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Effects of rosiglitazone on global ischemia-induced hippocampal injury and expression of mitochondrial uncoupling protein 2

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Abstract

We investigate the effect of rosiglitazone, a ligand for peroxisome proliferator-activated receptor-γ (PPARγ) with anti-inflammatory and anti-oxidative actions, on hippocampal injury and its roles in mitochondrial uncoupling protein 2 (UCP2) expression caused by transient global ischemia (TGI) in rats. Increased UCP2 expression was observed in mitochondria of hippocampal CA1 2–24 h after TGI/reperfusion, with maximal expression levels at 6–18 h. Administration of rosiglitazone to hippocampus 30 min prior to the onset of TGI further enhanced mitochondrial UCP2 expression 2–6 h following TGI/reperfusion. Rats subjected to TGI/reperfusion displayed a significant increase in lipid peroxidation, based on increased malondialdehyde (MDA) levels, in hippocampal CA1 mitochondria 2–6 h after reperfusion. Rosiglitazone significantly attenuated TGI/reperfusion-induced lipid peroxidation and suppressed hippocampal CA1 neuronal death based on the surviving neuronal counts. In conclusion, our results provide correlative evidence for the "PPARγ → UCP2 → neuroprotection" cascade in ischemic brain injury.

Keywords: Global ischemia; Hippocampus; Mitochondria; Peroxisome proliferator-activated receptor-γ; Rosiglitazone; Uncoupling protein 2

Transient global ischemia (TGI) may occur in serious cardiovascular disorders such as cardiac arrest, cardiogenic shock after myocardial infarction, or reversible severe hypotension [1]. A histological hallmark of TGI is the selective neuronal loss in CA1 field of hippocampus, which may occur days after the initial ischemic insult [2,3]. The underlying mechanism of delayed CA1 neuronal loss following TGI is not well understood. Free radicals have been implicated in ischemic neuronal death including that caused by TGI. Mitochondria are one of the major organelles that produce reactive oxygen species (ROS) within cells [4,5]. Excessive oxidative stress may lead to irreversible damage of mitochondrial DNA, membrane lipids, and proteins, thereby contributing to cell death [6]. Mitochondrial oxidative damage is considered to be a major factor in a wide variety of human disorders, including neurodegenerative diseases, aging, and cancers [7]. Recent evidence has suggested an intimate link between an excessive ROS production and the development of hippocampal neuronal death [8,9].

Uncoupling proteins (UCPs) are inner mitochondrial membrane proteins that dissipate the proton gradient, thereby releasing the stored energy as heat. Five related proteins have been cloned that were named UCP1, UCP2, UCP3, UCP4, and UCP5/BMCP1 [10]. Recent findings using *Ucp2*$^{-/-}$ mice have suggested that UCP2 might be implicated in decreasing the production of ROS [11], a role that has been ascribed to this protein capable of uncoupling respiration [12]. Studies support this notion

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showed that increase in UCP2 gene expression is related to a decreased generation of ROS or H_2O_2 [12,13]. Accumulating evidence indicates that UCP2 could be involved in neuroprotection in ischemic, traumatic, excitotoxic, and seizure-related brain injury [14–16].

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that may regulate lipid and lipoprotein metabolism, glucose homeostasis, cell proliferation, and differentiation, as well as apoptosis. Importantly, PPARs also modulate the inflammatory and oxidative response [17]. Evidence revealed that PPARs (PPARα and PPARγ) have beneficial effects in inflammatory diseases through regulation of cytokine production and adhesion molecule expression by interfering with the transactivation capacity for nuclear factor-κB, activator protein-1, and signal transducers and activators of transcription [17–19]. Several recent studies suggested that UCPs gene expression is regulated by PPARs [20,21], we therefore test the hypothesis that rosiglitazone, a PPARγ agonist, may attenuate TGI-induced lipid peroxidation in hippocampus through regulating the PPARγ-UCP2 cascade under ischemic condition.

Materials and methods

Animals and general preparations. The experimental procedures used in this study conformed to the guidelines of our Institutional Committee on Experimental Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. Adult male Sprague–Dawley rats (250–325 g) were purchased from the Experimental Animal Center, National Science Council in Taiwan. Animals were anesthetized initially with chloral hydrate (400 mg/kg, i.p.) to perform preparative surgery. The TGI model was performed as previously reported [22] with modifications. Briefly, the animals were subjected to a 10-min period of forebrain global ischemia by clamping both common carotid arteries and lowering the mean arterial blood pressure to 40 ± 5 mm Hg by withdrawing blood from a left femoral arterial catheter; blood pressure was restored by infusing the withdrawn blood afterwards. The right femoral arterial catheter was connected to a pressure transducer (Gould P23ID, Valley View, OH), and in turn to a universal amplifier (Gould G-20-4615-58), via which arterial blood pressure were amplified [23]. During anesthesia the core temperature was monitored and maintained at 37 ± 0.5 °C. On-line and real-time recording of the arterial blood pressure signals was conducted to keep mean arterial blood pressure in the designed range. After regaining consciousness, the animals were returned to the animal room for postoperative recovery in individual cages.

Microinjection of rosiglitazone into the hippocampal formation. Microinjection of test agents bilaterally into the hippocampal CA1 subfield was carried out sequentially with a stereotaxically positioned 27-gauge stainless steel needle connected to a 0.5-μl Hamilton microsyringe (Reno, NV, USA) [23]. The stereotaxic coordinates used for the hippocampal CA1 subfield were 2.3–3.2 mm posterior to the bregma, 3.6–4.4 mm from the cortical surface, and 1.5–2.4 mm lateral to the midline [23]. A total volume of 50 nl was delivered over 2 min to allow full diffusion of the injected solution. Rosiglitazone (Cayman Chemical, Ann Arbor, MI, USA) in a concentration of 50 nmol was freshly prepared during the experiment. Possible volume effect of microinjection was controlled by injecting the same amount of artificial cerebrospinal fluid (aCSF) as vehicle treatment. To avoid the confounding effects of drug interaction, only one treatment schedule was delivered to each animal.

Isolation of mitochondria from rat hippocampus. Rats were perfused intracardially with 100 ml of warm (37 °C) saline containing heparin (100 U/ml). The brain was rapidly removed under visual inspection and placed on a piece of gauze moistened with ice-cold 0.9% saline for the removal of bilateral CA1 subfields of hippocampus under dissecting microscope. Samples were immediately placed in ice-cold buffer containing 0.25 M sucrose, 0.5 mM EGTA, and 3 mM Hepes, pH 7.2 (SEH buffer). Isolation of rat mitochondria from hippocampus was carried out according to our previous report [23]. Tissues were gently homogenized in SEH buffer using a loose-fit 15 mL glass–Teflon homogenizer (Wheaton, Millville, NJ). The homogenate was centrifuged at 800*g* for 10 min at 4 °C, and the supernatant thus obtained was further centrifuged at 8000*g* for 10 min. The precipitate was collected and the above centrifugation was repeated. The final mitochondrial pellet was suspended in a minimal amount of SEH buffer and stored at −80 °C.

Western blotting. Total proteins in the mitochondrial suspension were estimated by the method of Bradford [24] with a protein assay kit (Bio-Rad, Hercules, CA, USA). Protein samples were subjected to 12% polyacrylamide gel electrophoresis followed by electroblotting onto a polyvinylidene difluoride membrane according to standard protocols. The primary antisera used included: goat polyclonal antiserum against UCP2 (SC-6526, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse monoclonal antiserum against β-actin (MAB1501, Chemicon, Temecula, CA, USA). The secondary antisera (Chemicon) used included: horseradish peroxidase-conjugated goat anti-rabbit (AP132A) or anti-mouse (AP124A) IgG. Specific antibody–antigen complex was detected using a BCIP/NBT Alkaline Phosphatase Substrate Kit (SK5400; Vector Laboratories, Burlingame, CA, USA). The amount of UCP2 was quantified by the ImageMaster Video Documentation System (Amersham–Pharmacia Biotech), and was expressed as the ratio to β-actin protein product.

Quantification of lipid peroxidation. An MDA-586 colorimetric assay kit (OXIS International, Portland, OR, USA) was used to quantitatively measure the end products malonaldehyde (MDA) derived from the lipid peroxidative reaction [25]. Briefly, samples with an aliquot of 200 μl of mitochondrial suspension from hippocampal CA1 were used to detect a stable chromophore produced by the reaction of a provided chromogenic reagent with MDA at 45 °C for 60 min. The absorbance of this chromophore was measured at 586 nm on a microtiter plate reader. Lipid peroxidation was determined according to a MDA standard curve and expressed in micromoles per milligram of proteins.

Quantitative assessment of CA1 neuronal loss. Animals were sacrificed 4 days after TGI by intracardiac perfusion with 200 ml of normal saline followed by 400 ml of formalin. Paraffin-embedded brain sections (10 μm) were deparaffinized in two exchanges of xylene for 5 min each, washed sequentially in 100%, 95%, and 70% ethanol, and stained with cresyl violet. Pyramidal neurons were counted in a double-blind manner in the middle 250-μm^2 CA1 region of the hippocampus. Twenty 250-μm^2 fields, five different sections with four areas per section on treatment with rosiglitazone and vehicles, were counted from each brain sample. The numbers of surviving neurons are denoted as Surviving Neurons/(250 $\mu m)^2$.

Statistical analysis. All values are expressed as means ± SE. One-way analysis of variance (ANOVA) was used to assess group means, followed by the Dunnett or Scheffé multiple range test for post hoc assessment of individual means. A value of $p < 0.05$ was considered statistically significant.

Results

Alterations of UCP2 expression in the mitochondria of hippocampus CA1 after TGI/reperfusion

UCP2, a mitochondrial protein, plays an important role in cells under oxidative stress in both physiological and pathological conditions. A potential neuroprotective effect of this protein was revealed based on the observation that UCP2 alleviated the oxidative stress under ischemic condition [10,14]. We therefore explored UCP2 expression in the mitochondria of hippocampal CA1 in the TGI/reperfusion

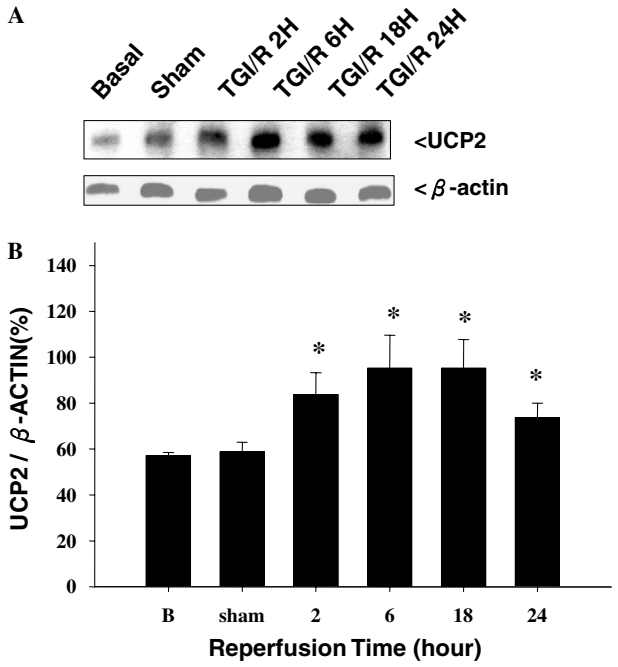

Fig. 1. UCP2 expression was increased in mitochondria of hippocampal CA1 after TGI/reperfusion. Rats were subjected to a 10-min global ischemia followed by reperfusion for 2, 6, 18, and 24 h. (A) Western blot showing temporal expression profile of mitochondrial UCP2 in hippocampal CA1 after TGI/reperfusion. The same blot was probed with a β-actin antibody to serve as an internal control for equal loading. (B) Semi-quantitative determination of UCP2 expression in relation to β-actin. Values are means ± SE. of triplicate analyses from 4 to 6 animals per experimental group. TGI, transient global ischemia, R, reperfusion. *$p < 0.05$ as compared to basal and sham-control group.

paradigm. Increased UCP2 expression was observed in the mitochondria prepared from hippocampal CA1 regions 2–24 h after TGI/reperfusion (Fig. 1A). A semi-quantitative measure of relative UCP2 contents using β-actin as an internal control was conducted to show the temporal profile of its expression pattern after TGI/reperfusion (Fig. 1B).

Rosiglitazone-enhanced UCP2 expression in hippocampus after TGI/reperfusion

As UCPs are regulated by PPARs [20,21], it is tempting to speculate that PPAR agonist can modulate the UCPs expression. Rosiglitazone, a PPAR agonist for treating diabetics in clinical practice as an insulin sensitizer, was reported to protect intestinal, renal, and myocardial damage from ischemia–reperfusion injury [26–28], which may be attributed to its potential anti-inflammatory and antioxidative effect. As UCP2 may have a pivotal role in ischemia–reperfusion injury in brain tissue, it is intriguing to know if rosiglitazone has effect over UCP2 expression in hippocampus after brain ischemia. Consistent with this contention, microinjection of rosiglitazone into hippocampal CA1 region 30 min before ischemia further increased the expression of UCP2 in mitochondria 2–6 h after TGI/reperfusion (Fig. 2).

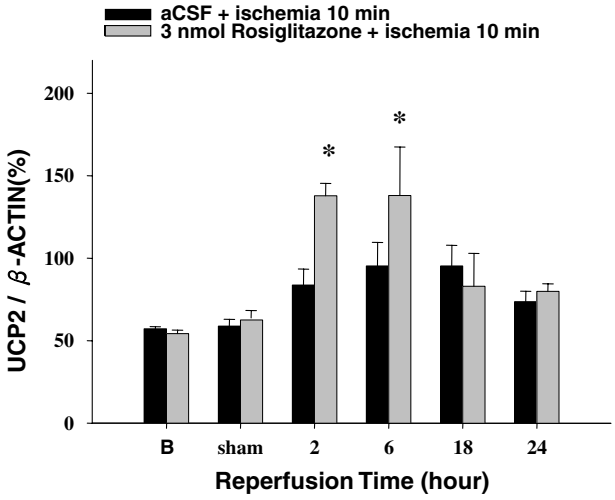

Fig. 2. Rosiglitazone further enhanced ischemia-induced UCP2 expression in mitochondria of hippocampal CA1 after TGI/reperfusion. Rats received microinjection of 50 nl rosiglitazone (3 nmol) or aCSF were subjected to a 10-min TGI followed by reperfusion for various periods of time. After reperfusion the hippocampal CA1 tissues were dissected for extraction of mitochondrial proteins in Western blot analyses to detect UCP2 expression. Note the rosiglitazone-enhanced UCP2 expression in TGI/reperfusion 2–6 h in mitochondria of hippocampal CA1. Values are means ± SE of triplicates from 4 to 6 animals per experimental group. *$p < 0.05$ as compared to vehicle-treated animals subjected to TGI/reperfusion.

Rosiglitazone effects on TGI/reperfusion-induced increases in lipid peroxidation in the mitochondria of hippocampal CA1 region

Lipid peroxidation, an index for cellular oxidative stress, contributes to tissue injury following cerebral ischemia and reperfusion. Lipid peroxides derived from polyunsaturated fatty acids are unstable and decompose to form a complex series of compounds. Malondialdehyde (MDA) is the most abundant reactive carbonyl compound as a result of fatty acid degradation. Therefore, measurement of MDA is widely used as an indicator for the extent of lipid peroxidation [29]. To further evaluate the effect of PPARγ agonist on neuroprotection, we examined whether rosiglitazone may alleviate ischemia-induced lipid peroxidation. A significant increase in MDA level was detected 2–6 h subsequent to TGI/reperfusion and returned to basal levels after 18–24 h (Fig. 3). Microinjection of 50 nl rosiglitazone (3 nmol) into the hippocampal CA1 subfield almost completely abolished TGI/reperfusion-induced increase in MDA contents (Fig. 3), suggesting a potential neuroprotective role in TGI-induced brain injury.

Effect of rosiglitazone on TGI-induced hippocampal neuronal injuries

TGI resulted in significant hippocampal CA1 neuronal loss as compared to the normal controls 4 days after ischemia/reperfusion (Fig. 4), a well-known phenomenon called delayed neuronal death. As shown in Fig. 3, TGI increased

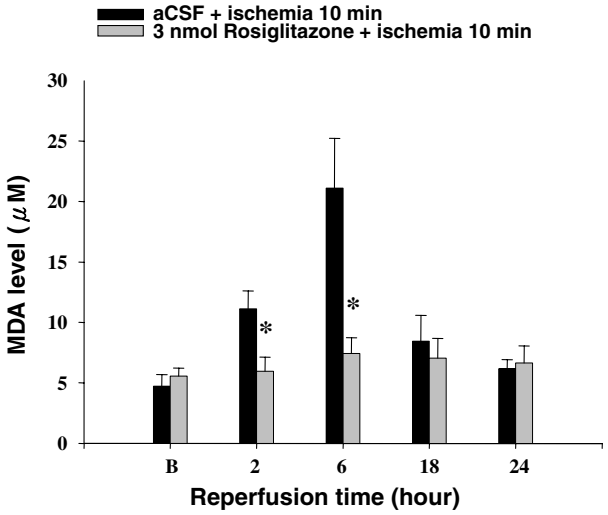

Fig. 3. Rosiglitazone attenuated TGI/reperfusion-induced lipid peroxidation in mitochondria of hippocampal CA1. Quantification of the extent of lipid peroxidation, as determined by measuring MDA levels, was conducted in control groups and in animals subjected to TGI with reperfusion for 2, 6, 18 or 24 h. Note increased MDA level in hippocampal mitochondria 2–6 h after reperfusion that was abolished by microinjection of rosiglitazone to hippocampus. Data shown are representative of three separate experiments with similar results. Values are means ± SE of triplicates from 4 to 6 animals per experimental group. $*p < 0.05$ as compared to vehicle-treated animals subjected to TGI/reperfusion.

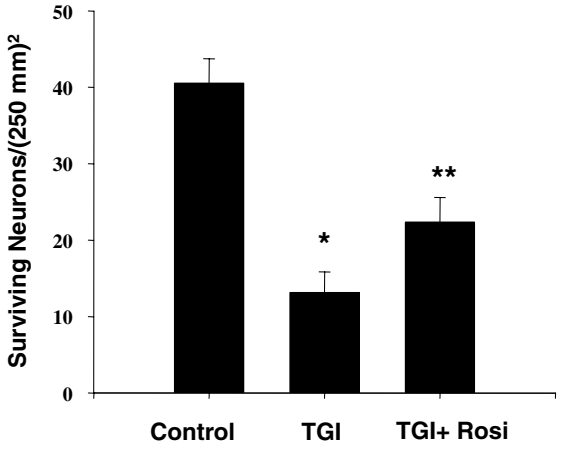

Fig. 4. Effects of rosiglitazone on the CA1 neuronal survival after TGI. Rats were subjected to TGI/reperfusion with or without rosiglitazone pretreatments under the identical experimental conditions as described in the legends of Fig. 2. The animals were sacrificed after 4 days to assess the effects of rosiglitazone on the numbers of surviving neurons in CA1 regions of hippocampus. Note the decreased neuronal survival in the animals with TGI and the recovery of surviving neuronal counts in the animals pretreated with rosiglitazone. $n = 6$–8 animals per group. Rosi, rosiglitazone $*p < 0.05$ as compared to the normal control animals. $**p < 0.05$ as compared to the TGI animals.

hippocampal CA1 mitochondrial lipid peroxidation that was substantially abolished by rosiglitazone. Since excessive oxidative stress may lead to irreversible damage of mitochondrial DNA, membrane lipids, and proteins, thereby contributing to cell death [4,5], we therefore examined whether rosiglitazone may also protect hippocampal CA1 neurons from TGI/reperfusion-induced injury. As shown in Fig. 4, the detrimental effect of TGI/reperfusion could be attenuated by rosiglitazone as evidence by an increase in neuronal survival, suggesting potential therapeutic implication of this PPARγ agonist in cerebral ischemia/reperfusion injury.

Discussion

Recent evidence has suggested an intimate link between an excessive generation of ROS and the development of hippocampal neuronal death [8,9,30]. ROS can damage various cellular components, such as proteins, lipids, and DNAs. Mitochondria are particularly vulnerable to oxidative stress because excessive ROS production constantly occurs during oxidative phosphorylation. UCP2, a mitochondrial protein with ability to dissipate proton to lessen ROS formation, is considered an endogenous neuroprotective agent [14–16,31]. In the present study, we showed that TGI/reperfusion-induced UCP2 expression in the mitochondria of hippocampal CA1 regions (Fig. 1), which likely represents a stress response of brain tissues to cope with ischemic injury. Rosiglitazone, a PPARγ agonist, further enhanced TGI/reperfusion-induced UCP2 expression (Fig. 2) and decreased MDA contents, a common index for lipid peroxidation (Fig. 3) [29]. More importantly, rosiglitazone attenuated TGI/reperfusion-dependent hippocampal neuronal death as shown in cresyl violet staining for neuronal survival (Fig. 4).

UCPs are known to be regulated by PPARs [20,21]. Recent studies also showed that pioglitazone-another PPARγ agonist, reduced the infarct size in transient focal cerebral ischemia that may relate to Cu/Zn-SOD as the mediator of this neuroprotection [32] or through prevention of COX-2 up-regulation [33]. However, the potential roles of PPARs-UCPs cascades in ischemia/hypoxia of central nervous system have not been reported. Hypoxia may decrease the expression of PPARs in various cell types [34,35]. PPARγ-deficient mice suffered from more severe damages in a model of intestinal ischemia–reperfusion (I/R) injury [26]. As hypoxia–ischemia results in change of redox state and ischemia/reperfusion causes inflammatory response, PPARs may play a significant role in these diseased states. Consistent with this contention, rosiglitazone, a PPARγ agonist, attenuated TGI-induced increase in MDA level (Fig. 3) and enhanced neuronal survival in TGI-induced neuronal death (Fig. 4). Rosiglitazone-mediated protective effects have been reported in other systems, such as gastric ischemia–reperfusion, by alleviating the inflammatory response and the oxidative events [36]. Rosiglitazone also protected the heart from ischemia/reperfusion-induced myocardial apoptosis [37]. In addition, rosiglitazone inhibited the expression of intercellular adhesion molecule-1 (ICAM-1) and reduced polymorphonuclear neutrophil (PMN) infiltration into renal tissues with subsequent reduction of oxidative damages during ischemia/reperfusion [27]. In this report, we demonstrated that

rosiglitazone enhanced UCP2 expression. These results, coupled with the finding that rosiglitazone attenuated TGI/reperfusion-induced lipid peroxidation in the mitochondria of hippocampal CA1 and increased neuronal survival, provide correlative evidence for the "PPARγ → UCP2 → neuroprotection" cascade in ischemic brain injury.

Acknowledgments

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