A Peroxisome Proliferator-Activated Receptor-γ Agonist Reduces Infarct Size in Transient but Not in Permanent Ischemia

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Background and Purpose—Activators of peroxisome proliferator-activated receptor-γ (PPARγ), a member of the PPAR family, increase levels of CuZn-superoxide dismutase (SOD) in cultured endothelium, suggesting a mechanism by which it may exert its protective effect within the brain. These properties raise the question of whether a PPARγ agonist may be neuroprotective in models of ischemia without reperfusion, in which oxidative injury is less prevalent.

Methods—In 2 groups of rats, 90 minutes of middle cerebral artery (MCA) occlusion was followed by 1 day of reperfusion, with 1 group receiving pioglitazone (a PPARγ agonist) starting 72 hours before MCA occlusion (MCAO) and continuing through the day of occlusion, whereas the other group received vehicle only. In 2 comparable groups, the MCA was occluded permanently. One day after occlusion, the animals were tested neurologically and infarct volumes were calculated. In a separate group, rats were treated with pioglitazone or vehicle for 4 days. Tissue was obtained from the cortex and the striatum 2 hours into reperfusion after 90 minutes of MCAO, and the tissue was examined for CuZn-SOD by Western blot.

Results—Results show a significant reduction in infarct size in the treated rats, with transient MCAO but not permanent MCAO. There was also an improvement in neurological score in the treated animals after transient MCAO. The level of CuZn-SOD was increased in the cortex in treated animals.

Conclusions—These data, which show that a PPARγ agonist reduces infarct size in transient but not permanent MCAO, suggest that the role of PPARγ is specific to events occurring during reperfusion. Our data point to CuZn-SOD as the mediator of this neuroprotection. (Stroke. 2005;36:353-359.)

Key Words: cerebral ischemia, focal ▪ reperfusion ▪ superoxide dismutase

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that function as ligand-activated transcriptional regulators of genes controlling lipid and glucose metabolism.1

There has been a recent appreciation that PPARs and their ligands may play an important role in brain. Whereas a handful of early reports described expression patterns of PPAR mRNAs and proteins in brain,2 recently, an increasing number of studies have reported on the effects of PPAR agonists in animal models of neurological damage and disease, including the excitatory damage that occurs in stroke.3–5

The transcriptional activity of PPARγ is induced by binding diverse ligands, including natural fatty acid derivatives, thiazolidinedione, and nonsteroidal anti-inflammatory drugs. Recent research has implicated PPARγ in macrophage biology, cell cycle regulation, cellular differentiation, and atherosclerosis. Together, these additional “insulin-independent” properties and functions of PPAR agonists and activation have sparked a rapidly increasing interest in their potential therapeutic use. In vivo, PPARγ agonists have been shown to modulate inflammatory responses in the brain6 and to reduce infarction size against transient focal ischemia.5 Cerebral ischemia is frequently accompanied by inflammation, which can worsen neuronal injury. Activation of PPARγ reduces inflammation and the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. In addition, PPARγ activators increase levels of CuZn-superoxide dismutase (SOD) in cultured endothelium, suggesting an additional mechanism by which it may exert protective effects within the brain.7

Because CuZn-SOD plays a significantly larger role in temporary ischemia than in ischemia without reperfusion,8,9 the purpose of this study was to determine whether a PPARγ
agonist may be neuroprotective in a model of permanent ischemia. To this end, we used the PPARγ agonist pioglitazone, a synthetic ligand of the thiazolidinedione class currently used as an antidiabetic agent because of its insulin-sensitizing effect. When administered orally, it is rapidly absorbed and crosses the blood–brain barrier.10

Materials and Methods
All experiments were performed on male Sprague-Dawley rats (weight 268 to 348 g; Charles River Laboratories, Inc, Wilmington, Mass). Procedures were approved by the institutional animal care and use committee of the University of Pennsylvania.

Treatment
Pioglitazone (ACTOS; Takeda Chemical Industries) was mixed with peanut butter and was given orally (20 mg/kg per day) to 22 rats daily, beginning 72 hours before middle cerebral artery (MCA) occlusion and continuing through the day of occlusion; 23 rats received the peanut butter only. This treatment regimen has been shown previously to be useful for investigating the PPARγ-mediated effects of pioglitazone.11

MCA Occlusion Experiments
Anesthesia was induced with halothane (1.0% to 1.5%) in a mixture of 70% nitrous oxide and 30% oxygen. Each rat was allowed to breathe spontaneously. The body temperature was monitored by a rectal probe and maintained at 37.0±0.5°C with a heating blanket regulated by a homoothermic blanket-control unit. In a subset of animals (n=5), temperature was also monitored in the temporalis muscle. A polyethylene catheter (PE-50) was placed into the tail artery, and blood pressure was continuously monitored and recorded on a Grass polygraph recorder (model 7D; Grass Instruments). Samples for blood gas analysis were taken from the tail artery catheter before and after the ischemic period and measured with a blood gas analyzer (i-STAT; Abbott Diagnostics).

Focal cerebral ischemia was induced using the filament model as described by Koizumi et al.12 Through a midline neck incision, the right external and internal carotid arteries were dissected from the surrounding connective tissue. A 4.0-nylon monofilament suture coated with silicone was inserted into the right common carotid artery. A polyethylene catheter (PE-50) was placed into the right internal carotid artery. The suture was advanced into the right internal carotid artery until mild resistance was felt, indicating that the filament was positioned properly to occlude blood flow to the MCA. After 90 minutes of occlusion, the suture was withdrawn to allow for cerebral reperfusion (vehicle 17; pioglitazone 16). In animals made permanently ischemic (vehicle 6; pioglitazone 6), the filament was left in place. After a period of observation, the arterial catheter was withdrawn, all wounds were closed, and animals were returned to their cages, where they were allowed free access to food and water. Postischemic temperature was controlled until animals recovered from anesthesia.

Cerebral Blood Flow Assessment During MCA Occlusion
The microvascular blood flow in the right frontal–parietal cortex (MCA territory) was monitored continuously with a laser-Doppler flowmeter (LDF; PeriFlux 4001; Perimed). The LDF probe (tip diameter 1 mm; fiber separation 0.25 mm) was attached to the thinned skull, and changes in cerebral blood flow (CBF) were monitored starting 15 minutes before MCA occlusion (MCAO) and extending until 10 minutes after reperfusion. In the animals with permanent MCAO, CBF was monitored for 60 minutes after occlusion.

Measurement of Damaged Volume
Rats were killed with an intraperitoneal injection of pentobarbital sodium (150 mg/kg) 24 hours after MCAO. Brains were carefully removed from the skull and cooled in ice-cold PBS for 15 minutes to facilitate cutting. They were sectioned in the coronal plane at 1-mm intervals with the use of a rodent brain matrix (RBm-4000C; ASI Instruments), and the brain slices were incubated in PBS containing 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) at 37°C for 10 minutes. The TTC-stained sections were photographed with a digital camera, and the damaged area was determined at each cross-sectional level selected by reference to the corresponding level in an atlas of the rat brain with use of a computer-based image analyzer (AIS 6.0; Imaging Research). To avoid artifacts attributable to edema, the damaged area was calculated by subtracting the area of the normal tissue in the hemisphere ipsilateral to the stroke from the area of the hemisphere contralateral to the stroke. Total lesion volumes in cortex and striatum were calculated by summation of the infarct areas of 10 brain slices and integrated by the thickness.

Neurological Evaluation
Neurological evaluation was performed before euthanization 1 day after MCAO according to the protocol of De Ryck et al.13 Briefly, postural reflex, visual placing in the forward and sideways directions, tactile placing of the dorsal and lateral paw surfaces, and proprioceptive placing were tested. These 6 tests were each scored from 0 to 2, and the behavioral deficit was calculated as the sum of the scores of the individual tests ranging from 0 (no deficit) to 12 (maximum deficit).

Western Blot Analysis
In separate groups of animals, rats were treated with pioglitazone (20 mg/kg per day) or vehicle for 4 days. Eleven rats underwent 90-minute MCAO 3 days after treatment started (vehicle 5; pioglitazone 6). The brain was removed 2 hours into reperfusion, and tissue was obtained from the cortex and the striatum from the nonischemic hemisphere and the ischemic hemisphere and examined for CuZn-SOD (SOD 1) level by Western blot.7 Protein was extracted from the brain tissue using a commercial kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents; Pierce). Briefly, small brain tissue samples (40 mg) were added to PBS buffer and homogenized. After centrifugation at 500g for 3 minutes, the supernatant was removed. The amount of protein, which was loaded for Western blotting, was 15 μg. To correct for loading, we used the expression of GAPDH as the housekeeping protein. Antibody against rat SOD 1 (FL-154; Santa Cruz Biotechnology) was used for Western blotting.14 After protein blotting, membranes were incubated with horseradish peroxidase-conjugated rabbit anti-goat monoclonal antibody. Antigen detection was performed with a chemiluminescence detection system, (ECL kit; Amersham), and signal detection was done by exposing to x-ray film and analyzed using a scanning densitometer and NIH image. Results were obtained by calculating a ratio of the SOD 1 protein levels to GAPDH protein levels and reported as relative optical density.

Statistical Analysis
Results were expressed as mean±SD. Significant differences between groups were determined with Student t test for infarct volume, physiological parameters, and ratio of optical density of SOD 1 protein levels/GAPDH protein levels by Western blot. Differences between groups were determined with 2-way repeated-measures ANOVA for mean arterial blood pressure, CBF, and infarction areas. When significant differences were found, the Tukey test was used to find at which time point or slice these differences occurred. Significant differences of neurological scores between groups were determined with the Mann–Whitney test.

Results
Physiological Variables
Data on blood gas analyses, plasma glucose, and mean arterial blood pressure 30 minutes before and 30 minutes after MCAO are summarized in the table. The values of these parameters were within the physiological range, with no
significant differences found either as a result of MCAO or among the groups. Temporalis muscle temperature mimicked body temperature in each animal. Of the 34 animals that had the MCA occluded for infarct volume determination, 6 developed subarachnoid hemorrhage (vehicle 4; treated 2) and were discarded from the analysis.

**CBF Assessment After MCAO**

In all groups, CBF decreased rapidly after occlusion, dropping to \( \approx 21\% \) to 26\% of control 1 minute after occlusion (Figure 1). Vehicle and pioglitazone-treated animals with permanent MCAO did not differ significantly in CBF after MCAO. There were no significant differences in CBF at any time between the permanent and transient MCAO groups. In the transient MCAO groups, the CBF rapidly increased during the first minute after reperfusion to 115.6\( \pm 25.7\% \) (vehicle) and 104.6\( \pm 34.3\% \) (pioglitazone treated) before decreasing to below the preischemic level 10 minutes after release of the occlusion. Only at 5 minutes after reperfusion was CBF of treated animals (74.6\( \pm 25.0\% \)) different from that of untreated animals (106.6\( \pm 32.7\% \); \( P < 0.01 \)).

**Histopathological Evaluation**

Effects of pioglitazone and vehicle on infarct area and volume in the transient and permanent MCAO groups are presented in Figure 2. Treatment with pioglitazone reduced the total infarct volume after transient MCAO compared with vehicle-treated animals (Figure 2A). Pioglitazone-treated animals had a total infarct volume of 71.3\( \pm 32.8 \) mm\(^3\), which was significantly lower than that in vehicle-treated animals (191.9\( \pm 55.1 \) mm\(^3\); \( P < 0.01 \)). The decrease in infarct size occurred in 8 of 10 sections examined (Figure 2C). The effect of pioglitazone on infarct volume was most dramatic in the cortex, where infarct volume was reduced from 134.7\( \pm 55.1 \) mm\(^3\) (vehicle) to...
29.0±23.5 mm³ (P<0.01; Figure 3A). The decrease in infarct size was significant for 9 of 10 sections examined (Figure 3B).

In the striatum, the infarct volume decreased from 57.1±14.5 mm³ (vehicle) to 42.2±12.5 mm³ (P<0.05; Figure 3C), with 3 of 10 sections exhibiting a significant decrease in infarct size.

In the permanent MCAO groups, the infarct volume in the pioglitazone-treated animals (293.3±47.8 mm³) was not different from the vehicle-treated animals (276.6±53.0 mm³; P>0.1; Figure 2B).

Neurological Evaluation

One day after transient MCAO, the pioglitazone group exhibited significant improvement in neurological score compared with the vehicle group (P<0.001; Figure 4). However, in permanent ischemia, there was no significant difference between the neurological score for the vehicle- and the pioglitazone-treated animals. This lack of an improvement in neurological score correlates with the lack of a difference in total infarction volume.

Western Analysis

Pioglitazone significantly increased SOD 1 protein level in the cortex of nonischemic rats (P<0.05) and ischemic rats (P<0.05; Figure 5). In contrast, the protein level of SOD 1 in the striatum was not different between the pioglitazone- and the vehicle-treated animals in either the nonischemic or the ischemic tissue.

Discussion

In the present study, we found that a PPARγ agonist (pioglitazone) reduces infarct size in transient but not permanent MCAO, suggesting that the neuroprotective role of PPARγ is specific to events occurring during reperfusion. Our data point to CuZn-SOD as the mediator of this neuroprotection because CuZn-SOD was significantly upregulated with pioglitazone treatment.

Data in the literature are consistent with our results. Preliminary data indicate that pioglitazone5 reduces infarction size in transient MCAO in the rat. Resveratrol, which is a selective activator of PPARα and PPARγ, reduces infarction in wild-type mice subjected to permanent MCAO but fails to offer neuroprotection in PPARα knockout mice, suggesting that PPARγ activation is not neuroprotective in permanent ischemia.15

The ligands/activators for PPARα and PPARγ increase CuZn-SOD in primary endothelial cells,7 indicating that the expression of CuZn-SOD, which scavenges free radicals,
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Figure 3. Treatment effects on infarct volume and areas in the cortex and striatum after transient MCAO. A, Cortical infarct volume. B, Cortical infarct areas for each slice. C, Striatal infarct volume. D, Striatal infarct areas for each slice. **P<0.01 vs vehicle; *P<0.05 vs vehicle.

Figure 4. Neurological score in animals treated with pioglitazone or vehicle after transient (A) or permanent (B) MCAO. ***P<0.001 vs vehicle.

might be regulated by PPAR. Kim et al. cloned the 1.5-kb upstream region of the rat CuZn-SOD gene and found a PPAR response element at the −797 region of this gene. PPARγ ligand/activator might activate the transcription of the CuZn-SOD gene by binding to the PPAR response element.

Reactive oxygen-induced free radicals play an important role as mediators of tissue injury associated with inflamma-
tory and ischemic states. Chan et al demonstrated that ischemic infarction is significantly reduced after transient focal cerebral ischemia in mice overexpressing CuZn-SOD compared with wild-type littermates. However, these animals were not protected against permanent MCAO, lending further support to the notion that oxidative stress plays a role in reperfusion injury.

We hypothesized that the PPARγ agonist pioglitazone induces CuZn-SOD, and it is this increase in CuZn-SOD that is responsible for the decrease in infarct size in transient but not in permanent ischemia. To test this hypothesis, we examined the effect of pioglitazone treatment on CuZn-SOD protein level in the brain and found that it was increased in the cortex in treated animals (although there was no significant increase of CuZn-SOD in the striatum).

As observed in the present study, pioglitazone strongly attenuated cortical damage in transient MCAO while having a less dramatic protective effect on striatal damage. It is likely that the beneficial effects in the cortex are attributable to neuronally specific events because pioglitazone did not appreciably alter local cortical blood flow either during or after MCAO. In the MCAO model, the cortex appears to have a larger salvageable penumbra that is supported by a collateral blood supply than does the basal ganglia.

Preliminary data from another laboratory have reported that the expression of PPARγ itself was increased in the brain as early as 6 hours after transient MCAO. This increase in PPARγ might induce CuZn-SOD in the ischemic brain, thereby offering some neuroprotection because reperfusion injury is thought to be largely attributable to oxidative stress concomitant with the resupply of oxygen during reperfusion.

Exogenous delivery of pioglitazone will further increase the level of CuZn-SOD, protecting the brain from ischemic damage. Although the largest change in CuZn-SOD with treatment occurred in the cortex, which also exhibited the greatest neuroprotection, mechanisms other than the upregulation of CuZn-SOD may still play a role in the neuroprotection seen with pioglitazone treatment. PPARγ agonists also have the ability to decrease reactive oxygen species generation. We have some preliminary data on the effects of pioglitazone on the expression of the superoxide generating enzyme NADPH oxidase in cerebral tissue. These data show that the 22-kDa and the 47-kDa subunit protein levels in NADPH oxidase in the ischemic cortex 2 hours after transient MCAO were significantly decreased by treatment with pioglitazone (P<0.05). Other potential mechanisms by which PPARγ agonists may offer neuroprotection include an upregulation of inducible NO synthase and alterations in other free radical scavengers or initiation of anti-inflammatory processes. The improvement in neurological score in the pioglitazone-treated group compared with vehicle-treated group subjected to transient MCAO correlates well with the decrease in infarction volume seen in the pioglitazone-treated animals. These data are consistent with the preliminary observations of Gamboa et al, who also report an improvement in neurological score in rats treated with pioglitazone.

Phase 2 trials to test the safety and efficacy of pioglitazone in neurodegenerative diseases have already begun. Because impaired insulin sensitivity is a potential risk factor for vascular disease, a study was undertaken recently to examine the effect of pioglitazone on insulin sensitivity in nondiabetic patients with either a recent stroke or transient ischemic attack. They found a >60% increase in insulin sensitivity after 3 months of treatment, suggesting that a prophylaxis for those at risk of ischemia-reperfusion injury could be achieved by PPARγ agonist treatment.

In conclusion, these data, which show that a PPARγ agonist reduces infarct size in transient but not permanent MCAO, suggest that the role of PPARγ is specific to events occurring during reperfusion. Our data point to CuZn-SOD as the mediator of this neuroprotection.

Acknowledgments

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