.

Among anti-platelet drugs, aspirin is a cyclo-oxygenase inhibitor that reduces thromboxane A_2 and subsequent inflammation. Clopidogrel is an adenosine diphosphate (ADP) P2Y12 receptor antagonist that has anti-platelet and vasodilating effects by increasing nitric oxide and prostaglandin I2.[6] Cilostazol is a phosphodiesterase III (PDE3) inhibitor that has not only anti-platelet effects but also anti-oxidative and vasodilation effects through increasing intra-cellular cyclic AMP (cAMP) and endothelial nitric oxide synthase (eNOS) activity. In the second Cilostazol Stroke Prevention Study (CSPS 2), cilostazol was more effective than aspirin for secondary prevention of stroke with fewer hemorrhagic events. Cilostazol also improved cognitive function for Alzheimer's disease in a mouse model.[7]

Therefore, in the present study, we first investigated the difference in efficacy of 3 anti-platelet drugs on stroke, motor and cognitive functions for SHR-SP in relation to oxidative stress markers and insulin-like growth factor 1 receptor (IGF-1R).

Materials and Methods

Experimental model

Seven-week-old male SHR-SP (Disease Model Cooperative Research

Association, Kyoto, Japan) were divided into 4 groups: vehicle-treated (0.5% carboxymethyl cellulose sodium salt), aspirin-treated (10 mg/kg), clopidogrel-treated (10 mg/kg), and cilostazol-treated (100 mg/kg) groups (n=7 in each group). Each dose was determined for obtaining effective blood levels of the respective drugs. A small burr hole (1.5 mm in diameter) was drilled at 2 mm posterior and 5 mm lateral to the bregma for regional cerebral blood flow (rCBF) measurements. The following week, at 8 weeks (W) of age, animals began to be fed a HFC diet with 1% NaCl water. Vehicle, aspirin and clopidogrel were given orally daily and cilostazol was given daily by intraperitoneal injection for the subsequent 14 days until 10 W. Body weight (BW), blood pressure (BP) and pulse rate (PR) were measured at 8, 9 and 10 W, and measurements for BP and PR were performed with the tail-cuff method (Softron BP98A, Tokyo, Japan). Regional CBF was measured using a laser-Doppler flowmeter (FLO-C1, Omegawave, Tokyo, Japan) at 8, 9 and 10 W. Changes in rCBF at 9 and 10 W were expressed as a percentage of rCBF at 8 W.

Serum triglyceride (TG), total cholesterol (T-cho), high-density lipoprotein cholesterol (HDL-cho), and low-density lipoprotein cholesterol (LDL-cho) levels were measured at 8 and 10 W. The animals were euthanized when stroke-like symptoms developed. Animals with no symptoms were euthanized at 10 W. They were

transcardially perfused with heparinized saline, followed by 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2). The whole brain was removed and immersed in the same fixation for 12h at 4°C. After washing with PBS, the tissues were transferred into graded sucrose of 10%, 20% and 30% and then embedded in powdered dry ice and stored a -80°C. Coronal brain sections with 12 μm thickness were prepared using a cryostat at -18°C, and mounted on a silane-coated glass. All experimental procedures were approved by the Animals Committee of the Graduate School of Medicine and Dentistry, Okayama University.

Motor behavior test with the rotarod and Morris water maze task

Motor coordination and balance were evaluated by measuring latency (sec) until falling off from a rotating rod (four-lane rotarod; UGO BASILE, Comerio, Italy) according to our previous report [8]. Briefly, rats were placed on the rod with the rotation speed accelerating from 4 to 40 rpm over the course of 5 min. This procedure was repeated 3 times per day in each rat, and averaged data were recorded as the rotarod time at 8, 9 and 10 W.

A circular water maze pool (150 cm diameter) was filled with opaque water at 25-26°C, which contained a platform submerged 2 cm below the surface of water to escape. The rat was placed in the water at one random start location. Rats were allowed

to find the submerged platform within 90 sec and rest on it for 15 sec after climbing up. If the rat failed to find the platform within 90 sec, it was placed on it for 15 sec. This procedure was repeated 4 times per day with 1 min intervals between trials for 3 consecutive days per week from 7 to 10 W. Averaged escape latency times needed to find the hidden platform were recorded.

Measurement of infarct volume

For evaluation of the infarct volume of spontaneous stroke, a set of sections (n=10 each) was immunohistochemically examined for neuronal nuclei (NeuN). Briefly, 5 coronal sections (12 μm) at 2-mm intervals of each rat were treated with anti-NeuN antibodies (1:200; MAB377; Chemicon, CA, USA). They were then incubated with biotinylated secondary antibodies and the signals were visualized with diaminobenzidine tetrahydrochloride (DAB). Areas devoid of NeuN staining were regarded as infarcted regions. The infarct volume of each animal was calculated by adding infarct areas in 5 sections (10 mm) and multiplying it by the distance between sections (2 mm).

Immunohistochemistry for oxidative stress markers and IGF-1R

We performed immunohistochemistry for *N*-(hexanonyl)lysine (HEL), 4-hydroxynonenal (4-HNE), advanced end glycation products (AGE), 8-hydroxy-

2-deoxyguanosine (8-OHdG) and IGF-1R. After fixation with 4% formaldehyde, sections were incubated in 0.3% hydrogen peroxidase/methanol for 10 min to block endogenous peroxidase activity and incubated with bovine serum albumin for 1 h. Sections were then incubated at 4°C overnight with primary antibody for HEL (1:50; MHL-021P; JAICA, Shizuoka, Japan), 4-HNE (1:50; MHN-100P; JAICA,), AGE (1:200;Transgenic, Kobe Japan), 8-OHdG (1:50; MOG-020; JAICA) and IGF-1R (1:300; ab39675; Abcam, Cambridge, UK). On the next day, the sections were incubated with biotinylated secondary antibody for 2 h at room temperature; biotinylated anti-mouse monoclonal antibody (1:500, Vector Laboratories, CA, USA) was used for HEL, 4-HNE, AGE, and 8-OHdG, and biotinylated anti-goat monoclonal antibody (1:500, Vector Laboratories, CA, USA) was used for IGF-1R. The sections were then incubated with avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories, CA, USA) for 30 min and visualized with DAB.

Cell density of the hippocampus and cell counts

A set of sections (n=7 each) was stained with 0.1% cresyl violet (CV) for histological examination of the cell density of the hippocampus. The numbers of positive cells for CV staining in the dentate gyrus (DG), CA3 and CA1 regions of the hippocampus were counted in 3 randomized 0.01 mm^2 areas.

The numbers of positive cells for HEL, 4-HNE, AGE and 8-OHdG at the boundary zone of spontaneous infarction were counted within the 3 randomized 0.5 mm distance areas from the border of the necrotic ischemic core. IGF-1R positive cells were quantified by counting stained cells in the DG, CA3, and CA1 regions of the hippocampus and expressed as the percentage of IGF-1R-positive cells over the total number of neurons stained with CV staining in those regions.

Western blot analysis

Western blot analysis was performed using the hippocampus of 5 rats from each group. Animals were anesthetized at 10 W and transcardially perfused with heparinized saline. Whole brains were then removed and hippocampal tissues were collected. Lysis buffer was added to each tube and it was homogenized at 4°C. The homogenate was centrifuged at 12,000 rpm at 4°C for 10 min and the supernatant fractions (S1) were collected. Protein concentrations of the S1 samples were determined by Lowry assay (Amersham Biosciences, Ultrospec 3100 pro). An amount equivalent to 20 μg of total protein for each sample was subjected to 8% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, IPVH00010). Membranes were blocked with 5% skimmed milk/0.1% Tween-20 in PBS (pH 7.4). We carried out western blot analysis using standard techniques with Super

Signal West Dura Extended Duration Substrate (Thermo scientific, 34075) according to our previous report.[9] The primary antibody was anti-IGF-1Rβ (1:1000; Cell Signaling Technology, MA, USA). We carried out densitometry analysis using Scion Image Beta 4.02 software and took the average value of the 5 rats.

Statistical analysis

All data are presented as mean±SD. Statistical analyses were performed using one-factor ANOVA, followed by a Tukey–Kramer post comparison. Differences with a probability value of $p<0.05$ were considered statistically significant.

Results

Physiological and biochemical parameters in SHR-SP

Mean survival times and BW were not significantly different among the 4 groups (Table 1). The time-dependent changes in systolic BP (SBP) and diastolic BP (DBP) in the 4 groups are shown in Fig. 1. In all 4 groups (n=7 in each group and time point), SBP increased with age. In the vehicle-treated group, SBP at 8 W was 190.4±6.7 mmHg (mean±SD), which progressively escalated at 9 and 10 W to 227.2±15.6 ($^{\dagger\dagger}p<0.01$ vs 8 W) and 243.6±23.9 ($^{\dagger\dagger}p<0.01$ vs 8 W), respectively. Aspirin-, clopidogrel-, and cilostazol-treated groups also showed similar SBP changes at 9 W ($^{\dagger\dagger}p<0.01$ vs 8 W) and 10 W ($^{\dagger}p<0.05$ vs 8 W) for the aspirin-treated group, at 9 W

(††p<0.01 vs 8 W) and 10 W (††p<0.01 vs 8 W) for the clopidogrel-treated group, and at 9 W (††p<0.01 vs 8 W) and 10 W (††p<0.01 vs 8 W) for the cilostazol-treated group. There were no differences in SBP among the 4 groups at 8, 9 or 10 W. DBP in the 4 groups showed similar significant increases (Fig 1), but these were not different among the groups. Mean BP (MBP) and PR were also not significantly different among the groups.

The time-dependent course of rCBF in the 4 groups is shown in Fig. 1. In the vehicle-treated group (n=7 at each time point in all groups), rCBF showed a progressive decrease from 8 W to 10 W (†p<0.05, 10 vs 8 W). In the aspirin-treated group, rCBF also showed a progressive decrease from 8 W to 10 W (†p<0.05, 10 vs 8 W), and the clopidogrel-treated group showed a progressive decrease from 8 W to 10 W. In contrast, the cilostazol-treated group showed a small increase in rCBF from 8 W to 9 W (107.4±15.0% of 8 W) and only a small decrease at 10 W (98.6±9.9% of 8 W). However, there was no significant difference in rCBF among the 4 groups at 8, 9, or 10 W (Table 1). Serum TG, T-cho, HDL-cho and LDL-cho levels were not significantly different among the 4 groups at 8 and 10 W (Table 1).

Rotarod and Morris water maze task

The mean times until falling off from the rotarod in the 4 groups are shown in

Fig. 2. In the vehicle-treated group (n=7 in each group), the drop-off times at 8 and 9 W were 138.0±38.6 sec and 138.4±26.3 sec, respectively, and this time was greatly decreased at 10 W to 58.7±53.5 sec ($^{\dagger\dagger}p<0.01$ vs 8 W, $^{\dagger}p<0.05$ vs 9 W). The aspirin- and clopidogrel-treated groups showed similar changes as the vehicle-treated group. In contrast, only the cilostazol-treated group showed preserved rotarod scores at 8, 9, and 10 W ($^{\S}p<0.05$ vs the other 3 groups).

The water maze task of the 4 groups is shown in Fig. 2. In the vehicle-treated group (n=7 in each group), the latency time showed a gradual extension in time from 8 W (11.1±5.7 sec) to 9 (11.5±7.7 sec) and 10 W (13.6±5.9 sec). In the aspirin- and clopidogrel-treated groups, the latency time showed no change at 8, 9, and 10 W. In contrast, only the cilostazol-treated group showed a gradual decrease in time from 8 W (9.8±7.1 sec) to 9 (7.4±1.4 sec) and 10 W (6.7±2.0 sec, $^{*}p<0.05$ vs vehicle).

Spontaneous infarct volume in SHR-SP

The spontaneous infarct volumes evaluated with NeuN staining are shown in Fig. 3. The infarct volumes in vehicle- and aspirin-treated groups were 4.4±1.5 mm^3 and 5.7±2.4 mm^3 (n=10 in each group). The infarct volume in the clopidogrel-treated group (n=10) showed a significant decrease (3.0±1.4 sec, $^{\#}p<0.05$ vs aspirin) with a further decrease in the cilostazol-treated group (1.8±1.0 sec, n=10) compared with both the

vehicle- and aspirin-treated groups ($^{*}p<0.05$ vs vehicle, $^{\#\#}p<0.01$ vs aspirin).

Oxidative stress markers

Typical staining patterns for HEL, 4-HNE, AGE and 8-OHdG at the boundary zone of spontaneous infarction are shown in Fig. 4. The aspirin-treated group did not show any difference in staining for these 4 oxidative stress markers compared with the vehicle-treated group. On the other hand, the clopidogrel-treated group tended to reduce staining strength with more evident reduction in the cilostazol-treated group.

For quantitative analyses, the numbers of positive cells for HEL in the vehicle- (n=7 in each group) and aspirin-treated groups were 121.4±27.7/mm^2 and 113.6±20.4/mm^2, respectively (Fig. 4). The clopidogrel-treated group tended to show a decrease in number to 89.4±19.3/mm^2 with a significant decrease in the cilostazol-treated group to 78.1±19.8/mm^2 ($^{**}p<0.01$ vs vehicle, $^{\#}p<0.05$ vs aspirin). The number of positive cells in vehicle-, aspirin-, clopidogrel- and cilostazol-treated groups (n=7 in each group) showed a significant reduction only in the cilostazol-treated group for 4-HNE ($^{*}p<0.05$ vs vehicle, $^{\#}p<0.05$ vs aspirin), AGE ($^{*}p<0.05$ vs vehicle, $^{\#}p<0.05$ vs aspirin), and 8-OHdG ($^{*}p<0.05$ vs vehicle, $^{\#}p<0.05$ vs aspirin).

Hippocampal histology, immunohistochemistry and western blotting

Typical CV staining for the hippocampus is shown in Fig. 5 including the DG,

CA3 and CA1 regions. Neuronal cell density in the hippocampus in vehicle-, aspirin-, clopidogrel- and cilostazol-treated groups (n=7 in each group) at 10 W was not significantly different among the 4 groups in the DG, CA3 or CA1 regions.

Immunoreactive IGF-1R staining is shown in Fig. 5. IGF-1R positive cell density of hippocampal neurons in vehicle-, aspirin-, clopidogrel- and cilostazol-treated groups (n=7 in each group) was shown in Fig. 5B. The ratio of IGF-1R positive cells to total cell number with CV staining in the DG region of vehicle-, aspirin- and clopidogrel-treated groups (n=7 in each group) was 23.1±6.4%, 23.9±6.4%, and 25.7±10.5%, respectively. The cilostazol-treated group tended to show an increase in this ratio in the DG (27.9±4.3, n=7) as well as in the CA3 region (n=7 in each group). In contrast, only the CA1 region showed a significant increase in this ratio in the cilostazol group (15.6±1.7%, $^{*}p<0.05$ vs vehicle, $^{\#}p<0.05$ vs aspirin) compared with vehicle- (11.9±2.7%), aspirin- (11.3±2.4%) and clopidogrel-treated (12.7±1.9%) groups (n=7 in each group).

Western blot results are shown in Fig. 5C. The ratios of IGF-1Rβ to β-tubulin in the hippocampus of vehicle-, aspirin- and clopidogrel-treated groups (n=5 each) were 0.41±0.16, 0.41±0.11, and 0.56±0.15, respectively. The cilostazol-treated group showed a significantly increase in this ratio (0.67±0.15, n=5) compared with the vehicle-treated

group ($^{*}p<0.05$ vs vehicle).

Discussion

The present study showed that among the 3 anti-platelet drugs, aspirin, cilostazol and clopidogrel, which are commonly used in the clinical setting, only cilostazol improved motor and cognitive functions with a significant increase in the IGF-1R positive ratio in hippocampal CA1 and IGF-1Rβ expression in the hippocampus. We also showed that both cilostazol and clopidogrel reduced spontaneous infarct volume more than aspirin. Additionally, only cilostazol reduced 4 oxidative stress markers in affected neurons in SHR-SP rats regardless of BP and serum lipid levels. SHR-SP develop an accelerated hypertension with subsequent cerebrovascular injury by loading salt and this serves as a good experimental model for spontaneous stroke.[2] The SHR-SP model is also known as cerebral small-vessel disease[4] and vascular dementia[5] models, which are characterized by multiple lacunar infarctions and white-matter lesions. As we previously reported, the double loading of salt and HFC strongly promote arteriosclerotic change and increase the risk of infarction.[3] Recent studies have shown that salt loading increases superoxide production and potentiates NADPH oxidase activity in the brain.[10] In the present study, we evaluated the end products of reactive oxygen species by immunohistochemistry for HEL, 4-HNE, AGE, and

8-OHdG, which represent oxidative stress markers of early lipid, late lipid, protein and DNA peroxidation, respectively, and found that only cilostazol reduced these oxidative stress markers in neurons of SHR-SP.

In addition to the primary pharmacological activity of cilostazol of inhibiting phosphodiesterase III for increasing intra-cellular cAMP and eNOS activity, cilostazol has a further anti-oxidative effect among the 3 anti-platelet drugs. Cyclic AMP activates protein kinase A (PKA)/phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways, which are important for cell survival.[11, 12] Produced eNOS protects vascular endothelium against oxidative damage and delays endothelial cellular senescence.[13] Similar to our findings on cilostazol, we have previously shown that statin has a pleiotropic anti-oxidative effect other than a direct cholesterol-lowing effect, resulting in the reduction of stroke volume.[14-16] In the present study, we showed a strong reduction in oxidative damage by cilostazol (Fig. 4), which accounted for the reduction in spontaneous infarct volume compared with the other anti-platelet drugs (Fig. 3). In CSPS 2, it was found that cilostazol is superior to aspirin for preventing stroke and reducing hemorrhagic complications with probable cerebrovascular endothelial protection.[17] On the other hand, in our study, the infarct volume of clopidogrel tended to be smaller than that in the vehicle-treated group and was smaller than the aspirin-treated

group (Fig. 3). This may represent a beneficial effect of clopidogrel on cerebrovascular endothelium as reported in the second Trial of Cilostazol in Symptomatic Intracranial Arterial Stenosis (TOSS 2) study for preventing intracranial atherosclerotic progression.[18]

Previous studies have shown that cilostazol increases rCBF in a rat model of chronic cerebral hypoperfusion,[19, 20] and that cilostazol improves rCBF in the penumbral region in a rat model of middle cerebral artery occlusion.[21] In clinical studies, cilostazol also increases rCBF compared with ticlopidine at the chronic stage of cerebral infarction with a vasodilating effect.[22] In the present study, cilostazol tended to maintain rCBF compared with vehicle and the other 2 anti-platelet drugs, (Fig. 1). This vasodilating effect of cilostazol could also play a part in reducing spontaneous infarct volume (Fig. 3).

The IGF-1/IGF-1R signaling pathway plays an important role in growth and anabolic effects[23] as well as in learning and memory.[24] A previous study showed that serum IGF-I levels in patients with Alzheimer's disease were lower than those in normal controls.[25] Mice with reduced serum IGF-1 levels have an impaired spatial learning, and this effect is partially reversed by administrating IGF-1 subcutaneously.[26] A previous study showed that cilostazol has a beneficial effect against the decline in spatial learning

by increasing cAMP and IGF-1 levels in the hippocampus,[27] and IGF-I exerts beneficial effects by increasing synaptic plasticity, neurotransmission and neurogenesis in the hippocampus.[28, 29] IGF-1 binds to tyrosine kinase receptor (IGF-1R), which is structurally similar to the insulin receptor.[30] Because blocking IGF-1R by β-amyloid (1-42) results in preventing memory function,[31] the present results of cilostazol up-regulating IGF-1R positive cells in hippocampal CA1 and increasing IGF-1R expression in the hippocampus (Fig. 5) may partly account for the memory improvement (Fig. 2). A very recent study showed an improved cognitive function in Alzheimer's disease model mice by decreasing β-amyloid accumulation by cilostazol.[7]

In the present study, a possible pleiotropic effect of cilostazol resulted in the reduction of spontaneous infarct volume and preservation of motor and spatial cognitive functions. An increase in IGF-1R positive cells and IGF-1R expression in the hippocampus could also play an important role in preserving spatial cognitive function. These pleiotropic effects of cilostazol may partly account for the recent clinical study on cilostazol (CSPS 2, 2010; TOSS 2, 2011).

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Disclosures

None

References

Figure Legends

Fig. 1. The time-dependent changes of systolic blood pressure (SBP), diastolic blood pressure (DBP) and regional cerebral blood flow (rCBF) in the 4 groups. SBP and DBP showed similar elevations among the 4 groups at 8, 9 and 10 W. Regional CBF showed a progressive decrease with age in the vehicle-, aspirin- and clopidogrel-treated groups, but it was preserved in the cilostazol-treated group (p =n.s. among the 4 groups at 8, 9, and 10 W).

Fig. 2. The mean time until falling off from the rotarod (A) and mean escape latency in the water maze task (B) in the 4 groups. In the vehicle-, aspirin- and clopidogrel-treated groups, the drop off time was greatly decreased at 10 W. In contrast, only the

cilostazol-treated group had a preserved rotarod score at 10 W compared with the other 3 groups ($^{\S}p<0.05$ vs the other 3 groups). In the water maze task, only the cilostazol-treated group showed a gradual decrease in time taken with age and was significantly shorter than that in the vehicle-treated group at 10 W ($^{*}p<0.05$ vs vehicle).

Fig. 3. Spontaneous infarct volume with NeuN staining in SHR-SP in the 4 groups. White lines show the border of the infarction in the upper figures. Quantitative analysis showed that the infarct volume in the clopidogrel-treated group was significantly decreased with a further decrease in the cilostazol-treated group compared with that in the vehicle- and aspirin-treated groups. $^{*}p<0.05$ vs vehicle, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs aspirin.

Fig. 4. Immunohistochemistry for HEL, 4-HNE, AGE, and 8-OHdG at the boundary zone of spontaneous infarction in the 4 groups (A), and quantitative analysis of the positive cells (B). The number of positive cells tended to decrease in the clopidogrel-treated group and was significantly decreased in the cilostazol-treated group compared with that in the vehicle- and aspirin-treated groups for all the 4 oxidative stress markers. $^{*}p<0.05$, $^{*}p<0.01$ vs vehicle, $^{\#}p<0.05$ vs aspirin; scale bars = 50 μm, 10 μm (inset).

Fig. 5. Cresyl violet (CV) staining (A, a to d), immunohistochemistry for IGF-1R in the dentate gyrus (DG), CA3, and CA1 regions of the hippocampus (A, e to p), quantitative

analysis of the IGF-1R positive cell density ratio (B) and western blotting for IGF-1Rβ in the hippocampus (C) in the 4 groups. The ratio of IGF-1R positive cells to total cell number with CV staining in the CA1 region showed a significant increase in the cilostazol group compared with that in the vehicle- and aspirin-treated groups. The ratio of IGF-1Rβ to β-tubulin in the hippocampus showed a significant increase in the cilostazol-treated group compared with that in the vehicle-treated group. $^{*}p<0.05$ vs vehicle, $^{\#}p<0.05$ vs aspirin; scale bars = 500 μm (d), 100 μm (p), 10 μm (p, inset).

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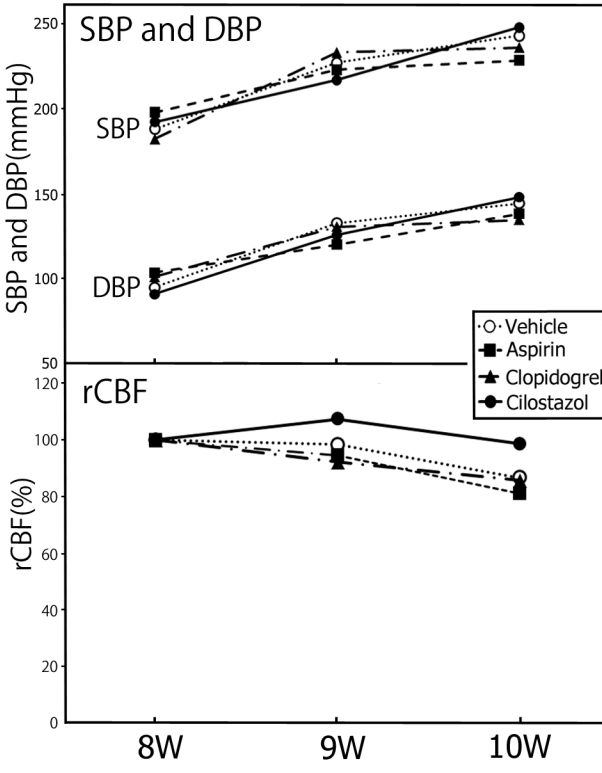
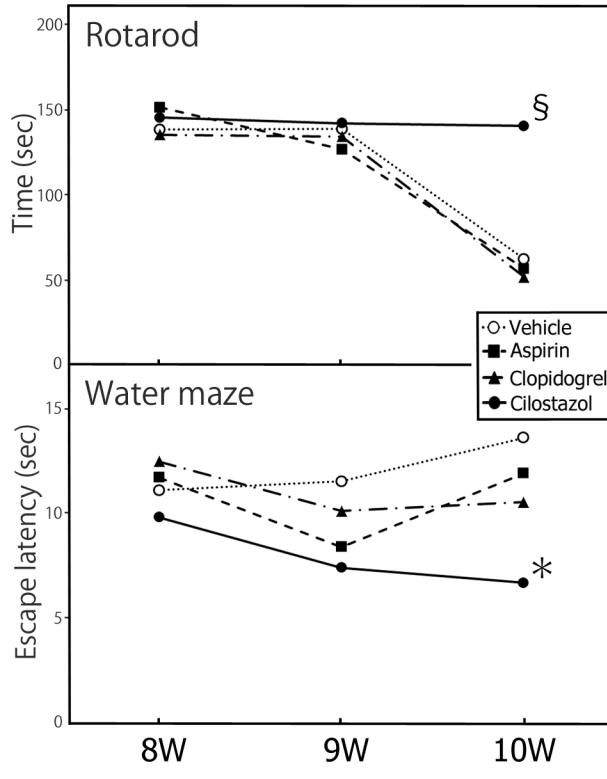
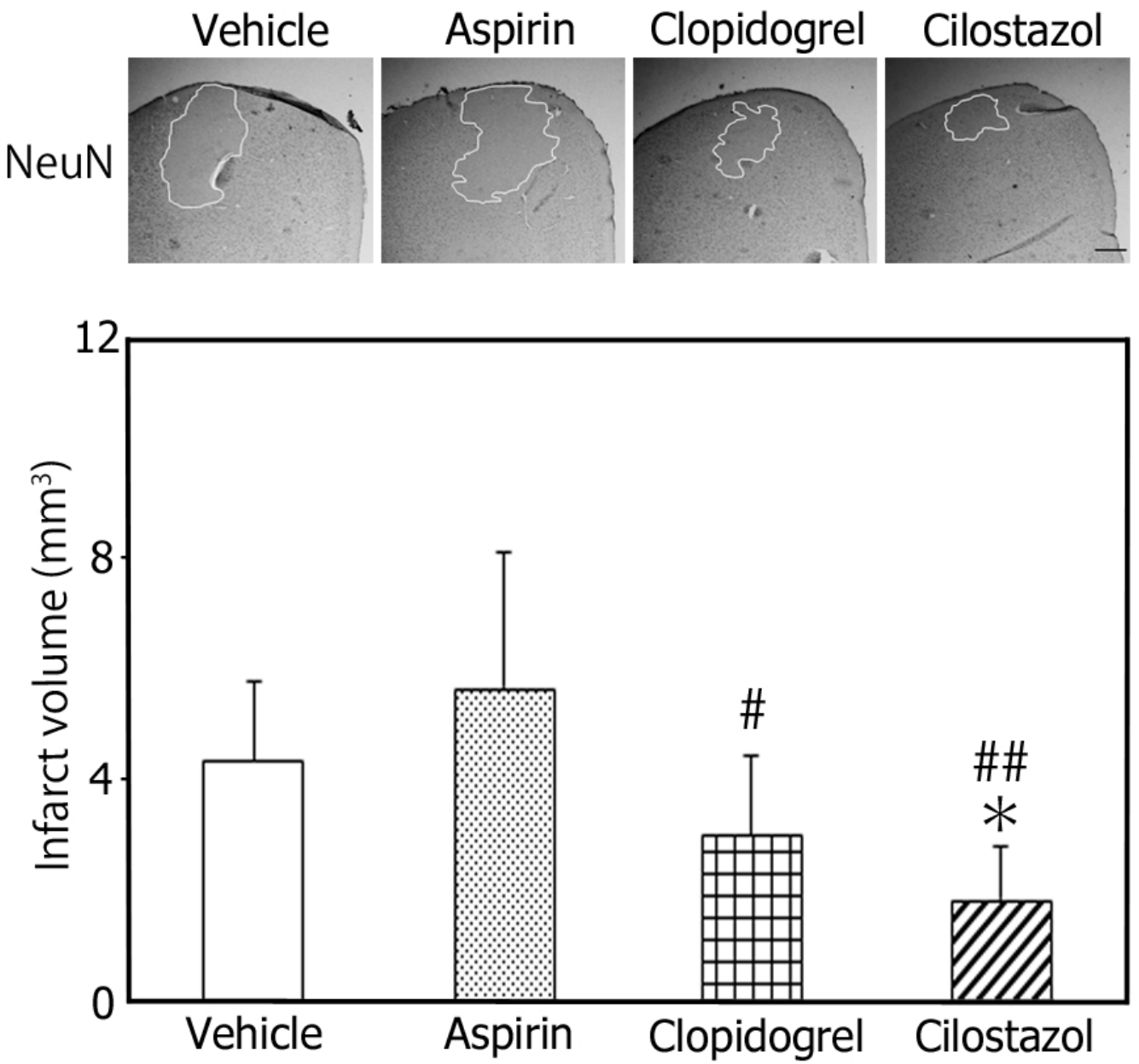
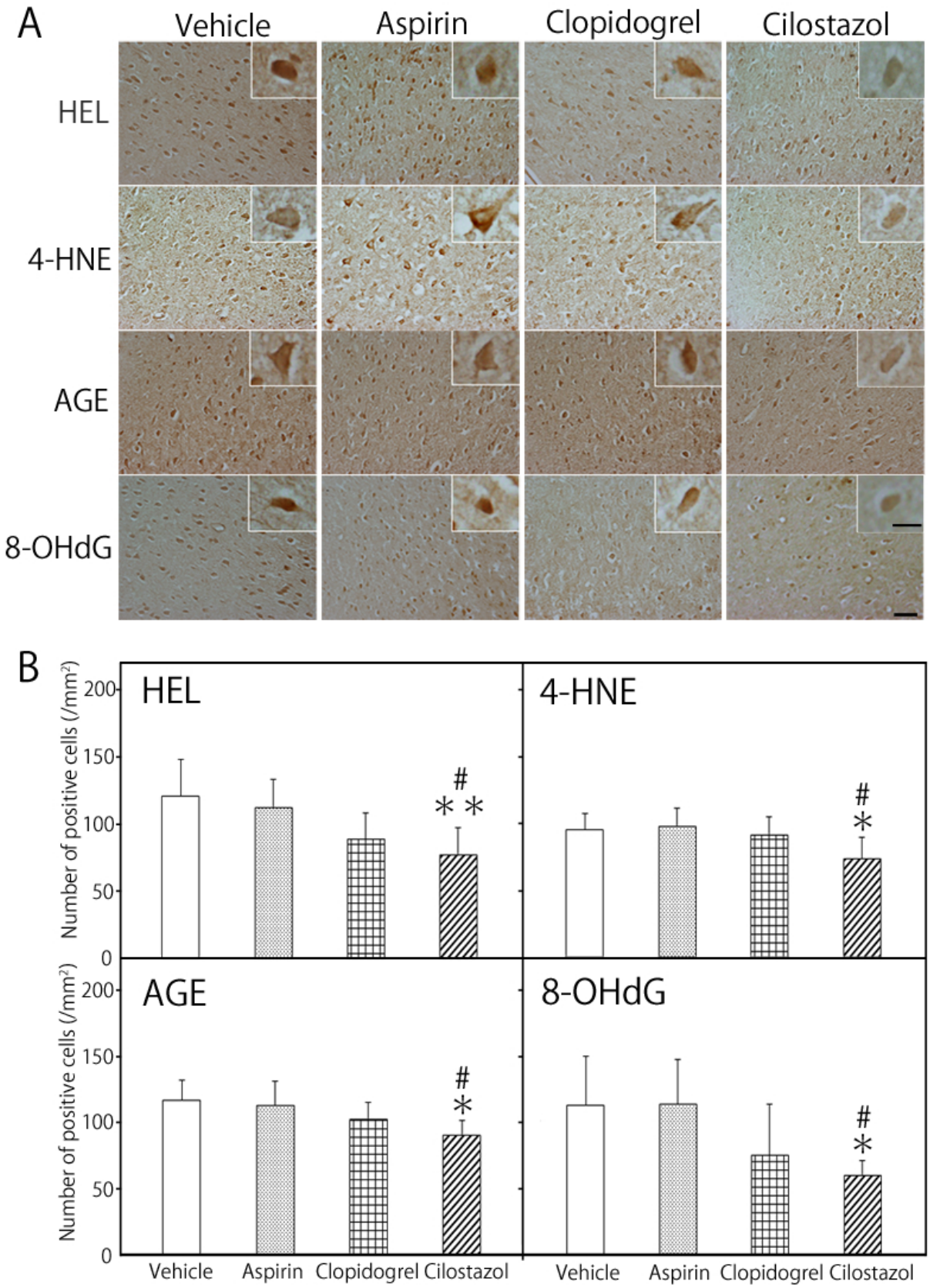
Table 1 Physiological and Biochemical Parameter at 10W

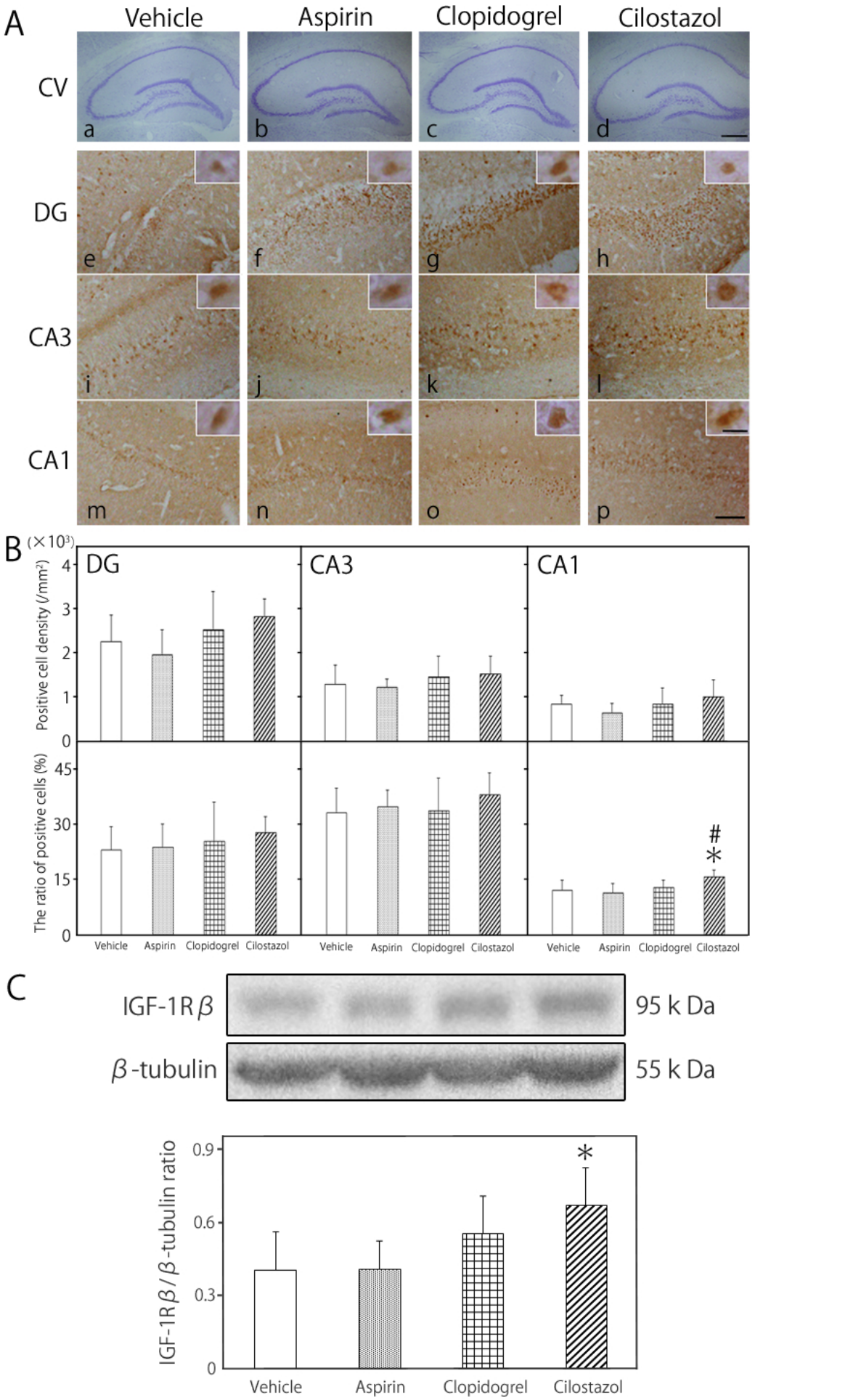
	Vehicle (n=7)	Aspirin (n=7)	Clopidogrel (n=7)	Cilostazol (n=7)
Body weight (g)	177.1 ± 9.0	171.3 ± 15.2	181.1 ± 5.3	179.4 ± 11.5
Systolic BP (mm Hg)	243.6 ± 23.9	231.9 ± 23.1	234.7 ± 29.3	248.1 ± 32.9
Diastolic BP (mm Hg)	144.1 ± 19.3	135.7 ± 10.4	134.3 ± 13.5	147.9 ± 36.7
Mean BP (mmHg)	177.2 ± 19.3	167.7 ± 9.7	167.6 ± 14.3	181.1 ± 34.7
Pulse rate (/min)	463.3 ± 46.4	470.1 ± 51.4	450.7 ± 55.4	475.7 ± 48.8
Survival time (d)	14.3 ± 1.3	14.3 ± 1.9	14.3 ± 1.5	14.6 ± 1.1
rCBF (ml/min/100g)	33.8 ± 5.6	33.5 ± 6.3	34.6 ± 4.2	37.6 ± 3.8
TG (mg/dL)	159.9 ± 134.4	83.7 ± 77.8	220.7 ± 163.3	98.6 ± 49.0
T-cho (mg/dL)	288.1 ± 197.0	216.7 ± 138.8	349.3 ± 126.6	268.0 ± 106.7
HDL-cho (mg/dL)	39.9 ± 4.7	34.4 ± 7.8	35.4 ± 9.9	33.4 ± 6.4
LDL-cho (mg/dL)	92.7 ± 92.8	64.7 ± 63.0	126.6 ± 65.8	92.7 ± 56.1


SBP and DBP
SBP and DBP(mmHg)
250
200
150
100
50
SBP
DBP
Vehicle
Aspirin
Clopidogrel
Cilostazol
rCBF
rCBF(%)
120
100
80
60
40
20
0
8W
9W
10W


Rotarod
Time (sec)
200
150
100
50
0
§
Vehicle
Aspirin
Clopidogrel
Cilostazol
Water maze
Escape latency (sec)
15
10
5
0
*
8W
9W
10W


Vehicle
Aspirin
Clopidogrel
Cilostazol
NeuN
12
8
4
0
Infarct volume (mm³)
#
##
*
Vehicle
Aspirin
Clopidogrel
Cilostazol


A
Vehicle
Aspirin
Clopidogrel
Cilostazol
HEL
4-HNE
AGE
8-OHdG
B
HEL
4-HNE
AGE
8-OHdG
Number of positive cells (/mm²)
0
50
100
150
200
#
*
**
Vehicle
Aspirin
Clopidogrel
Cilostazol


A
Vehicle
Aspirin
Clopidogrel
Cilostazol
CV
DG
CA3
CA1
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
B
(×10³)
Positive cell density (/mm²)
The ratio of positive cells (%)
DG
CA3
CA1
Vehicle
Aspirin
Clopidogrel
Cilostazol
#
*
C
IGF-1Rβ
β-tubulin
95 k Da
55 k Da
IGF-1Rβ/β-tubulin ratio
0.9
0.6
0.3
0
*
Vehicle
Aspirin
Clopidogrel
Cilostazol