Activation of Signal Transducer and Activator of Transcription-3 by a Peroxisome Proliferator-Activated Receptor Gamma Agonist Contributes to Neuroprotection in the Peri-Infarct Region After Ischemia in Oophorectomized Rats

Tomoya Kinouchi, MD; Keiko T. Kitazato, BS; Kenji Shimada, MD; Kenji Yagi, MD, PhD; Yoshiteru Tada, MD, PhD; Nobuhisa Matsushita, MD; Manabu Sumiyoshi, MD; Junichiro Satomi, MD, PhD; Teruyoshi Kageji, MD, PhD; Shinji Nagahiro, MD, PhD

Background and Purpose—The role of the phosphorylated signal transducer and activator of transcription-3 (p-STAT3) after cerebral ischemia by the peroxisome proliferator-activated receptor γ (PPARγ) agonist pioglitazone (PGZ) remains controversial. Whether the increase in p-STAT3 by estrogen is mediated by the estrogen receptor α is also obscure. We examined the role of p-STAT3, PPARγ, and estrogen receptor α against ischemic brain damage after PGZ treatment.

Methods—Female Wistar rats subjected or not subjected to bilateral oophorectomy were injected with 1.0 or 2.5 mg/kg PGZ 2 days, 1 day, and 1 hour before 90-minute middle cerebral artery occlusion–reperfusion and compared with vehicle-control rats.

Results—The cortical infarct size was larger in ovarietomized than in nonovarietomized rats; it was reduced by PGZ treatment. Inversely with the reduction of the infarct size, PPARγ and p-STAT3 but not estrogen receptor α in the peri-infarct area were increased in PGZ-treated compared with vehicle-control rats. The increase in PPARγ and p-STAT3 was associated with the transactivation of antiapoptotic and survival genes and the reduction of caspase-3 in this area. Inhibitors of PPARγ or STAT3 abolished the PGZ-induced neuroprotection and the increase in p-STAT3. More importantly, p-STAT3 increased by PGZ was bound to PPARγ and the complex translocated to the nucleus to dock to the response element through p-STAT3.

Conclusions—Our findings suggest that the activation in the peri-infarct region of p-STAT3 and PPARγ by PGZ is essential for neuroprotection after ischemia and that PGZ may be of benefit even in postmenopausal stroke patients. (Stroke. 2012;43:478-483.)

Key Words: cerebral ischemia ■ ERα ■ neuroprotection ■ OVX ■ PPARγ ■ p-STAT3

Stroke has devastating sequelae and is a major cause of death.1 The lack of ovarian steroid hormones renders postmenopausal women more vulnerable to cerebrovascular disease than cycling women. Estrogen exerts various vascular and neuroprotective effects2 and is thought to be mediated by estrogen receptors. The signal transducer and activator of transcription-3 (STAT3) is affected by estrogen3; it is activated through phosphorylation of tyrosine kinases-2, a member of the Janus family, in response to a wide variety of external stimuli, including cytokines, hormones,4 growth factors,5 and epidermal growth factor-α 7 nicotinic-, interleukin-, and erythropoietin receptor pathways.6 Phosphorylated STAT3 (p-STAT3) dimerizes and translocates to the nucleus where it binds to specific DNA regions, the STAT-inducible elements, ultimately leading to an increase in gene transcription.7

Dziennis et al8 demonstrated that estradiol upregulated the antiapoptotic gene Bcl-2 and the survival marker MAP2 through an increase in p-STAT3 in the peri-infarct region, thereby inhibiting cerebral ischemic damage in female rats subjected to oophorectomy. They also suggested that this neuroprotective effect may be mediated by estrogen receptor α (ERα). On the other hand, Tureyen et al9 showed that in male rats, the peroxisome proliferator-activated receptor γ (PPARγ) agonists rosiglitazone and pioglitazone (PGZ) exerted protection against ischemic brain damage and that the prevention of STAT3 phosphorylation by rosiglitazone dampened interleukin-6 signaling in infarct regions. The

Received March 5, 2011; final revision received September 6, 2011; accepted September 26, 2011.
From the Department of Neurosurgery, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan. The online-only Data Supplement is available at http://stroke.ahajournals.orglookup/suppl?doi:10.1161/STROKEAHA.111.618926/-DC1. Correspondence to Tomoya Kinouchi, MD, Department of Neurosurgery, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15, Kuramoto-cho, Tokushima 770-8503, Japan. E-mail k-tomoya@xg8.so-net.ne.jp © 2011 American Heart Association, Inc.

Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.111.618926
discrepancy between these studies regarding the role of p-STAT3 points to the diverse effects of p-STAT3 in cerebral ischemia. We hypothesized that the role of p-STAT3 would be different depending on the ischemic region and that the neuroprotection by the PPARγ ligand may be associated with the upregulation of ERα in female animals. To investigate the relationship among ERα, PPARγ, and p-STAT3 as molecular mechanisms underlying neuroprotection after ischemia, we used nonovariectomized (OVX−) and ovariectomized (OVX+) rats treated or not treated with PGZ.

We provide new evidence that PGZ reduces the cortical infarct size in OVX+ rats susceptible to cerebral ischemic damage and that the increase in p-STAT3 by PPARγ activation in the peri-infarct area is associated with the transactivation of neuroprotective genes. Unexpectedly, PGZ did not affect ERα. More notably, the binding p-STAT3 and PPARγ was increased and the resulting complex translocated to the nucleus to dock to the response element through p-STAT3.

Under estrogen-deficient conditions, the activation of p-STAT3 and PPARγ by PGZ may play an essential role in neuroprotection in the peri-infarct area.

Materials and Methods

Animals and Agents

All experiments were performed on 10-week-old female Wistar rats (Charles River Laboratories, Japan) weighing 250 to 280 g; they were anesthetized with 2% isoflurane in 30% oxygen and 70% nitrous oxide. Four weeks before the experiments they were subjected to bilateral oophorectomy (OVX−) or sham OVX (OVX+). The OVX− and OVX+ rats subjected to 90-minute middle cerebral artery occlusion–reperfusion (MCAO-R) were divided into three groups; 1 group was injected intraperitoneally with 1.0 mg/kg PGZ, the other with 2.5 mg/kg PGZ at 2 days and 1 day and 1 hour before MCAO-R; the third group served as the vehicle control (VC). The effect of PGZ, a gift from the Takeda Pharmaceutical Company, was examined 24 hours post-MCAO-R. It was dissolved in dimethylsulfoxide and diluted ×3 with saline just before the injection of 0.4 mL/kg. The PPARγ antagonist GW9662 and the STAT3 inhibitor cucubitacin1 (JSI-124) were purchased from Cayman Chemicals (Ann Arbor, MI) and Merck Chemicals (Tokyo, Japan), respectively. Like PGZ, they were dissolved in dimethylsulfoxide; GW9662 was injected (4 mg/kg, intraperitoneally) 1 hour before MCAO-R and JSI-124 (0.5 mg/kg, intraperitoneally) 15 minutes after MCAO.

For other detailed descriptions of Materials and Methods, see http://stroke.ahajournals.org.

Results

PGZ Reduced the Cortical Infarct Size in OVX+ Rats Susceptible to Cerebral Ischemic Damage

As shown in Figure 1, at 24 hours after MCAO-R, the cortical infarct volume was significantly larger in OVX+ than OVX− rats (P<0.01). In OVX− and OVX+ rats treated with 1.0 (PGZ 1.0) and 2.5 mg/kg PGZ (PGZ 2.5), the infarct volume in the cortex but not the basal ganglia was reduced in a PGZ dose-dependent manner compared with the VC (PGZ 1.0, P<0.05; PGZ 2.5, P<0.01 versus VC; PGZ 1.0 versus PGZ 2.5, P<0.05), respectively. Together with the reduction in the cortical infarct size, the neurological score at 24 hours after MCAO-R was significantly lower in PGZ 2.5 than VC rats (2.16±0.40 versus 3.00±0.68, P<0.05), indicating the amelioration of neurological dysfunction.

There was no significant difference in the cerebral blood flow, blood glucose levels, and blood pressure among the 4 groups (OVX−, OVX+ rats treated with VC, PGZ 1.0, and PGZ 2.5 rats) during and after MCAO (data not shown). The plasma level of estrogen in OVX+ rats (13.1±3.4 pg/mL; n=8) was significantly lower than in OVX− rats (31.3±14.1 pg/mL; n=8; P<0.01); PGZ treatment did not affect the estrogen level.

Activation of STAT3 and PPARγ Was Associated With Apoptosis Inhibition in the Peri-Infarct Region

Next, we focused on p-STAT3, PPARγ, and ERα, molecules that play a role in cortical neuroprotection.

In the peri-infarct region, immunohistochemical studies showed that the expression of PPARγ (Figure 2A) and ERα (Supplemental Figure S1A) was increased in OVX− rats 24 hours after MCAO-R; it was reduced in OVX+ rats as was the level of p-STAT3 (Figure 2A; Supplemental Figure S1A). On the contralateral side without ischemia, these molecules were low level. PPARγ and p-STAT3 but not ERα were increased in OVX+ rats treated with PGZ 2.5 (Figure 2A; Supplemental Figure S1A–B); p-STAT3 peaked at 24 hours after MCAO-R (Supplemental Figure S2A). PPARγ- and p-STAT3-positive cells were found among endothelial cells and neurons (Figure 2C). As the level of PPARγ and p-STAT3 increased, the level of cleaved caspase-3 decreased (Figure 2A). The PPARγ, p-STAT3, and caspase-3 level in the peri-infarct area (Figures 3A, 3B, and 3D), determined by Western blot analysis, reflected our immunohistochemical results in the same region (Figure 2A); total STAT3 was not different among the groups (Figure 3C).
In the infarct core (Figure 2B), immunopositivity for PPARγ and p-STAT3 was not necessarily correlated with the ischemic damage; the expression of cleaved caspase-3 was not different among the 4 groups (OVX−, OVX+ rats treated with VC, PGZ 1.0, or PGZ 2.5). These results suggested that the increase in p-STAT3 and PPARγ in the peri-infarct region but not in the ischemic core is associated with neuroprotection afforded by PGZ.

Furthermore, in the peri-infarct regions, the mRNA level of PPARγ, antiapoptotic Bcl-2 and Bcl-xL, and survival-related MAP2 was reduced in OVX+ rats compared to OVX− rats (Figure 4A–D); there was an inverse correlation with the expression of caspase-3. In OVX+ rats treated with PGZ,

Figure 2. Representative immunohistochemistry. The expression of PPARγ, p-STAT3, and caspase-3 in the peri-infarct region (A) and ischemic core (B) of VC-treated OVX− and OVX+ rats and PGZ-treated OVX+ rats. Double immunohistochemistry staining (C) for PPARγ (red) and p-STAT3 (red) with NeuN (green) in the peri-infarct region shows the colocalization of PPARγ- and p-STAT3-positive cells in neurons. Scale bars, 20 μm. Representative hematoxylin and eosin staining indicates the peri-infarct region (#) in the ipsilateral cortex and ischemic core (*). PPARγ indicates peroxisome proliferator-activated receptor γ; p-STAT3, phosphorylated signal transducer and activator of transcription-3; VC, vehicle control; OVX, ovariectomized; NeuN, neuronal nuclei.

Figure 3. Expression of PPARγ (A), p-STAT3 (B), t-STAT3 (C), and caspase-3 (D) in the peri-infarct region. Each protein level was detected by Western blot and analyzed using Image J software. All values were normalized by setting the densitometry of the control sample to 1.0. Each bar represents the mean±SD (each group n=4). *P<0.05 vs VC in OVX− rats by analysis of variance followed by the Scheffe test. PPARγ indicates peroxisome proliferator-activated receptor γ; t-STAT3, total signal transducer and activator of transcription-3; p-STAT3, phosphorylated signal transducer and activator of transcription-3; VC, vehicle control; OVX, ovariectomized.

Figure 4. mRNA level of PPARγ and of antiapoptotic- and survival-related genes in the peri-infarct region. The mRNA level of PPARγ (A) Bcl-2 (B), Bcl-xL (C), and MAP2 (D) was determined by quantitative real-time PCR. Their expression in each group of rats was compared with the VC in OVX− rats. Data are the mean±SD from 8 rats per group. *P<0.05, **P<0.01 vs OVX−/VC rats by analysis of variance followed by the Scheffe test. PPARγ indicates peroxisome proliferator-activated receptor γ; PCR, polymerase chain reaction; VC, vehicle control; OVX, ovariectomized.
these neuroprotective molecules were upregulated and caspase-3 was reduced. The increase in \( p-\text{STAT3} \) and PPAR/\( \gamma \) in this region appeared to contribute to the transcriptional activation of neuroprotective molecules.

**Inhibition of p-STAT3 Abolished Neuroprotection Afforded by PGZ**

To assess whether the reduction in p-STAT3 affects the neuroprotection afforded by PGZ and renders neurons susceptible to damage, we used the PPAR/\( \gamma \) antagonist GW9662 and the STAT3 inhibitor JSI-124. Although the infarct size in OVX rats treated with GW9662 or JSI-124 was similar to that in nontreated OVX rats, GW9662 or JSI-124 abolished the PGZ-induced reduction in the cortical infarct size (Figure 5A; \( P<0.01 \)). This was associated with a decrease in p-STAT3 and an increase in caspase-3 in the peri-infarct area (Figure 5B). The PGZ-induced mRNA expression of Bcl-2, Bcl-xL, and MAP2 in the peri-infarct area was abrogated. Each bar represents the mean \( \pm \) SD from 2 areas in each of 4 rats. \( *P<0.05, **P<0.01 \) vs PGZ 2.5 alone by analysis of variance followed by the Scheffe test. PPAR/\( \gamma \) indicates peroxisome proliferator-activated receptor \( \gamma \); STAT3, signal transducer and activator of transcription-3; PGZ, pioglitazone; MCAO, middle cerebral artery occlusion; p-STAT3, phosphorylated signal transducer and activator of transcription-3; DAPI, 4',6-diamidino-2-phenylindole.

**PPAR\( \gamma \) Facilitated the Binding of p-STAT3 to the Response Element in DNA**

To verify that there was a causal link between p-STAT3 and PPAR\( \gamma \), we examined the interaction of these molecules. In cell lysates from the peri-infarct area of PGZ 2.5-treated compared with VC rats, p-STAT3 in PPAR\( \gamma \) immunoprecipitates were elevated (Figure 6A), suggesting that PGZ promotes the binding of p-STAT3 to PPAR\( \gamma \). Furthermore, in DNA binding assay using nuclear extracts obtained from the peri-infarct area, docking to the response element through p-STAT3 was increased by PGZ treatment (Figure 6B). On the other hand, binding to the response element through PPAR\( \gamma \) was not affected by PGZ. These observations suggest that in the peri-infarct area, PPAR\( \gamma \) activated by PGZ facilitates the binding of p-STAT3 to specific DNA regions and contributes to the transactivation of neuroprotective molecules.

**Discussion**

The incidence of stroke in women dramatically increases after menopause and it further rises with age. However, there are few studies on female mammals that mimic the postmenopausal state in women. We provide new evidence that under conditions of hypoestrogenicity, the cortical infarct size was increased and that it was reduced by PGZ treatment. As the size of the infarct area decreased, PGZ increased the level of p-STAT3 and PPAR\( \gamma \) but not in the peri-infarct area. The PGZ-induced expression of p-STAT3 was abolished by PPAR\( \gamma \) and STAT3 inhibitors, resulting in a decrease in the expression of neuroprotective genes and an increase in apoptosis in this area. More importantly, p-STAT3 increased by PGZ treatment was bound to PPAR\( \gamma \) and promoted docking to the DNA response element through p-STAT3. These results suggest that the activation of PPAR\( \gamma \)
is attributable to STAT3 phosphorylation in the peri-infarct area and that the activation of STAT3 and PPARγ by PGZ contributes, at least partly, to neuroprotection after cerebral ischemia under estrogen-deficient conditions.

Several studies found that STAT3 activated by granulocyte colony stimulating factor, NRG-1β, or secretoneurin exerted neuroprotective effects. Although the mechanisms underlying the activation of p-STAT3 may be different, these studies support our hypothesis that the activation of p-STAT3 and PPARγ in the peri-infarct region is essential for neuroprotection against ischemic brain damage.

PPARγ transduces signals as an obligate heterodimer with the retinoid X receptor. These nuclear receptors include the common domains; the N-terminal autonomous activation function domain that interacts with transcriptional coregulator proteins and the DNA binding-, the hinge-, and the C-terminal ligand binding domain that contains a ligand-regulated activation function. Agonist ligands for PPARγ activate transcription by promoting the recruitment of coactivators. In contrast, antagonist ligands contain an extended pendant group that is found in corepressors such as the silencing mediator for retinoid and thyroid hormone and the nuclear receptor corepressor silence transcription. In our study, the transcriptional activation of neuroprotective genes by p-STAT3 through PPARγ in the peri-infarct area may reflect the recruitment of coactivators.

Various interactions between p-STAT3 and PPARγ agonists have been documented in multiple myeloma cells. In neurons, the administration of rosiglitazone increased the translocation of PPARγ to the nucleus. PPARγ expression after focal ischemia was increased, especially in the peri-infarct area, but surprisingly, the DNA-binding activity of PPARγ was reduced. These earlier studies support our hypothesis that the activation of p-STAT3 and PPARγ in the peri-infarct area increased complex formation with p-STAT3 and that the complex translocated to the nucleus to bind the DNA response element through p-STAT3 but not PPARγ. Furthermore, inhibitors of PPARγ or p-STAT3 abolished the transcriptional activation of neuroprotective genes by PGZ in the peri-infarct area. Taken together, the activation of PPARγ by PGZ may facilitate the transactivation of neuroprotective genes by p-STAT3. To resolve the discrepancy between our earlier studies by Tureyen et al regarding p-STAT3, we examined p-STAT3 level in preliminary studies (Supplemental Figure S2B). Compared with the ischemic core, p-STAT3 was increased in the peri-infarct region in male rats as well as in OVX rats by PGZ treatment. Lo suggested the penumbra as an area of damaged brain tissue that continues to be viable after focal ischemia and posited that the presence of a penumbra may render therapeutic salvage theoretically possible. Although the role of p-STAT3 may be different depending on the brain area, the PGZ-induced increase of p-STAT3 in the peri-infarct region may play a crucial role for neuroprotection.

Estradiol exerted profound neuroprotective actions in a model of cerebral ischemic injury and its protective actions involved the neuronal antiapoptotic pathway and alterations in the expression of multiple genes in an ERα-dependent manner. Based on the suggestion by Dziennis et al that the estradiol-induced increase in p-STAT3 in the peri-infarct area is mediated by ERα, we examined whether the elevation of p-STAT3 by the PPARγ ligand is associated with ERα. In the presence of estrogen, ERα was increased in the penumbra area, as were p-STAT3 and PPARγ, compared with the nonischemic region and the hypoestrogenic condition. However, unexpectedly, ERα in PGZ-treated OVX rats was not affected despite the increase in p-STAT3 after MCAO. This phenomenon was also observed in PGZ-treated OVX rats (data not shown). Elsewhere, we demonstrated that in rats, the activation of ERα by an angiotensin type 1 receptor blocker, olmesartan, was neuroprotective against ischemic brain damage; olmesartan, on the other hand, did not affect PPARγ (data not shown). The activation of PPARγ by the soy phytoestrogen genistein at high doses downregulated its estrogen transcriptional activity, whereas the activation of ERα at low-dose genistein downregulated PPARγ transcriptional activity. Wang et al found that estrogen-activated ERα did not associate directly with STAT3 in multiple myeloma cells and Houston et al reported that the PPARγ ligand mediated the inhibition of ER-responsive gene trans-

Figure 6. Interactions between PPARγ and p-STAT3 in the course of transactivation after PGZ treatment in the absence of estrogen. Immunoprecipitates with PPARγ antibody in tissue lysates (A) from the peri-infarct area were prepared using the Catch and Release Version 2.0 immunoprecipitation kit and p-STAT3 was detected by Western blot analysis. Data are the mean ± SD from 6 rats per group. *P < 0.05 vs VC rats by Student t test. Binding to DNA response element by p-STAT3 or the PPARγ in nucleus extracts (B) from the peri-infarct area was assayed with an enzyme-linked immunosorbent assay (ELISA)-based transactivation TransAM p-STAT3 or PPARγ kit. Data are the mean ± SD from 6 rats per group. *P < 0.05 vs VC rats by Student t test. PPARγ indicates peroxisome proliferator-activated receptor γ; p-STAT3, phosphorylated signal transducer and activator of transcription-3; PGZ, pioglitazone; VC, vehicle control.
activation and ER-induced protein expression. Although ER and PPAR signaling pathways may be regulated differently in different cell types, the negative crosstalk between ERα and PPARγ supports our findings that the activation of p-STAT3 by PGZ is ERα-independent.

Although we cannot rule out other mechanisms, our findings provide new insights that activation of STAT3 by PPARγ in the salvageable peri-infarct region may be essential for the prevention of neuronal cell death and PGZ appears to play a role in the transcriptional activation of neuroprotective genes by p-STAT3 through PPARγ in this area even under ER-deficient conditions. Therefore, PGZ may provide neuroprotection against cerebral ischemic damage. To assess the usefulness of PGZ for stroke prevention based on a risk–benefit assessment, additional clinical studies are required.

**Acknowledgments**

We are indebted to Professor Masaaki Uno, Department of Neurosurgery, Kawasaki Medical School, for helpful comments.

**Sources of Funding**

This study was supported in part by a grant from the Ministry of Education, Culture, Sports, Science & Technology of Japan and in part by Takeda pharmaceutical Co Ltd.

**Disclosures**

None.

**References**

Activation of Signal Transducer and Activator of Transcription-3 by a Peroxisome Proliferator-Activated Receptor Gamma Agonist Contributes to Neuroprotection in the Peri-Infarct Region After Ischemia in Oophorectomized Rats
Tomoya Kinouchi, Keiko T. Kitazato, Kenji Shimada, Kenji Yagi, Yoshiteru Tada, Nobuhisa Matsushita, Manabu Sumiyoshi, Junichiro Satomi, Teruyoshi Kageji and Shinji Nagahiro

Stroke. 2012;43:478-483; originally published online November 10, 2011;
doi: 10.1161/STROKEAHA.111.618926
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/43/2/478

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2011/11/17/STROKEAHA.111.618926.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/
Activation of STAT3 by PPARγ agonist contributes to neuroprotection in the peri-infarct region after ischemia in oopherectomized rats

Tomoya Kinouchi, MD; Keiko T. Kitazato, BS; Kenji Shimada, MD; Kenji Yagi, MD, PhD; Yoshiteru Tada, MD, PhD; Nobuhisa Matsushita, MD; Manabu Sumiyoshi, MD; Junichiro Satomi, MD, PhD; Teruyoshi Kageji, MD, PhD; Shinji Nagahiro, MD, PhD

Department of Neurosurgery, Institute of Health Biosciences, The University of Tokushima Graduate School, JAPAN

Correspondence should be addressed to Dr. Tomoya Kinouchi, Department of Neurosurgery, Institutes of Health Biosciences, The University of Tokushima Graduate School, 3-18-15, Kuramoto-cho, Tokushima 770-8503, Japan
Phone, +81-88-633-7149,
Fax, +81-88-632-9464
e-mail; k-tomoya@xg8.so-net.ne.jp
Supplemental Materials and Methods

Our study was approved by the Ethics Committee of the Institute of Health Biosciences of the University of Tokushima Graduate School; all procedures were performed according to a protocol approved by the Animal Care Committee of Tokushima University Medical School. The rats received standard chow and were housed in conventional rat cages at a 12-hr inverted light/dark cycle.

Focal cerebral ischemia
During all surgical procedures the rats were under anesthesia with 2% isofluorane in 30% oxygen and 70% nitrous oxide; their rectal temperature was monitored with a thermometer (KN-91, Natsume) and maintained at 37 ± 0.5°C with a warming plate. For 90-min MCAO we inserted an intraluminal filament as described previously.\textsuperscript{1,2} To block major collateral flow the pterygopalatine artery was ligated at its origin. The internal- and common carotid artery were transiently occluded with loosely tied 3-0 silk sutures; a silicon-coated 4-0 nylon thread was introduced into the external carotid artery and advanced into the internal carotid artery to occlude the proximal orifice of the MCA. To confirm MCAO, bloodflow was measured from the temporal bone surface at a site 1 mm posterior to the bregma and 3 mm inferior to the temporal line using a laser-Doppler flow probe (Unique Medical, Osaka, Japan). After MCAO it was reduced to 20-30% of the baseline. Rats with successful MCAO consistently exhibited circling behavior, decreased resistance to lateral push, forelimb flexion, and shoulder adduction. We performed MCAO on 120 rats and excluded around 10% because MCAO was incomplete. Blood glucose levels were determined in whole venous blood with an automatic glucose meter (Accu-check Aviva blood glucose meter; Roche Diagnostics, Tokyo, Japan). The blood pressure was measured by telemetry (Data Science Inc. MN55126, USA) before, during, and after MCAO and recorded using the Dataquest Advanced Research Technologies Acquisition program (Unique Medical). Electrochemiluminescence immunoassay (SRL, Japan) was used to assess the plasma estradiol level.

Measurement of the infarct volume
At 24 hr after MCAO-R, the rat brains were extracted and equal 2-mm spaced slices and 6 coronal blocks were prepared immediately using a brain matrix (Bioresearch Center, Nagoya, Japan); the samples did not contain olfactory tissue or tissue from the cerebellum. They were immersed in a 2,3,5-triphenyltetrazolium
chloride (TTC) solution in phosphate-buffered saline (PBS). Third coronal sections were stored at -80°C until Western blot analysis and determination of the mRNA level. The extent of ischemic infarction was traced and the integrated volume was calculated using NIH 1.36b Image J software. Artifact from brain edema was reduced by the indirect measurement method based on the contralateral brain volume.

Neurological assessment

Neurological deficits were assessed by an examiner who was blinded to the treatment the rats had undergone. The neurological scoring system described by Huang et al³ and Yang et al⁴ was used; findings were recorded as 0 = normal motor function, 1 = flexion of the torso and contralateral forelimb upon lifting the rat by the tail, 2 = circling to the contralateral side upon holding the rat by the tail on a flat surface with normal posture at rest, 3 = leaning to the contralateral side at rest, and 4 = no spontaneous motor activity. The rats were evaluated immediately after successful MCAO and 24 hr after MCAO-R.

Immunohistochemistry

The rat brains were transcardially perfused with 4% paraformaldehyde in PBS on ice. After fixing, 6-µm-thick frozen sections were mounted on Matsunami adhesive silane (MAS)-coated glass slides (Matsunami Glass, Tokyo, Japan). After blocking with serum-free protein block (DakoCytomation), the slides were incubated with primary antibodies diluted with Canget signal immunostain (Toyobo, Osaka, Japan). The antibodies were rabbit polyclonal antibody against PPARγ (Santa Cruz Biotechnology Inc., CA), -against cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), -against p-STAT3 (Cell Signaling Technology, Beverly, MA), and -against ERα (Santa Cruz Biotechnology); we used mouse monoclonal antibody against neuronal nucleus (NeuN) (Millipore, Tokyo, Japan). The tissue samples were mounted with Vectashield (Vector Laboratories Inc. Burlingame, CA). Visualization was with Alexa Fluor 594 donkey anti-rabbit IgG or 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR); the slides were examined under a fluorescence microscope (IX71SIF-2, Olympus, Tokyo, Japan). To examine the specificity of the immunoreactivity, the primary antibody was omitted to provide a nonspecific control. A parallel set of tissue sections was subjected to hematoxylin and eosin (H&E) staining to identify the infarction core and the peri-infarct region. As p-STAT3 and caspase-3 immunopositive cells were heterogeneously distributed in the brain, we counted all cells.
positive for p-STAT3- and caspase-3 in the peri-infarct area. Two areas containing positive cells in 150 x 150-μm fields around the peri-infarct area were assessed in each animal. Tissue samples from 4 rats in each group were analyzed.

Western blot analysis

Brain tissue in the peri-infarct area was homogenized and sonicated in RIPA buffer (Thermo Scientific, Rockford, IL) containing phosphatase- and protease inhibitor (Roche, Tokyo, Japan), and centrifuged. Total protein in the supernatant was measured with the BCA protein assay kit (Pierce, Rockford, IL). Protein was separated by 7.5% or 12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. After blocking with 5% skim milk or BSA in Tris-buffered saline solution-Tween 20 (T-TBS), the membrane was incubated with the primary antibodies in Canget signal immunostain or T-TBS. The same primary antibodies as used for the immunohistochemical studies, rabbit polyclonal antibody against t-STAT3 (Santa Cruz Biotechnology), and mouse monoclonal anti-β-actin (Sigma, Tokyo Japan), were used. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare, Buckinghamshire, UK), signals were detected by chemiluminescence using an ECL-plus kit (GE Healthcare). Images were analyzed with Image J software and quantified as a relative increase over the controls after normalization with β-actin.

Immunoprecipitation

Tissues were prepared as described for Western blots. The immunoprecipitation (IP) kit Catch and Release v2.0 (Upstate Signaling Solutions, CA) was used according to the manufacturer’s instructions. Briefly, 500 μg of wild whole-cell lysates, 2.5 μl of antibody against PPARγ (Santa Cruz Biotechnology) and 10 μl of antibody capture affinity ligand were mixed and placed in a catch and release v2.0 spin column containing 0.5 ml of prepacked IP capture resin. After 30 min end-over-end shaking, the column was centrifuged, washed 3 times, and eluted with 70 μl elution buffer. Immunoprecipitation was analyzed by immunoblotting using primary antibody against PPARγ (Santa Cruz Biotechnology), p-STAT3 (Cell Signaling Technology).

Nuclear extracts and DNA binding assay

Nuclear protein from tissue samples in the peri-infarct area was extracted using the nuclear complex co-immunoprecipitation kit (ActiveMotif, Carlsbad, CA) according
to the manufacturer’s protocol. PPARγ and STAT3 activation were assayed with ELISA-based transactivation transAM PPARγ- and transAM STAT3 kits (Active Motif) using the manufacturer’s protocol. Binding to each oligonucleotide of PPARγ and STAT3 in nuclear extracts was detected with antibody against PPARγ and STAT3 and HRP-conjugated secondary antibody, and quantified by spectrophotometry.

Quantitative real-time PCR
Total RNA obtained from the peri-infarct area was isolated with the BioRobot EZ1 and EZ1 universal tissue kit (Qiagen, Tokyo, Japan). RNA was converted to cDNA using the transcript first-strand cDNA synthesis kit (Qiagen). Quantitative real-time PCR of each sample was on LightCyclerFastStart DNA Master SYBR Green I- and Roche LightCycler 2.0 (Roche Diagnostics, Tokyo, Japan) instruments. Primers for Bcl-2 and GAPDH were from Roche and used according to the manufacturer’s directions. The other primers were 5'-GCCAGGCTTGCTGAACGTGA-3' and 5'-TTGGCGAACAGCTGGGAGGA-3'; 112 bp for PPARγ, 5'-CCA AATGTGGCTCTAA AGA AC 3' and 5'-AGGTACATGGGTGAGCATTTGTC 3'; 91 bp for microtubular associated protein-2 (MAP2), 5'-CTACCTGGAGTCTGGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGA91

Statistical analysis
Data were expressed as the mean ± SD. Statistical analysis was with analysis of variance (ANOVA) followed by Scheffe’s test. The plasma level of estrogen and neurological score were analyzed with the Man-Whitney U-test. Differences of p<0.05 were considered statistically significant.

Supplemental References


**Supplemental Figure S1**

A

![Immunohistochemical images of ERα expression](image1)

B

![ERα mRNA expression](image2)

**Supplemental Figure Legends S1**

Protein and gene expression of ERα.

Immunohistochemically, we observed a significant increase in the expression of ERα in the peri-infarct- compared to the contralateral region of OVX+/VC rats (A). Scale bar, 20 μm. The mRNA level of ERα (B) in OVX+/VC rats was significantly lower than in OVX−/VC rats. There was no significant difference in the expression of ERα between OVX+/VC rats and OVX+ rats treated with 2.5 mg/kg PGZ. Data are the mean ± SD from 8 rats per group. **p<0.01 vs OVX+/VC rats by ANOVA followed by Scheffé’s test.

ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OVX, oophorectomy; PGZ, pioglitazone
Supplemental Figure Legends S2

**Time course (A) and gender difference (B) of p-STAT3 level in the peri-infarct region by PGZ**

A. OVX+ rats were treated with PGZ 2.5 mg/kg for 3 days and sacrificed 6-, 12-, or 24 hours after MCAO-R. Changes in p-STAT3 were determined by western blot analysis and compared with the vehicle control rats (VC) at 24 hour. Each datum represents the mean ± SD from 5 rats in each group. * p<0.05 vs VC by ANOVA followed by Scheffe’s test.

B. The increase in p-STAT3 in the peri-infarct region and ischemic core in male rats treated with PGZ 2.5 mg/kg for 3 days before MCAO-R was compared with OVX+ rats treated under the same protocol. In both male and OVX+ female rats treated with PGZ, p-STAT3 was markedly higher in the peri-infarct region than in the ischemic core. Each datum represents mean ± SD from 5 rats in each peri-infarct region (peri) and ischemic core (core). ** p<0.01 vs each ischemic core by the Mann-Whitney U-test.