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

Yoshio Omote, MD; Kentaro Deguchi, MD; FengFeng Tian, BS; Hiromi Kawai, BS; Tomoko Kurata, MD; Toru Yamashita, MD, PhD; Yasuyuki Ohta, MD, PhD; Koji Abe, MD, PhD

Background and Purpose—Cerebral infarction is a major cause of death or decreasing activities of daily living. This study aimed to investigate the efficacy of commonly used antiplatelet 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 were treated with vehicle, aspirin, clopidogrel, and cilostazol from 8 to 10 weeks of age. Physiological parameters, regional cerebral blood flow, and serum lipids were examined. Motor and cognitive functions were evaluated weekly by the Rotorod 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-1R expression in the hippocampus was assessed by Western blotting.

Results—The antiplatelet 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 stroke-prone spontaneously hypertensive rats regardless of blood pressure, regional cerebral blood flow, or serum lipid levels.

Conclusions—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 stroke-prone spontaneously hypertensive rats. (Stroke. 2012;43:1639-1646.)

Key Words: cerebral infarction ● cilostazol ● IGF-1R ● oxidative stress ● SHR-SP

Cerebral infarction is a major cause of death and decreasing the activities of daily living, and prevention of stroke is an important problem that needs to be solved. Antiplatelet 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 to cause stroke, vascular dementia, and Alzheimer disease. Recent studies have reported that improvement of such vascular risk factors reduces risks for both stroke and dementia. Among antiplatelet drugs, aspirin is a cyclo-oxygenase inhibitor that reduces thromboxane A2 and subsequent inflammation. Clopidogrel is an adenosine diphosphate P2Y12 receptor antagonist that has antiplatelet and vasodilating effects by increasing nitric oxide and prostaglandin I2. Cilostazol is a phosphodiesterase III inhibitor that has not only antiplatelet effects, but also antioxidative and vasodilating effects through increasing intracellular cAMP and endothelial nitric oxide synthase 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 disease in a mouse model. Therefore, in the present study, we first investigated the difference in efficacy of 3 antiplatelet 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 next week, at 8 weeks of age, animals began to be fed a high fat and cholesterol 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 weeks. Body weight, blood pressure (BP), and pulse rate were measured at 8, 9, and 10 weeks, and measurements for BP and pulse rate were performed with the tail-cuff method (Softron BP90A, Tokyo, Japan). Regional cerebral blood flow was measured using a laser-Doppler flowmeter (FLO-C1; Omegawave, Tokyo, Japan) at 8, 9, and 10 weeks. Changes in rCBF at 9 and 10 weeks were expressed as a percentage of rCBF at 8 weeks.

Motor Behavior Test With the Rotorod and Morris Water Maze Task
Motor coordination and balance were evaluated by measuring latency (seconds) until falling off from a rotating rod (4-lane Rotorod; UGO BASILE, Comerio, Italy) according to our previous report. Briefly, rats were placed on the rod with the rotation speed accelerating from 4 to 40 rpm over the course of 5 minutes. This procedure was repeated 3 times per day in each rat, and averaged data were recorded as the Rotorod time at 8, 9, and 10 weeks.

A circular water maze pool (150 cm diameter) was filled with opaque water at 25°C to 26°C, which contained a platform submerged 2 cm below the surface of water to escape. The rat was placed in the water at 1 random start location. Rats were allowed to find the submerged platform within 90 seconds and rest on it for 15 seconds after climbing up. If the rat failed to find the platform within 90 seconds, it was placed on it for 15 seconds. This procedure was repeated 4 times per day with 1-minute intervals between trials for 3 consecutive days per week from 7 to 10 weeks. 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. Briefly, 5 coronal sections (12 μm) at 2-mm intervals of each rat were treated with antineuronal nuclei antibodies (1:200; MAB377; Chemicon). They were then incubated with biotinylated secondary antibodies and the signals were visualized with dianinobenzidine tetrahydrochloride. Areas devoid of neuronal nuclei 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-(hexanoyl)lysine (HEL), 4-hydroxynonanal (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 peroxide/methanol for 10 minutes to block endogenous peroxidase activity and incubated with bovine serum albumin for 1 hour. 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 hours at room temperature; biotinylated antimouse monoclonal antibody (1:500; Vector Laboratories) was used for HEL, 4-HNE, AGE, and 8-OHdG, and biotinylated antigoat monoclonal antibody (1:500; Vector Laboratories) was used for IGF-1R. The sections were then incubated with avidin–biotin–peroxidase complex (Vectastain ABC kit; Vector Laboratories) for 30 minutes and visualized with diaminobenzidine tetrahydrochloride.

Cell Density of the Hippocampus and Cell Counts
A set of sections (n=7 each) was stained with 0.1% cresyl violet for histological examination of the cell density of the hippocampus. The numbers of positive cells for cresyl violet staining in the dentate gyrus (DG), CA3, and CA1 regions of the hippocampus were counted in 3 randomized 0.01-mm² 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 cresyl violet 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 weeks 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 minutes 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 phosphate-buffered saline (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. The primary antibody was anti-IGF-1Rβ (1:1000; Cell Signaling Technology). 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 1-factor analysis of variance followed by a Tukey–Kramer postcomparison. 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 body weight were not significantly different among the 4 groups (Table). The time-dependent changes in systolic BP and diastolic BP in the 4 groups are shown in Figure 1. In all 4 groups (n=7 in each group and time point), systolic BP increased with age. In the vehicle-treated group, systolic BP at 8 weeks was 190.4±6.7 mm Hg (mean±SD), which progressively escalated at 9 and 10 weeks to 227.2±15.6 (††P<0.01 versus 8 weeks) and 243.6±23.9 (††P<0.01 versus 8 weeks), respectively. Aspirin-, clopidogrel-, and cilostazol-treated groups also showed similar systolic BP changes at 9 weeks (††P<0.01 versus 8 weeks) and 10 weeks (†P<0.05 versus 8 weeks) for the aspirin-treated group, at 9 weeks (††P<0.01 versus 8 weeks) and 10 weeks (††P<0.01 versus 8 weeks) for the clopidogrel-treated group, and at 9 weeks (††P<0.01 versus 8 weeks) and 10 weeks (††P<0.01 versus 8 weeks) for the cilostazol-treated group. There were no differences in systolic BP among the 4 groups at 8, 9, or 10 weeks. Diastolic BP in the 4 groups showed similar significant increases (Figure 1), but these were not different among the groups. Mean BP and pulse rate were also not significantly different among the groups.

The time-dependent course of rCBF in the 4 groups is shown in Figure 1. In the vehicle-treated group (n=7 at each time point in all groups), rCBF showed a progressive decrease from 8 weeks to 10 weeks (†P<0.05, 10 versus 8 weeks). In the aspirin-treated group, rCBF also showed a progressive decrease from 8 weeks to 10 weeks (†P<0.05, 10 versus 8 weeks), and the clopidogrel-treated group showed a progressive decrease from 8 weeks to 10 weeks. In contrast, the cilostazol-treated group showed a small increase in rCBF from 8 weeks to 9 weeks (107.4%±15.0% of 8 weeks) and only a small decrease at 10 weeks (98.6%±9.9% of 8 weeks). However, there was no significant difference in rCBF among the 4 groups at 8, 9, or 10 weeks (Table). Serum triglyceride, total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol levels were not significantly different among the 4 groups at 8 and 10 weeks (Table).

Rotorod and Morris Water Maze Task

The mean times until falling off from the Rotorod in the 4 groups are shown in Figure 2. In the vehicle-treated group (n=7 in each group), the dropoff times at 8 and 9 weeks were
138.0±38.6 seconds and 138.4±26.3 seconds, respectively, and this time was greatly decreased at 10 weeks to 58.7±53.5 seconds (††P<0.01 versus 8 weeks, †P<0.05 versus 9 weeks). The aspirin- and clopidogrel-treated groups showed similar changes as the vehicle-treated group. In contrast, only the cilostazol-treated group showed preserved Rotorod scores at 8, 9, and 10 weeks (§P<0.05 versus the other 3 groups).

The water maze task of the 4 groups is shown in Figure 2. In the vehicle-treated group (n=7 in each group), the latency time showed a gradual extension in time from 8 weeks (11.1±5.7 seconds) to 9 (11.5±7.7 seconds) and 10 weeks (13.6±5.9 seconds). In the aspirin- and clopidogrel-treated groups, the latency time showed no change at 8, 9, and 10 weeks. In contrast, 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 weeks (P<0.05 versus vehicle).

**Spontaneous Infarct Volume in SHR-SP**

The spontaneous infarct volumes evaluated with neuronal nuclei staining are shown in Figure 3. The infarct volumes in vehicle- and aspirin-treated groups were 4.4±1.5 mm³ and 5.7±2.4 mm³ (n=10 in each group). The infarct volume in the clopidogrel-treated group (n=10) showed a significant decrease (3.0±1.4 seconds, #P<0.05 versus aspirin) with a further decrease in the cilostazol-treated group (1.8±1.0 seconds, n=10) compared with both the vehicle- and aspirin-treated groups (*P<0.05 versus vehicle, ##P<0.01 versus 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 Figure 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/mm² and 113.6±20.4/mm², respectively (Figure 4). The clopidogrel-treated group tended to show a decrease in number to 89.4±19.3/mm², and a significant decrease in the cilostazol-treated group to 78.1±19.8/mm² (***P<0.01 versus vehicle, #P<0.05 versus 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 versus vehicle, #P<0.05 versus aspirin), AGE (*P<0.05 versus vehicle, #P<0.05 versus aspirin), and 8-OHdG (*P<0.05 versus vehicle, #P<0.05 versus aspirin).
Hippocampal Histology, Immunohistochemistry, and Western Blotting

Typical cresyl violet staining for the hippocampus is shown in Figure 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 weeks was not significantly different among the 4 groups in the DG, CA3, or CA1 regions.

Immunoreactive IGF-1R staining is shown in Figure 5. IGF-1R-positive cell density of hippocampal neurons in vehicle-, aspirin-, clopidogrel-, and cilostazol-treated groups (n=7 in each group) is shown in Figure 5B. The ratio of IGF-1R-positive cells to total cell number with cresyl violet 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 versus vehicle, #P<0.05 versus 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 Figure 5C. The ratio 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 versus vehicle).

Discussion

The present study showed that among the 3 antiplatelet 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-1Rβ-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-SPs develop an accelerated hypertension with subsequent cerebrovascular injury by...
loading salt and this serves as a good experimental model for spontaneous stroke. The SHR-SP model is also known as cerebral small-vessel disease and vascular dementia models, which are characterized by multiple lacunar infarctions and white matter lesions. As we previously reported, the double loading of salt and high fat and cholesterol strongly promotes arteriosclerotic change and increases the risk of infarction. Recent studies have shown that salt loading increases superoxide production and potentiates reduced nicotinamide-adenine dinucleotide phosphate oxidase activity in the brain.

In the present study, we evaluated the end products of reactive oxygen species by immunohistochemistry for HELP, 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-SPs.

In addition to the primary pharmacological activity of cilostazol of inhibiting phosphodiesterase III for increasing intracellular cAMP and endothelial nitric oxide synthase activity, cilostazol has a further antioxidative effect among the 3 antiplatelet drugs. cAMP activates protein kinase A/phosphatidylinositol 3-kinase/Akt signaling pathways,

Figure 5. Cresyl violet (CV) staining (A, a–d); immunohistochemistry for IGF-1R in the dentate gyrus (DG), CA3, and CA1 regions of the hippocampus (A, e–p); quantitative analysis of the IGF-1R-positive cell density ratio (B); and Western blotting for IGF-1R 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-1R/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 versus vehicle, #P<0.05 versus aspirin; scale bars=500 μm (d), 100 μm (p), 10 μm (p, inset). IGF-1R indicates insulin-like growth factor 1 receptor.
which are important for cell survival. Produced endothelial nitric oxide synthase protects vascular endothelium against oxidative damage and delays endothelial cellular senescence. Similar to our findings on cilostazol, we have previously shown that statin has a pleiotropic antioxidative effect other than a direct cholesterol-lowering effect, resulting in the reduction of stroke volume. In the present study, we showed a strong reduction in oxidative damage by cilostazol (Figure 4), which accounted for the reduction in spontaneous infarct volume compared with the other antiplatelet drugs (Figure 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. 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 (Figure 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.

Previous studies have shown that cilostazol increases rCBF in a rat model of chronic cerebral hyperperfusion and that cilostazol improves rCBF in the penumbra region in a rat model of middle cerebral artery occlusion. In clinical studies, cilostazol also increases rCBF compared with ticlopidine at the chronic stage of cerebral infarction with a vasodilating effect. In the present study, cilostazol tended to maintain rCBF compared with vehicle and the other 2 antiplatelet drugs (Figure 1). This vasodilating effect of cilostazol could also play a part in reducing spontaneous infarct volume (Figure 3).

The IGF-1/IGF-1R signaling pathway plays an important role in growth and anabolic effects as well as in learning and memory. A previous study showed that serum IGF-1 levels in patients with Alzheimer disease were lower than those in normal control subjects. Mice with reduced serum IGF-1 levels have an impaired spatial learning, and this effect is partially reversed by administrating IGF-1 subcutaneously. 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, and IGF-1 exerts beneficial effects by increasing synaptic plasticity, neurotransmission, and neurogenesis in the hippocampus. IGF-1 binds to tyrosine kinase receptor (IGF-1R), which is structurally similar to the insulin receptor. Because blocking IGF-1R by β-amyloid (1–42) results in preventing memory function, the present results of cilostazol upregulating IGF-1R-positive cells in hippocampal CA1 and increasing IGF-1R expression in the hippocampus (Figure 5) may partly account for the memory improvement (Figure 2). A very recent study showed an improved cognitive function in an Alzheimer disease model mice by decreasing β-amyloid accumulation by cilostazol.

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
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Abstract

背景および目的：脳梗塞は死亡や日常生活の活動性低下の重大な原因となっている。本研究では、広く用いられている抗血小板薬が脳卒中ならびに運動および認知機能に及ぼす影響を、酸化ストレスマーカーおよびインスリン様成長因子1受容体（IGF-1R）と関連づけて検討することを目的とした。

方法：脳卒中易発症-高血圧自然発症ラットに、賦形剤、アスピリン、クロピドグレルおよびシロスタゾールを生後8週から10週まで投与した。生理学的パラメータ、局所脳血流量および血清脂質値を測定した。運動および認知機能は、ロータロッドテストと水迷路試験で週1回評価した。自然発症梗塞の容積、自然発症梗塞の虚血境界域における脂質、蛋白およびDNAの酸化ストレスマーカー、および海馬におけるIGF-1R陽性細胞の比率を、脳切片で免疫組織化学的に評価した。海馬におけるIGF-1Rの発現を、ウェスタンブロット法で評価した。

結果：抗血小板薬シロスタゾールおよびクロピドグレルは自然発症梗塞の容積をアスピリンよりも減少させた。シロスタゾールのみが運動機能と認知機能を改善し、海馬の記憶関連IGF-1Rの陽性率およびIGF-1Rβの発現を有意に上昇させた（p < 0.05）。シロスタゾールは脳卒中易発症-高血圧自然発症ラットの罹患したニューロンにおいて血圧、局所脳血流量、または血清脂質値に関わりなく、4種類の酸化ストレスマーカーを減少させた。

結論：本研究の結果は、シロスタゾールの潜在的な多面発現性効果は自然発症梗塞の容積を縮小させ、運動ならびに空間認知機能を維持することを示唆している。脳卒中易発症-高血圧自然発症ラットにおける空間認知機能の保存は、海馬CA1領域におけるIGF-1R陽性細胞の増加によって、部分的説明できると考えられる。

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