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PPARγ agonist pioglitazone reduces matrix metalloproteinase-9 activity and neuronal damage after focal cerebral ischemia

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ABSTRACT

Pioglitazone, a peroxisome proliferator-activated receptor gamma (PPARγ) agonist, has shown protective effects against ischemic insult in various tissues. Pioglitazone is also reported to reduce matrix metalloproteinase (MMP) activity. MMPs can remodel extracellular matrix components in many pathological conditions. The current study was designed to investigate whether the neuroprotection of pioglitazone is related to its MMP inhibition in focal cerebral ischemia. Mice were subjected to 90 min focal ischemia and reperfusion. In gel zymography, pioglitazone reduced the upregulation of active form of MMP-9 after ischemia. In *in situ* zymograms, pioglitazone also reduced the gelatinase activity induced by ischemia. After co-incubation with pioglitazone, *in situ* gelatinase activity was directly reduced. Pioglitazone reduced the infarct volume significantly compared with controls. These results demonstrate that pioglitazone may reduce MMP-9 activity and neuronal damage following focal ischemia. The reduction of MMP-9 activity may have a possible therapeutic effect for the management of brain injury after focal ischemia.

Stroke is the one of the leading causes of death and neurological dysfunctions in the industrialized countries. Complex biochemical cascades are involved in the neuronal damage process in cerebral ischemia. Intracellular and extracellular processes have been suggested to explain the causative pathophysiology of cerebral ischemic insult. In addition to intracellular pathologic events, the dysregulation of the pericellular environment such as cell-to-matrix or cell-to-cell interactions has been suggested as a possible background leading to neural tissue damage in cerebral ischemia [1,2]. In particular, structural alterations of neurovascular units, such as blood–brain barrier (BBB) degradation, may contribute to the significant pathologies in focal cerebral ischemia [3–5]. MMPs are a family of zinc-dependent endopeptidases that are capable of degrading most components of the extracellular matrix. Abnormal elevations in the expression and activity of MMPs may contribute to the various types of brain diseases [6]. Matrix metalloproteinases (MMPs) become upregulated and play an important role in the pathologic processes of focal cerebral ischemia [7,8]. In particular, MMP-9 and MMP-2, degrade the matrix components of the basement membrane maintaining the cerebral vasculature integrity, which results in neuroinflammation, brain edema, and hemorrhagic transformation in focal cerebral ischemia [8–10]. Genetic and pharmacological reductions of MMP-9 have been shown to reduce neuronal damage in global and focal brain ischemia [7,11,12].

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcriptional factors that belong to the nuclear hormonal receptor superfamily and are key regulators of glucose and lipid metabolism [13,14]. Pioglitazone, one of the synthetic antidiabetic thiazolidinediones, has been identified as an agonist for PPARγ. Pioglitazone has shown to have important pharmacological effects, such as anti-oxidant [15,16], and anti-inflammatory [17,18] activities. In particular, pioglitazone has been shown to have neuroprotective effects against focal and global brain ischemia [12,19–21].

The inhibitory effects of pioglitazone on the MMP activities are well documented. Alterations in MMP activity and expression may contribute to a number of pathologic conditions including extracellular matrix breakdown and tissue destruction. Pioglitazone has been shown to inhibit MMPs in a variety of experimental models [9,22–24]. To our knowledge, however, there are no previous studies on the effect of pioglitazone against MMP activity following transient focal cerebral ischemia. Here, we examine the effect of pioglitazone on the MMP-9 activity and neuronal damage following transient focal cerebral ischemia.

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Materials and methods

Animals and production of focal cerebral ischemia. All protocols and procedures were approved by the Institutional Animal Care and Use Committee of the Keimyung University School of Medicine. Male C57BL/6 mice (Koatec-Harlan, Korea) weighing 25–30 g were used in this study. Mice were kept in cages under a 12:12 light-dark cycle with free access to food and water. Mice were anesthetized with 3% isoflurane in a mixture of 70% N_2O and 30% O_2 and anesthesia was maintained with 1.5–2.0% isoflurane. After neck skin incision, the bifurcation of the common carotid artery was exposed. The external carotid artery was ligated and the internal carotid artery carefully isolated from the vagus nerve. A microvascular clip was placed across the right common carotid artery. Through the external carotid stump, a 7–0 surgical nylon monofilament with silicon coat was advanced into the right internal carotid artery up to the origin of the middle cerebral artery (MCA). Laser Doppler flowmetry (Perimed 5000 system, Järfälla, Sweden) was used to confirm adequate induction of focal cerebral ischemia. During surgery, rectal temperature was monitored and maintained at 37 ± 0.5 °C with a feedback-controlled heating-pad (CMA 150, Stockholm, Sweden). The filament was left in place for 90 min and then reperfusion was produced by withdrawal of the monofilament suture. The restoration of the blood flow was confirmed by laser Doppler flowmetry. Animals were then placed in a warm box at 30 °C temperature condition for 3 h to avoid introducing bias to the results due to hypothermia. At the end of the reperfusion period (24 h after reperfusion following 90 min MCAO), the mice were killed for the subsequent experiments.

Drug treatment. Pioglitazone (40 mg/kg/day, as a suspension in 0.5% carboxymethylcellulose, provided by Takeda Pharmaceutical Co. LTD, Osaka, Japan) was administered to mice twice daily via oral ingestion during the three days before ischemia and twice daily from ischemia until sacrifice, respectively. Carboxymethylcellulose solution was administered to mice as a vehicle according to the same volume and time schedule of pioglitazone. According to our pilot study, the pioglitazone treatment protocol in the present study did not significantly lower blood glucose levels in mice compared with vehicle-treated mice.

Gelatin gel zymography. Mice were anesthetized deeply with ethyl ether and then perfused transcardially with ice-cold PBS (pH 7.4). The brains were removed quickly, divided into ipsilateral and contralateral hemispheres on ice, and stored at −80 °C. Brain sample extracts were prepared as described previously [11]. Briefly, brain tissues were homogenized in lysis buffer including protease inhibitors on ice. After centrifugation, the supernatant was collected, and the total protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Prepared protein samples were loaded and separated by 10% Tris–glycine gel with 0.1% gelatin as substrate. After separation by electrophoresis, the gel was washed with renaturing buffer for 1 h and then incubated with developing buffer at 37 °C for 24 h. After developing, the gel was stained with 0.5% Coomassie Blue R-250 for 30 min and then destained appropriately. For band identification and standardization, MMP-2 and MMP-9 standards were loaded in each gel. Proteolytic band intensities were quantified by scanning densitometry (Quantity one, Bio-Rad).

In situ zymography. *In situ* zymography was performed as described previously [2]. The gelatin with a fluorescent tag remains caged (no fluorescence) until the gelatin is cleaved by gelatinase activity. Fresh ischemic brain slices (14 μm) were incubated with reaction solution including FITC-labeled gelatin (Molecular Probes, Eugene, OR, USA) and either vehicle or pioglitazone (1 mM) to test the direct inhibitory effect of pioglitazone on the *in situ* gelatinase activity after ischemia. A broad-spectrum MMP inhibitor doxycycline (200 μM) was used as a standard metalloproteinase inhibitor. The *in situ* gelatinolytic activity was revealed by the appearance of fluorescent brain constituents. Reaction products were visualized by fluorescence microscope with Leica IM50 system (Leica Microsystems, Heerbrugg, Switzerland).

Measurement of infarct volume. Mice were killed for the determination of infarct volume 24 h after production of focal cerebral ischemia. The brains were removed and then sliced into six 1-mm coronal sections. The sections were incubated in 2% solution of 2,3,5-triphenyl tetrazolium chloride monohydrate (TTC, Sigma) for 30 min at 37 °C. When the stain had developed, the sections were fixed with 10% formalin solution [25]. TTC-stained coronal sections were then photographed by a digital camera (Nikon, Coolpix 5200, Tokyo, Japan). The infarct volume was calculated with the Image J program (National Institute of Health) version 1.30, by an investigator blind to the animal treatments (H.Y.K.) (n = 8, vehicle-treated animals; n = 9, pioglitazone-treated animals). To avoid artifacts in volume measurement from brain edema within the infarcted tissue, the corrected infarct volume was calculated by measuring and subtracting the volume of the uninfarcted part of the ipsilateral hemisphere from the volume of the contralateral hemisphere [26].

Statistical analysis. Data were expressed as mean ± SE. Statistical analyses were performed by the Mann-Whitney U-test as nonparametric analyses. A value of $P < 0.05$ was considered significant.

Results

Gelatin gel zymography

Gelatin gel zymography was performed to evaluate the protein levels of MMP-9 and MMP-2 in the ipsilateral hemisphere. Within the limits of our sensitivity, sham-operated animals showed very low levels of the active form of MMP-9 (97 kDa) and the latent form of MMP-2 (72 kDa) (Fig. 1A). After transient focal cerebral ischemia,

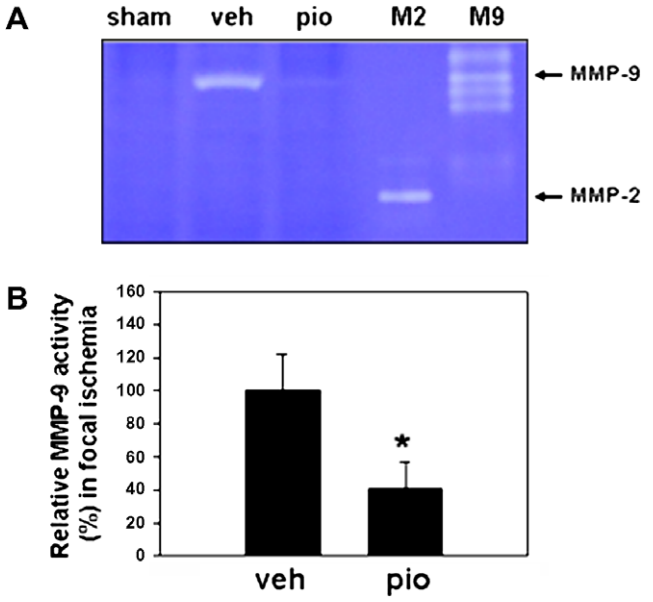

Fig. 1. Representative gelatin gel zymogram of the ipsilateral hemisphere following transient focal cerebral ischemia. Sham-operated animals showed very low levels of the active form of MMP-9 (97 kDa) and the latent form of MMP-2 (72 kDa). The active form of MMP-9 was increased following transient focal cerebral ischemia. Pioglitazone administration reduced the increase of MMP-9 activity in the ipsilateral hemisphere (A). Bar graph of measurement of relative optical density of MMP-9 bands. The increased optical density of MMP-9 band (97 kDa) was attenuated by pioglitazone administration (mean ± S.E.; n = 7) (B). Sham: sham-operated animals, veh: vehicle-treated animals, pio: pioglitazone-treated animals. M2: MMP-2 standard marker, M9: MMP-9 standard marker. $^{*}P < 0.05$ versus vehicle-treated animals.

the active form of MMP-9 in the ipsilateral hemisphere of vehicle-treated animals increased markedly, and pioglitazone administration significantly inhibited the induction of the active form of MMP-9 (Fig. 1A and B, $p < 0.05$). Although both MMP-2 and MMP-9 may contribute to gelatinase activity, the amount of the latent form of MMP-2 (72 kDa) was not affected by ischemia, and there was no active form of MMP-2 in the present study (Fig. 1).

In situ zymography

To study the histological distribution of gelatinolytic activity, *in situ* gelatin zymography was performed with fresh postischemic brain slices. FITC signal representing gelatinase activity was clearly observed in the damaged cortical and striatal areas of the ipsilateral hemisphere. Gelatinase activity was reduced in the ipsilateral hemisphere of pioglitazone-treated animals (Fig. 2).

To examine whether pioglitazone shows direct inhibitory effects on gelatinase activity in our model, we also tested its effect on *in situ* gelatinase activity in postischemic brain slices. Pioglitazone (1 mM) co-incubation clearly reduced the intensity and density of gelatinase activity (Fig. 3). Doxycycline (200 μM), a broad-spectrum potent MMP inhibitor was used as a standard metalloproteinase inhibitor (Fig. 3).

Effect of pioglitazone on the infarct volume

We measured the brain infarct volume with TTC staining to test the effect of pioglitazone. Pioglitazone administration significantly reduced the infarct volume compared with vehicle-treated animals ($p < 0.01$) (Fig. 4).

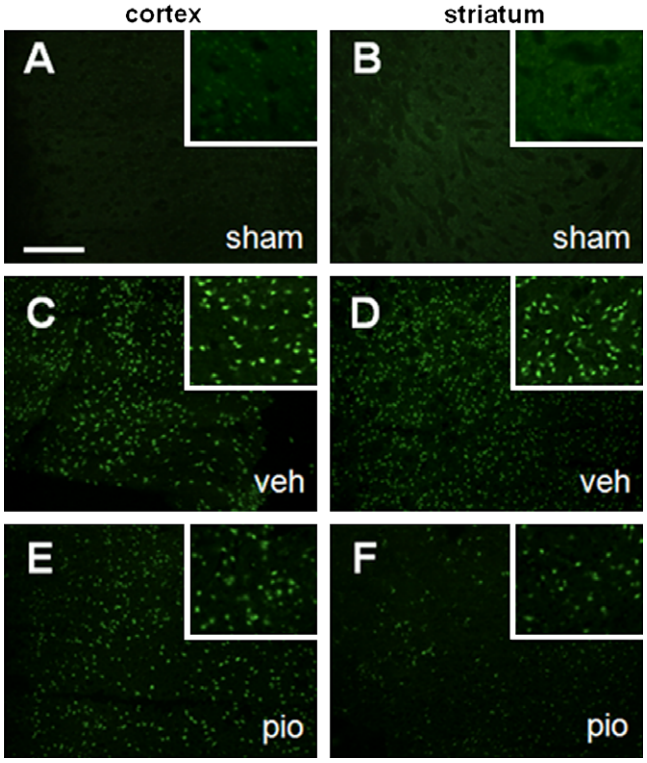

Fig. 2. Representative *in situ* gelatin zymograms in the cortex and striatum following transient focal cerebral ischemia. Gelatinase activity was very weak in the contralateral cortex and striatum in sham-operated animals (A and B). *In situ* gelatinase activity markedly increased in the ipsilateral cortex and striatum following transient focal cerebral ischemia (C and B). Pioglitazone administration markedly reduced the *in situ* gelatinase activity in the ipsilateral cortex and striatum (E and F). Sham: sham-operated animals, veh: vehicle-treated animals, pio: pioglitazone-treated animals. Scale bar = 100 μm.

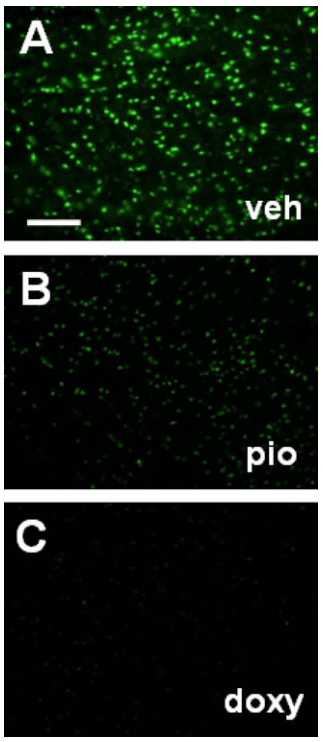

Fig. 3. Direct inhibitory effect of pioglitazone on the increased *in situ* gelatinase activity induced by transient focal cerebral ischemia. Transient focal cerebral ischemia-induced gelatinase activity in the ipsilateral cortex (A). Suppression of gelatinolytic activity in the postischemic cortex after co-incubation with 1 mM of pioglitazone (B) or 200 μM of doxycycline, a broad-spectrum MMP inhibitor (C). Veh: vehicle, pio: pioglitazone, doxy: doxycycline. Scale bar = 100 μm.

Discussion

Studies have shown that MMPs play an important role in cerebral ischemia [4,8,27,28]. It has been suggested that transient focal ischemia-induced neuronal injury coincides with dysregulated pericellular proteolysis involving MMP enzymes [1,11,29]. MMPs, especially the gelatinases MMP-2 and -9, have been shown to be clearly increased in animal models of focal cerebral ischemia [9,10,29] and in human focal ischemic stroke [30]. Gelatinase-induced breakdown of the neurovascular matrix and the extracellular matrix leads to edema, bleeding, increased inflammatory influx, and neuronal death in cerebral ischemia [10–12]. Another possible MMP-mediated pathophysiology in ischemia is the neuronal anoikis, which is induced by degradation of the cell-to-matrix interaction in brain parenchyma after focal ischemia [1]. In transient global cerebral ischemia, in particular, the MMP-induced degradation of perineuronal matrix proteins also plays a role in the production of anoikis-type neuronal cell death [2,12]. Our *in situ* zymography data showed that *in situ* MMP activity signals are mainly located in neuronal cells and small vasculatures. MMPs from neuronal cells may play a role in the injury of neurons and capillaries. Previous reports also showed that ischemia produced MMP activity in injured neuronal cells [5,31,32]. At least in mouse systems, a dominant role has been attributed to MMP-9 because MMP-9 knock-out mice showed a neuroprotection against trauma and ischemia [11,33], whereas MMP-2 knock-out mice did not show a neuroprotection against acute brain damage after focal cerebral ischemia [34]. In the previous study, transient focal cerebral ischemia can induce MMP-9 expression in the damaged area, while pharmacological inhibition or MMP-9 depletion reduces focal ischemia-induced infarction [11]. In order to test the histological distribution of gelatinolytic activity, we performed *in situ* zymography. Although both of MMP-2 and MMP-9 are known to

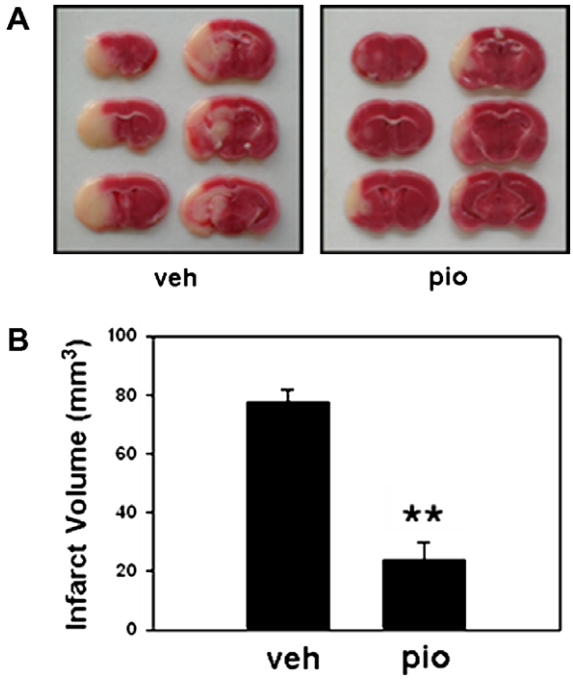

Fig. 4. Pioglitazone administration reduced infarct volume after 90 min focal ischemia and 24 h reperfusion. Representative sections of the brains stained with TTC showing infarction areas in the animals of vehicle-treated animals (A) and pioglitazone-treated animals (B). Quantitative data of infarct volume in the vehicle-treated control and pioglitazone-treated animals (C). Veh: vehicle-treated animals, pio: pioglitazone-treated animals. The data were expressed as mean ± S.E., $n = 8$ vehicle-injected animals; $n = 9$ pioglitazone-injected mice per group, $^{**}P < 0.01$.

contribute to gelatinolytic activity, MMP-9 might be a dominant gelatinase in mouse brain ischemia [12]. Here, in gelatin gel zymography, the active form of MMP-9 increased markedly after focal ischemia and pioglitazone reduced the MMP-9 activity. There were no active form MMP-2 bands in our gel zymography data. It is controversial whether MMP-2 is activated after brain ischemia. Several studies showed the upregulation of active MMP-2 after brain ischemia [9,10], but others did not show active MMP-2 [2,11,12,29].

Pioglitazone is a synthetic agonist of PPARγ that has been shown to reduce brain damage and recover neurological function after focal ischemia [21,35]. A number of papers have shown the neuroprotective role of pioglitazone. Pioglitazone inhibits harmful biochemical events that play important roles in ischemic stroke. Pioglitazone reduces interleukin-1β, cyclooxygenase-2, and inducible nitric oxide synthase expressions [21]. Pioglitazone also inhibits macrophage and microglia accumulation cells [35]. It also shows antioxidant effects, increasing the levels of CuZn-superoxide dismutase [36] and decreasing oxidative stress in the postischemic hippocampus [37]. As previously mentioned, pioglitazone has been known to inhibit MMP enzyme activity and expression in a number of experimental models. Pioglitazone inhibits MMP-2 activity in ischemia-reperfusion injury of the heart [24], MMP-9 levels in diabetic nephropathy [17] and MMP-9 expression in human bronchial epithelial cells [38]. Previously, we also reported the inhibitory effect of pioglitazone on MMP-9 activity in transient global cerebral ischemia [2]. Based on these MMP inhibitory effects of pioglitazone, it is possible that pioglitazone can reduce the tissue damage due to MMP activation. To our knowledge, there were no previous reports on the effect of pioglitazone on gelatinase activity after transient focal cerebral ischemia.

Based on the previous reports, MMP activation can be modulated by various mechanisms. After secreting from cytoplasm as an inactive proenzyme state, MMPs require an activation process by other types of proteolytic enzyme and free radicals. Free radical reaction has been known to be an important process of MMP activation [39]. Endogenous reactive oxygen species are necessary in the fibronectin fragment-induced MMP activation [39]. It is thus no surprise that the anti-oxidant and/or anti-inflammatory effects of pioglitazone may affect MMP activities and show neuroprotection in cerebral ischemia. MMP activity may be inhibited by the systemic administration of pioglitazone, which has been known to have neuroprotective effects. According to our *in situ* zymography data and previously reported studies on various types of disease, it is apparent that pioglitazone has a direct inhibitory effect on MMP activities. In the current study, we performed *in situ* zymography with co-incubation of pioglitazone on the postischemic brain section in which gelatinase was already activated, and found that pioglitazone inhibited gelatinase activity directly. Although we did not quantify the FITC signals for gelatinase activity in this study, it was obvious that pioglitazone co-incubation markedly reduced the intensity and density of gelatinase activity in the postischemic brain section.

The present results show the neuroprotective and the MMP inhibiting effects of pioglitazone in transient focal cerebral ischemia. In addition to the previously reported pathways for this neuroprotection, MMP-9 inhibition might be a possible mechanism, for the neuroprotective effect of the PPARγ agonist pioglitazone. Further study is necessary to clarify how pioglitazone reacts with MMP-9 induced by ischemic stroke.

Acknowledgments

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