Background and Purpose—Very recent subgroup analysis from the PROspective pioglitAzone Clinical Trial In macroVascular Events has shown that pioglitazone reduces the risk of recurrent stroke in type 2 diabetic patients. However, the underlying mechanism of stroke prevention by pioglitazone is unknown. Our aim was to examine the effect of pioglitazone on hypertension-based stroke in rats.

Methods—Pioglitazone (1 mg·kg⁻¹·d⁻¹) was orally administered to stroke-prone spontaneously hypertensive rats (SHRSP) to examine the effect on incidental stroke, cerebrovascular injury, brain inflammation, oxidative stress, and vascular endothelial dysfunction induced by hypertension.

Results—Treatment of SHRSP with pioglitazone for 4 weeks, without affecting blood pressure and blood glucose values, improved vascular endothelial dysfunction (P < 0.05), suppressed remodeling of the middle cerebral artery (P < 0.05) and brain microvessels (P < 0.05), and inhibited brain macrophage infiltration (P < 0.05) and the upregulation of brain monocyte chemoattractant protein-1 and tumor necrosis factor-α expression (P < 0.01). Furthermore, pioglitazone treatment significantly delayed the onset of stroke signs and death in SHRSP (P < 0.05). These beneficial effects of pioglitazone on cerebrovascular injury and stroke in SHRSP were associated with a reduction of brain and vascular superoxide via the inhibition of NADPH oxidase activity.

Conclusions—Our work provides the first evidence that pioglitazone significantly protects against hypertension-induced cerebrovascular injury and stroke by improving vascular endothelial dysfunction, inhibiting brain inflammation, and reducing oxidative stress. These beneficial effects of pioglitazone were independent of blood pressure or blood sugar values. Thus, pioglitazone appears to be a potential therapeutic agent for stroke in type 2 diabetes with hypertension.

Key Words: endothelium ▪ hypertension ▪ inflammation ▪ stroke

Pioglitazone is an agonist of peroxisome proliferator–activated receptor-γ (PPAR-γ) and is a useful drug for treatment of type 2 diabetes. The PROspective pioglitAzone Clinical Trial In macroVascular Events (PROACTIVE) was a large, prospective study whose goal was to ascertain whether pioglitazone reduces macrovascular morbidity and mortality in high-risk patients with type 2 diabetes. Very recently, the PROACTIVE investigators conducted analyses in patients who had entered the PROACTIVE with or without a history of stroke and found that pioglitazone significantly reduced the risk of recurrent stroke in high-risk patients with type 2 diabetes, supporting the notion that pioglitazone may be useful for prevention of stroke in diabetic patients. However, because the benefit of glucose-lowering therapy in stroke prevention is unclear, the underlying mechanism of prevention of stroke by pioglitazone in the PROACTIVE study is unknown. Furthermore, even in experimental animals, the effect of pioglitazone on prevention of stroke remains to be defined.

Hypertension is well established to be the strongest predictor for stroke in patients with diabetes as it is in the general population. Very interestingly, in a subanalysis of the PROACTIVE study, 83% of patients with previous stroke had a history of hypertension. Therefore, it is crucial to examine whether or not pioglitazone is effective for the prevention of hypertension-based stroke. The stroke-prone spontaneously hypertensive rat (SHRSP) is regarded as a useful model of human hypertensive encephalopathy, char-
acterized by cerebral hemorrhage and infarction, is extensively used for study of the effect of drugs on stroke. In the present study, we have obtained the first evidence that pioglitazone significantly prevents cerebrovascular injury and incidental stroke in SHRSP, independent of blood pressure or blood sugar values.

Materials and Methods

Animals
Male SHRSP and Wistar-Kyoto rats (WKY) were purchased from Japan SLC (Shizuoka, Japan). WKY rats are genetic controls for SHRSP. They were fed standard laboratory rat chow (CE2 Clea, Japan) and given tap water ad libitum. All procedures were in accordance with institutional guidelines for the care and use of laboratory animals.

Treatment of SHRSP With Pioglitazone
Eleven-week-old SHRSP were randomly assigned to 2 groups and were orally given vehicle (0.5% carboxymethylcellulose) or pioglitazone (1 mg·kg⁻¹·d⁻¹) for 4 weeks. Pioglitazone or vehicle was given to SHRSP by gastric gavage once a day. Blood pressure and heart rate were measured before and 1, 2, and 4 weeks after the start of drug treatment. Blood pressure of conscious rats was measured by the tail-cuff method (BP-98A; Softron Co, Tokyo, Japan). After 4 weeks of treatment, SHRSP and control age-matched WKY were anesthetized with ether, and blood was collected by cardiac puncture to measure blood glucose and plasma insulin values. After perfusion with phosphate-buffered saline, the brain, heart, carotid artery, aorta, and kidney were rapidly excised from SHRSP and WKY for plasma insulin levels were quantified by using the commercial reagent (Nippon Gene), according to the manufacturer’s instructions. One microgram of RNA sample was reverse-transcribed to first-strand cDNA with the QuantiTect reverse transcription kit (Qiagen), according to the manufacturer’s recommended protocol. The Thermal Cycler Dice real-time system (Takara) was used for 2-step reverse transcription–polymerase chain reaction. cDNA was amplified with SYBR Premix ExTaQ with specific oligonucleotide primers available online at http://stroke.ahajournals.org) or glyceraldehyde 3-phosphate dehydrogenase.

Brain and Vascular NADPH Oxidase Activity
The brain cortex and aortic tissues were homogenized with an Ultraturrax T8 and centrifuged, and NADPH oxidase activity of the resulting supernatant was measured by lucigenin chemiluminescence in the presence of 10 μmol/L NADPH and 10 μmol/L lucigenin as the electron acceptor, as described in detail by us. Protein concentrations were measured by the method of Bradford.

Measurement of Brain and Vascular Superoxide
The brain cortex and carotid arteries removed from SHRSP or WKY, were immediately frozen in Tissue-Tek OCT embedding medium (Sakura Finetek) and sectioned (10 μm) with a cryostat directly onto chilled microscope slides. Dihydrothiouridum was used to evaluate superoxide levels in the brain cortex and carotid artery in situ, as described in detail by us. Western blot analysis

Preparation of Brain Protein Extracts and Western Blot Analysis
Our detailed method has been described previously. In brief, after protein extracts of brain cortex and vascular tissues were subjected to sodium dodecy sulfates–polyacrylamide gel electrophoresis and electric transfer to polyvinylidene difluoride membranes, they were probed with a specific rabbit anti-p22 phox (×2000, Santa Cruz B), anti–Cu/Zn superoxide dismutase (SOD) antibody (×5000, Stressgen Biotechnologies), and then an anti–glyceraldehyde 3-phosphate dehydrogenase antibody (×10 000, Santa Cruz Biotechnologies). In individual samples, each value was corrected against that of glyceraldehyde 3-phosphate dehydrogenase.

NOS Activity (Arginine-to-Citrulline Conversion)
The activity of Ca²⁺–dependent nitric oxide synthase (NOS) (neuronal NOS and endothelial NOS (eNOS)) of the brain cortex and aorta was determined by measuring the conversion of [¹⁴C]arginine to [¹⁴C]citrulline with an NOS assay kit (Cayman Chemical).

Histologic Examination and Immunohistochemistry
The brain was sliced into horizontal sections, fixed with 10% formalin overnight, embedded in paraffin, cut into 4-μm-thick sections, and stained with hematoxylin-eosin or elastica van Gieson. Determination of the ratio of the lumen to wall area in the middle cerebral artery was performed in horizontal 4-μm-thick sections. Measurements were performed in the right hemispheres of 2 consecutive horizontal sections for each animal. The ratio of lumen to wall area of microvessels (intraparenchymal arterioles 30 to 50 μm in minimum transverse diameter with a round circumference) was calculated, as previously described. Three to 5 vessels per section, 2 sections per animal, were randomly selected, and the ratio of the lumen to wall area of each vessel was averaged for each animal.

Assessment of brain macrophage infiltration, the brain sections were immunostained with anti–ED-1 antibody (BMA Biomedicals AG; working dilution, 1:500) for identification of monocytes/macrophages, as described by us. The number of ED-1–positive cells was counted in 10 sections in individual rats; and the average ED-1–positive cell number was obtained for each rat.

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction
Total RNA was extracted from brain tissue with the use of Trizol reagent (Nippon Gene), according to the manufacturer’s instructions. One microgram of RNA sample was reverse-transcribed to first-strand cDNA with the QuantiTect reverse transcription kit (Qiagen), according to the manufacturer’s recommended protocol. The Thermal Cycler Dice real-time system (Takara) was used for 2-step reverse transcription–polymerase chain reaction. cDNA was amplified with SYBR Premix ExTaQ with specific oligonucleotide primers for target sequences of monocytic/monocytes/macrophages, as described by us. The number of ED-1–positive cells was counted in 10 sections in individual rats; and the average ED-1–positive cell number was obtained for each rat.

Measurement of Plasma Insulin
Plasma insulin levels were quantified by using the commercial ELISA kits from Morinaga.

Effect of Pioglitazone on Stroke and Survival Rate in SHRSP
To examine the effect of pioglitazone on incidental stroke in SHRSP, 11-week-old SHRSP were fed a high-sodium diet (8% NaCl diet) to accelerate the onset of stroke. They were given vehicle (n = 25) or pioglitazone (1 mg·kg⁻¹·d⁻¹) (n = 23) by gastric gavage once a day for 1 month (31 days). Blood pressure was measured by the tail-cuff method. The appearance of 1 or more of major stroke-associated signs, including paralytic gait, reduced motor activity, and
sudden death, was carefully monitored every day for 1 month (31 days). When 1 or more of these signs occurred in SHRSP, they were regarded as stroke sign–positive.

Statistics

Results are expressed as mean ± SEM. Statistical significance was determined by 1-way ANOVA, followed by Fisher’s protected least significant difference test, with StatView for Windows (SAS Institute, Inc, Cary, NC). The onset of stroke signs and death was analyzed by a standard Kaplan-Meier analysis with a log-rank test with a significant difference test, with StatView for Windows (SAS Institute, Inc, Cary, NC). The onset of stroke signs and death was carefully monitored every day for 1 month (31 days). When 1 or more of these signs occurred in SHRSP, they were regarded as stroke sign–positive.

Effect of Pioglitazone on Brain Superoxide, NADPH Oxidase Activity, p22<sub>phox</sub>, Cu/Zn SOD, and NOS Activity of SHRSP

As shown in Figure 1, brain cortical superoxide levels, NADPH oxidase activity, p22<sub>phox</sub>, and Cu/Zn SOD in SHRSP were 1.6-, 2.2-, 1.3-, and 1.4-fold, respectively, higher than those in WKY, whereas brain cortical Ca<sup>2+</sup>-dependent NOS activity (eNOS and neuronal NOS) in SHRSP was smaller than that in WKY. Pioglitazone treatment significantly alleviated the increase in brain cortical superoxide, NADPH oxidase activity, and p22<sub>phox</sub> of SHRSP by 49% (P<0.01), 35% (P<0.05), and 81% (P<0.01), respectively. However, brain cortical Cu/Zn SOD or NOS activity in SHRSP was not altered by pioglitazone treatment.

Effect of Pioglitazone on Brain Cortical Macrophage Infiltration and Expression of MCP-1 and TNF-α in SHRSP

As shown in Figure 2, brain cortical macrophage infiltration was significantly enhanced in SHRSP relative to WKY (P<0.01), and brain MCP-1 and TNF-α mRNA expressions in SHRSP were 2.5- (P<0.01) and 2.2-fold (P<0.01), respectively, higher than those in WKY. Pioglitazone treatment significantly reduced brain macrophage infiltration in SHRSP (P<0.01), and this effect of pioglitazone was associated with a significant attenuation of brain MCP-1 and TNF-α mRNA expression.

Effect of Pioglitazone on Remodeling of the Middle Cerebral Artery and Brain Arterioles of SHRSP

As shown in Figure 3, the ratio of lumen to wall area of the middle cerebral artery (0.32±0.01 vs 0.48±0.02, P<0.01) and arterioles (0.20±0.02 vs 0.40±0.03, P<0.01) was significantly smaller in SHRSP than WKY. Pioglitazone treatment significantly increased the ratio of lumen to wall area of the middle cerebral artery (P<0.05) and arterioles (P<0.01), indicating the suppression of cerebrovascular remodeling by pioglitazone.
Effect of Pioglitazone on Vascular Endothelial Function, Vascular Superoxide, NADPH Oxidase Activity, p22phox, and eNOS activity of SHRSP

As shown in Figure 4A, carotid arterial endothelium-dependent relaxation by Ach was significantly impaired in SHRSP relative to WKY (P<0.01), indicating significant impairment of vascular endothelial function in SHRSP. Pioglitazone treatment significantly improved Ach-induced vascular relaxation in SHRSP (P<0.05). Carotid arterial endothelium-independent relaxation by sodium nitroprusside did not significantly differ among WKY and SHRSP treated with vehicle and pioglitazone (data not shown).

As in the brain cortex, vascular superoxide levels, NADPH oxidase activity, and p22 phox in SHRSP were 2.6-fold (P<0.01), 1.9-fold (P<0.01), and 6.1-fold (P<0.01), respectively, higher than those in WKY (Figure 4B through 4D). Pioglitazone treatment significantly reduced vascular superoxide levels by 42% (P<0.01), and this effect was associated with significant attenuation of vascular NADPH oxidase activity and p22phox protein levels by pioglitazone (P<0.01). Although carotid arterial eNOS activity of SHRSP was lower than that of WKY (P<0.01), pioglitazone treatment did not alter vascular eNOS activity of SHRSP (Figure 4E).

Stroke and Survival Rate of Salt-Loaded SHRSP

To examine the preventive effect of pioglitazone against stroke in SHRSP, we examined the effect of pioglitazone on...
to stroke prevention by pioglitazone. The potential mechanism behind the reduction of stroke by pioglitazone in the PROACTIVE study remains to be elucidated.

Several previous investigations have addressed the effect of pioglitazone and other PPAR-γ agonists on focal, transient, cerebral acute ischemia, produced by occlusion of the middle cerebral or common carotid artery followed by reperfusion.14–16 The findings of those works have indicated that pioglitazone and other PPAR-γ agonists reduce infarct size and improve neurologic function in a transient, acute cerebral ischemia model.14–16 Previous experimental work on the

Discussion
To the best of our knowledge, our present work provides the first evidence that pioglitazone protects against hypertension-based stroke, independently of blood pressure or blood glucose values. Recent subanalysis from the PROACTIVE study2 supports the concept that pioglitazone seems to be a potential therapeutic agent for stroke in patients with type 2 diabetes. It has been shown that glucose-lowering therapy with other pharmacologic treatment (sulfonylurea or insulin) in type 2 diabetic patients does not significantly affect the incidence of stroke,3 and a 1% reduction in glycosylated hemoglobin is associated with only a 4% estimated decrease in risk of stroke (P=0.44).13 Thus, the marked reduction of stroke by pioglitazone in the PROACTIVE study cannot be explained by the magnitude of glycemic control by pioglitazone. Furthermore, blood pressure lowering by pioglitazone was not statistically significant in the PROACTIVE study,2 providing no evidence for the contribution of blood pressure

salt-loaded SHRSP, because salt loading is well known to markedly accelerate the onset of stroke in SHRSP.9,11,12 The blood pressure of vehicle-treated and pioglitazone-treated SHRSP was 223±5 and 226±3 mm Hg, respectively, at 1

week, and 235±2 and 231±4 mm Hg, respectively, at 2

weeks after the start of treatment. Thus, pioglitazone did not alter blood pressure of salt-loaded SHRSP, which was in good agreement with the lack of alteration of blood pressure in non–salt-loaded SHRSP by pioglitazone, as described earlier.

The appearance of 1 or more major stroke-associated signs was carefully monitored every day for 1 month (31 days), as described in Methods. As shown in Figure 5A, pioglitazone treatment significantly delayed the appearance of stroke signs in SHRSP (P<0.05). Furthermore, as shown in Figure 5B, pioglitazone also significantly prolonged the survival rate of SHRSP (P<0.05).
effect of pioglitazone on brain ischemia has been limited to a transient, acute cerebral ischemia model followed by reperfusion. However, the pathophysiologic characteristics of incidental stroke caused by risk factors, such as hypertension, markedly differ from those of stroke caused by transient, acute cerebral ischemia reported in previous work.\textsuperscript{14–16} To the best of our knowledge, there is no report evaluating the effect of pioglitazone on stroke caused by risk factors such as hypertension. Interestingly, \approx\,80\% of patients in the PROACTIVE study had a history of hypertension.\textsuperscript{2} Because hypertension is well established to be the major risk factor for stroke,\textsuperscript{4} it is a very critical question whether pioglitazone protects against hypertension-based stroke or not. Taken together with the fact that pioglitazone has multiple pleiotropic effects beyond the improvement of insulin resistance and a blood glucose–lowering effect,\textsuperscript{17} these findings encouraged us to examine the effect of pioglitazone on hypertension-induced stroke in SHRSP.

In this work, the dose of pioglitazone used was 1 mg·kg\textsuperscript{-1}·d\textsuperscript{-1}. In the present work, we did not examine the effect of pioglitazone on the PPAR-\gamma receptor due to technical difficulty. However, Sugiyama et al.\textsuperscript{18} previously examined the effect of various doses of pioglitazone on Wistar fatty rats, which are a useful model of type 2 diabetes, obesity, and moderate hypertension, and found that a dose of 1 mg·kg\textsuperscript{-1}·d\textsuperscript{-1} pioglitazone significantly improved insulin resistance, lowered blood glucose values, increased body weight, and reduced blood pressure in Wistar fatty rats. These findings strongly support that the 1 mg·kg\textsuperscript{-1}·d\textsuperscript{-1} dose of pioglitazone used in this study was sufficient to activate the PPAR-\gamma receptor in vivo. Therefore, the 1 mg·kg\textsuperscript{-1}·d\textsuperscript{-1} pioglitazone in this study appears to be an optimal and clinically relevant dose.

SHRSP are extensively studied as a useful animal model of hypertension-induced stroke characterized by hemorrhage and infarction.\textsuperscript{6} In the present study, treatment of SHRSP with pioglitazone at 1 mg·kg\textsuperscript{-1}·d\textsuperscript{-1} did not alter blood glucose, plasma insulin, body weight, and blood pressure values of SHRSP throughout the treatment. These observations are in good agreement with a previous report that this dose of pioglitazone did not alter plasma glucose, insulin, body weight, or blood pressure in the rat without marked insulin resistance and obesity.\textsuperscript{19} Therefore, our present experimental protocol allowed us to examine the potential effect of pioglitazone on hypertension-based stroke, independently of blood pressure or glycemic control.

Vascular endothelial dysfunction and remodeling\textsuperscript{20} and brain inflammation\textsuperscript{21,22} play a causative role in the onset of stroke in hypertension. Therefore, we investigated the effect of pioglitazone on vascular endothelial function, cerebrovascular remodeling, and inflammation in SHRSP. In this work, we found that pioglitazone markedly inhibited remodeling of a large cerebral artery and microvessels induced by hypertension, as assessed by histologic analysis, and ameliorated the impairment of carotid arterial endothelial function by hypertension, as assessed by Ach-induced vascular relaxation. Furthermore, pioglitazone also attenuated macrophage infiltration in the brains of SHRSP, which was associated with the suppression of MCP-1 and TNF-\alpha gene expressions by pioglitazone in SHRSP. These results show the marked anti-inflammatory action of pioglitazone in the brain of hypertensive rats. Moreover, pioglitazone treatment significantly delayed the onset of incidental stroke signs in SHRSP and prolonged the survival rate of SHRSP without affecting blood pressure. Collectively, these observations provide the first evidence on the protective effect of pioglitazone against cerebrovascular injury and stroke caused by hypertension.

Reactive oxygen species (ROS) and NO play a counter-regulatory role in brain or vascular injury.\textsuperscript{23,24} Hypertension is well known to enhance the production of superoxide in the brain or vascular tissues by causing the activation of NADPH oxidase, which is the major enzyme synthesizing ROS. The increased ROS cause brain and vascular injury by accelerating the impairment of vascular endothelial function, vascular remodeling, and neuronal damage. On the other hand, NO produced by vascular eNOS or brain NOS plays a protective role against ROS-mediated brain and vascular injuries.\textsuperscript{25,26} Therefore, to elucidate the mechanism responsible for the improvement of hypertension-mediated cerebrovascular injury by pioglitazone, we investigated the effect of pioglitazone on these parameters. Notably in this work, we found that pioglitazone significantly diminished superoxide levels in the brain and vascular tissues of SHRSP. Therefore, taken together with our previous report that ROS is implicated in brain injury and stroke in SHRSP,\textsuperscript{8} the protective role of pioglitazone in cerebrovascular injury and stroke in SHRSP seems to be mediated by the reduction of ROS by pioglitazone. Furthermore, this reduction of ROS by pioglitazone was associated with significant inhibition of brain and vascular NADPH oxidase activity and p22phox (a major NADPH oxidase subunit) upregulation by pioglitazone. Given that macrophage infiltration in SHRSP was limited to the perivascular area, the reduction of ROS in the brain and vascular tissues by pioglitazone in SHRSP appears to be mainly attributed to the inhibition of NADPH oxidase activity rather than the inhibition of inflammation. On the contrary, brain NOS activity and vascular endothelial NOS activity were not affected by pioglitazone treatment, providing no evidence for an important role for NOS in pioglitazone-induced cerebrovascular protection in SHRSP. Thus, the molecular mechanism underlying the protection of stroke by pioglitazone in SHRSP is different from that by statins in SHRSP, because eNOS is reported to play a major role in the protective effect of statins against stroke.\textsuperscript{27,28}

In this work, we examined the effect of pioglitazone on SHRSP after only 4 weeks of treatment, which did not permit us to elucidate the initiating event leading to brain protection by pioglitazone. However, it has been well established that vascular injury, including vascular endothelial function and remodeling, plays a major role in the mechanism of stroke in SHRSP. Taken together with the present findings on the significant protective effect of pioglitazone against vascular injury in SHRSP, vascular protection by pioglitazone in SHRSP seems to be the initiating event leading to the prevention of stroke. However, further study is needed to demonstrate our assumption.

In summary, in the present experimental work, we first investigated the effect of pioglitazone on incidental stroke
caused by hypertension and obtained the first evidence that pioglitazone, independently of blood pressure or blood sugar control, directly prevented the onset of stroke in hypertensive rats. Furthermore, this protective effect of pioglitazone against hypertension-induced stroke was attributed to the suppression of cerebrovascular remodeling, the improvement of vascular endothelial function, the inhibition of brain inflammation, and the reduction of ROS via inhibition of NADPH oxidase activity. Our present work highlights pioglitazone as a potential therapeutic agent for stroke in high-risk patients with type 2 diabetes and hypertension.

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Disclosures

None.

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Pioglitazone Exerts Protective Effects Against Stroke in Stroke-Prone Spontaneously Hypertensive Rats, Independently of Blood Pressure
Taishi Nakamura, Eiichiro Yamamoto, Keiichiro Kataoka, Takuro Yamashita, Yoshiko Tokutomi, Yi-Fei Dong, Shinji Matsuba, Hisao Ogawa and Shokei Kim-Mitsuyama

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