Protective Effects of Pioglitazone Against Global Cerebral Ischemic-Reperfusion Injury in Gerbils

Ravinder K. Kaundal, Seethalakshmi Iyer, Ashutosh Kumar, and Shyam S. Sharma

Molecular Neuropharmacology Laboratory, Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER), Sector- 67, S.A.S. Nagar, Punjab- 160062, India

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Abstract. Despite of the huge socio-economic burden, stroke still represents an unmet therapeutic need. Researchers failed to reproduce preclinical efficacy in subsequent clinical development. To bridge this translation failure, the Stroke Therapy Academic Industry Round Table (STAIR) has suggested a rigorous, robust, and detailed preclinical evaluation in at least 2 species and multiple cerebral ischemia models to avoid the clinical failure. Considering these recommendations, in the present study, we have investigated the effects of pioglitazone in global model cerebral ischemic-reperfusion (IR) injury in gerbils. Global cerebral IR injury, produced by bilateral carotid artery occlusion for 5 min, was characterized by neurological deficits, hyperlocomotion, and neurodegeneration in the hippocampal CA1 region. Global ischemia was also associated with oxidative stress and DNA fragmentation as evident from increased malondialdehyde (MDA) levels and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling)-positive cells. Global cerebral IR injury associated neurological damage was significantly attenuated by pioglitazone pretreatment as evident from reduction in neurological symptoms, hyperlocomotion, and CA1 hippocampal neuronal damage in IR-challenged gerbils. Pioglitazone pretreatment also attenuated the oxidative stress and DNA fragmentation after cerebral IR injury. Pioglitazone post-treatment has also significantly reduced the CA1 hippocampal neuronal damage and DNA fragmentation after cerebral IR injury in IR-challenged gerbils. This study demonstrates the neuroprotective activity of pioglitazone in global cerebral IR injury and its neuroprotective effects may be attributed to reduction in oxidative stress and DNA fragmentation.

Keywords: pioglitazone, bilateral carotid artery occlusion, TUNEL, peroxisome proliferator–activated receptor (PPAR), gerbil

Introduction

Stroke, a major cause of mortality and morbidity worldwide, is an acute neurological injury resulting from interruption of blood supply to the brain (1, 2). Conditions like cardiac arrest, coronary artery bypass surgery, cardio-respiratory failure, and others that lead to drastic reduction of blood flow to the brain results in global cerebral ischemia. Approximately 10% – 20% of cardiac arrest patients who recover show neurological symptoms like impaired learning and memory deficits because of ischemic neurodegeneration in certain areas of the brain (3, 4). Global cerebral ischemia, even for a short period of time, results in selective neurodegeneration in vulnerable brain regions, for example, CA region of the hippocampus. Particularly, pyramidal neurons of the CA1 field are among the most vulnerable cells to ischemic-reperfusion (IR) injury. Severe loss of CA1 hippocampal neurons have been shown to occur after transient global cerebral ischemia as a consequence of immediate, maturational, and delayed neuronal death (5 – 9).

Multiple pathophysiological events identified over the last decade are thought to involved in ischemic neurodegeneration (10, 11). Excessive releases of excitatory neurotransmitters trigger the metabolic cell death cascade. Several other factors can also instigate secondary auto-destructive reactions within the CNS, including free...
 radicals, eicosanoids, lipid degradation products, inflammation, and immune responses. The secondary injury factors are released or activated over a period of time starting from within few seconds to days after the primary ischemic insult and may act either sequentially or in parallel to cause cell death (10, 12). Oxidative stress, inflammation, and apoptosis are documented to dominate the pathophysiology of the ischemic injury (11, 13, 14). A growing body of evidence has suggested that peroxisome proliferator–activated receptors (PPARs) play a crucial role in pathogenesis of cerebral IR injury by interfering with the above mentioned phenomenon (15 – 17). Recent evidences from animal experiments indicate that PPARγ agonists confer neuroprotection and neurological improvement following cerebral ischemia (18, 19). Importantly, the PPAR-γ agonist pioglitazone is already prescribed to diabetic patients and has recently shown neuroprotective potential in rat model cerebral ischemia (17, 20, 21).

Several drug molecules have earlier also shown fruitful results in preclinical studies, but all failed subsequently in clinical development. This translational failure is a great challenge in stroke research, and several recommendations, based on lessons learned from past failure, have attempted to bridge this translation failure. Foremost among these are the Stroke Therapy Academic Industry Round Table (STAIR) recommendations that suggest that the success of a neuroprotective agent in clinical trial is a mere speculation without rigorous, robust, and detailed preclinical evaluation in at least 2 species and multiple cerebral ischemia models (22). Hence considering the recommendation of Lestage et al. (23) and the Stroke Therapy Academic Industry Roundtable (22), regarding rigorous, robust, and detailed preclinical evaluation in multiple cerebral ischemia model and at least in 2 species, in the present study we have investigated the effects of pioglitazone in global model cerebral IR injury in gerbils.

**Materials and Methods**

**Animals**

Adult male Mongolian gerbils (*Meriones unguiculatus*) weighing 50–70 g were obtained from Central Animal Facility of National Institute of Pharmaceutical Education and Research, India. Animals were housed in a room at controlled temperature of 22 ± 1°C with 12-h light/12-h dark cycle and allowed free access to food and water. All procedures used in this study were approved by Institutional Animal Ethics Committee, NIPER.

**Induction of transient global cerebral IR injury**

Global cerebral ischemia was induced by occluding the common carotid arteries bilaterally for 5 min (5). Sham-operated animals underwent the same surgical procedures, except common carotid arteries were not occluded.

**Neurological function**

At 4 h after IR injury, gerbils were assessed for neurological symptoms, according to the following stroke index (24): no symptom = 0, hunched posture or hair roughed up = 1, ptosis = 2, circling behavior = 3, splayed-out hind limb = 4, and seizures = 5.

**Hyperlocomotion**

Spontaneous hyperlocomotion was recorded using Opto-Varimex (Columbus Instruments, Columbus, OH, USA). Gerbils were placed individually in each cage and were acclimatized for 5 min before the study. For the time-course study, hyperlocomotion was assessed for 15 min at one and four days after the onset of reperfusion (25).

**Histological studies**

The gerbils were euthanized by decapitation at 96 h after the onset of reperfusion. The brains were removed, fixed in 10% formalin, and paraffin embedded. Brain coronal sections (5 μm) at level of 1.5 to 1.7 mm posterior to the bregma were taken with a microtome (Leica, Benshein, Germany). Sections were stained with 1% celestine blue and 1% acid fuchsin (26). In each gerbil brain, viable, dead, and total neurons were counted from representative sections of the hippocampus CA1 region.

**Assay for lipid peroxidation**

After 4 h of reperfusion, animals were sacrificed and brains were homogenized (5:1 v/w) in ice-cold 0.1 M phosphate buffer, pH 7.4. An aliquot (100 μL) of homogenate was added to a reaction mixture containing 100 μL of 8.1% sodium dodecyl sulphate, 750 μL of 20% acetic acid (pH 3.5), 750 μL of 0.8% thiobarbituric acid, and 300 μL distilled water. Samples were then heated in boiling water for 1 h and centrifuged at 4,000 × g for 10 min. Malondialdehyde (MDA) content was estimated in the supernatant at 532 nm. Quantification was done based on the standard curve generated using authentic MDA (26). The MDA content was expressed as μM/mg tissue protein. Total protein concentration of brain homogenate was estimated by the Lowry method (27).
DNA fragmentation detection
Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay was carried out to identify the extent of DNA fragmentation according to the previously reported method (28). Briefly brains were in situ fixed with 4% buffered paraformaldehyde (pH 7.4). The brain sections from in situ fixed brains were prepared for the labeling reaction by re-hydration followed by nuclear stripping (with proteinase K). The 3’ end of the fragmented DNA was labeled using Fluorescein-FragEL DNA Fragmentation detection kit (Oncogene Research Products, San Diego, CA, USA). The sections were mounted and were observed under fluorescent microscope (Leica).

Treatment schedule
Pioglitazone was freshly prepared in DMSO and administered intraperitoneally (i.p.) during bilateral carotid artery occlusion (BCAO) in the single-dose study and in the multiple-dose administration study, pioglitazone (3 mg/kg, i.p.) was administered 1, 24, 48, and 72 h after BCAO. Each group consisted of 5 – 6 animals, unless otherwise stated.

Statistical analyses
Statistical analysis was performed using statistical analysis software Sigma Stat 2.0. All the results are expressed as the mean ± S.E.M. unless otherwise stated. All parameters, except neurological score, were analyzed using one-way analysis of variance (ANOVA) followed by the multiple comparison test. Neurological scores were expressed as the median and analyzed using Kruskal-Wallis one-way analysis of variance on ranks test followed by post-hoc Dunn’s multiple comparison test. Differences were considered to be significant if \( P<0.05 \).

Results

Effect on neurological score
Pioglitazone treatment (10 and 30 mg/kg, i.p.) during BCAO produced significant reduction in neurological score as compared to the vehicle-treated group (Fig. 1), whereas pioglitazone at 3 mg/kg (single and multiple administration) did not cause significant reduction in neurological deficits.

Effect on locomotor activity
Locomotor activity was assessed on day 1 and 4 after reperfusion to assess the motor functions. IR-challenged animals showed significant increase in locomotor activity. Pioglitazone (3 mg/kg, single dose and multiple doses) treatment failed to show significant reduction in hyper-locomotion. Pioglitazone (10 and 30 mg/kg, single dose) treatment reduced locomotor activity significantly as compared to the vehicle-treated group on day 1 (Fig. 2).

Effect on neuronal damage
Histological examination of the CA1 region of the hippocampus showed a significant \( (P<0.001) \) reduction in the number of surviving neurons in IR-challenged gerbils. Hippocampal neuronal damage was significantly \( (P<0.01) \) reduced by pioglitazone treatment (3, 10, and 30 mg/kg; single administration, and 3 mg/kg; multiple administration) (Fig. 3: A and B).
Effect on lipid peroxidation

Gerbils subjected to global IR injury exhibited a significant \((P<0.001)\) increase in the brain MDA levels as compared to sham-operated animals. Significantly reduction in brain MDA were observed with pioglitazone treatment (3, 10, and 30 mg/kg; single administration, and 3 mg/kg; multiple administration) in the IR-injured group as compared to the vehicle-treated, IR-challenged group (Fig. 4).

Effect on DNA fragmentation

Significant number of TUNEL-positive cells were observed in the hippocampal CA1 region of IR-challenged gerbils after 4 days of BCAO, which indicates massive DNA fragmentation. The number of TUNEL-positive cells in IR-challenged gerbils were significantly reduced with pioglitazone treatment (10 and 30 mg/kg, single administration; 3 mg/kg, multiple administration) as compared to vehicle-treated, IR-challenged group (Fig. 5). Pioglitazone treatment (3 mg/kg, single) did not cause any reduction in the number of TUNEL-positive cells.

Discussion

This study demonstrates the neuroprotective potential of pioglitazone in global cerebral IR injury in gerbils. An incomplete Circle of Willis makes Mongolian gerbils more suitable for inducing global cerebral IR injury as they develop features of global cerebral ischemia just...
after occluding carotid arteries for 5 min, bilaterally. In accordance with the earlier studies (5, 28 – 30) we have also observed that bilateral carotid artery occlusion for 5 min followed by 96-h reperfusion results in selective hippocampal neurodegeneration, particularly in the CA1 region as evident from histology, along with neurological deficits and hyperlocomotion. Cerebral IR-induced hyperlocomotion has been correlated with hippocampal neuronal damage and reduction in the animal’s ability to habituate or to form spatial maps (31). Based on the literature evidence, hyperlocomotion was assessed one and four days after onset of reperfusion, and it was observed at peak after one day of reperfusion (30). Pioglitazone (10 and 30 mg/kg, single dose) treatment significantly decreased the neurological score and hyperlocomotion. The number of live hippocampal CA1 cells was significantly increased on single- or multiple-dose administration of pioglitazone. These results indicate the neuroprotective effect of pioglitazone in global cerebral IR injury even when it was administered in the post ischemic phase. Beneficial effects of pioglitazone in other models of neurodegeneration further support our results (16, 18, 32).

Oxidative stress predominates in the pathophysiology of IR injury and exerts its deleterious effects by oxidizing various cellular components. Peroxidation of lipid bi-layer, a marker of oxidative stress, can be estimated by measuring the MDA levels. Consistent with earlier reports, we have observed significant increase in lipid peroxidation, as evident from increased MDA levels, in IR-challenged gerbils (28, 33). MDA levels were significantly reduced with pioglitazone treatment after cerebral IR injury. Collino et al. has also demonstrated reduction in lipid peroxidation after pioglitazone treatment and has suggested that direct ROS scavenging activity along with its ability to increase the endogenous antioxidant levels may be responsible for reduction in oxidative stress (21). Several other studies have also shown the antioxidant potential of PPAR-γ agonists including pioglitazone in various diseased animal models. TUNEL-positive cells are a well characterized feature of apoptotic cell death, which is thought to play a key role in delayed neuronal cell death after global cerebral IR injury (28, 30, 34). In agreement with histological data, IR-challenged gerbils showed increased number of TUNEL-positive cells in the hippocampal CA1 region, which indicates apoptotic DNA fragmentation. These results further support the literature evidence regarding involvement of apoptosis in cerebral IR injury. This apoptotic DNA fragmentation was attenuated with pioglitazone treatment as evident from reduction in the number of TUNEL-positive cells. More prominent reduction in TUNEL-positive cells was observed when pioglitazone was administered at multiple time points in the post ischemic phase. Pereira et al. has also shown attenuation of apoptotic cell death in cerebral IR injury after treatment with a PPAR-γ agonist (19). The reduced lipid peroxidation and DNA fragmentation may be attributed to the antioxidant activity of pioglitazone. Secondly, whatever the aetiology, neuronal death is mostly associated with inflammatory and oxidative processes with a cross talk between the two phenomena.

![Representative photographs showing TUNEL-positive cells in the upper panel and total cells in the lower panel. Photographs were taken from the hippocampus CA1 region of gerbils 4 days after 5 min of global cerebral ischemia. Pioglitazone treatment has reduced the number of TUNEL-positive cells.](image-url)
The ability of PPAR ligands to beneficially modulate the mediators of inflammation (COX-2, iNOS, MMP-9, etc.) has been well documented (15, 21, 35). Lee et al. have also shown metalloproteinase inhibitory activity of pioglitazone (36). Studies have also shown that pioglitazone exhibits anti-inflammatory effects by attenuation of NFκB activation after IR injury (37). Pereira et al. have shown the direct inhibitory effects of L-796449, a nonthiazolidinedione PPAR-γ agonist, on NFκB activation (19). Their findings suggest that the NFκB inhibitory activity of PPAR-γ agonists is independent of the PPAR-γ– and thiazolidinedione-like properties of these compounds. Hence based on these evidences the anti-inflammatory potential of pioglitazone by reducing NFκB activation may play a role in the above-observed effects.

In summary, the present study demonstrates neuroprotective effects of pioglitazone in global cerebral IR injury in gerbils. The neuroprotective potential of pioglitazone may be attributed to attenuation of lipid peroxidation and DNA fragmentation after cerebral IR injury. This study further suggests the therapeutic potential of pioglitazone in cerebral ischemic-reperfusion injury.

References

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