Modulation of the oxidative stress and inflammatory response by PPAR-γ agonists in the hippocampus of rats exposed to cerebral ischemia/reperfusion

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Abstract

Agonists of the peroxisome proliferator-activated receptor-γ (PPAR-γ) exert protective effects in several models of ischemia/reperfusion injury, but their role in stroke is less clear. The study investigates the effects of two PPAR-γ agonists, rosiglitazone and pioglitazone, on oxidative stress and inflammatory response induced by ischemia/reperfusion in the rat hippocampus. Common carotid artery occlusion for 30 min followed by 1 h reperfusion resulted in a significant increase in the generation of reactive oxygen species, nitric oxide and the end products of lipid peroxidation as well as markedly reduced endogenous antioxidant glutathione levels and up-regulated superoxide dismutase activity. Western blot analysis showed that ischemia/reperfusion lead to an increase in cyclooxygenase-2 (COX-2) expression, as well activating p38 and p42/44 mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB). Pre-treatment with either rosiglitazone or pioglitazone significantly reduced oxidative stress, COX-2 protein expression and activation of MAPKs and NF-κB. Taken together, the results provide convincing evidence that PPAR-γ agonists exert protective effects in a rat model of mild forebrain ischemia/reperfusion injury by inhibiting oxidative stress and excessive inflammatory response.

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1. Introduction

It is becoming increasingly clear that oxidative stress and excessive inflammatory response are implicated in the pathogenesis of ischemic and reperfusion injury to many organs, including the brain (Schaller and Graf, 2004). Reactive oxygen species have been indicated as one of the earliest and most important components of tissue injury after reperfusion of ischemic organ and the extent of brain injury appears to depend on the experimental pattern of ischemia/reperfusion: free radical production is continuous during ischemia, while during reperfusion it is primarily confined to the early stage when fresh oxygen is supplied to the ischemic region (Nita et al., 2001). The brain is very susceptible to the damage caused by oxidative stress, due to the high rate of oxidative metabolic activity, high polyunsaturated fatty acid contents, relatively low antioxidant capacity and inadequate neuronal cell repair activity (Traystman et al., 1991). Overproduction of reactive oxygen species results in oxidative damage, including lipid peroxidation, protein oxidation and DNA damage, which can lead to cell death (Floyd, 1999; Love, 1999; Phillis, 1994). Furthermore, reactive oxygen species can activate diverse downstream signalling pathways, such as mitogen-activated protein kinases (MAPKs) or the transcription factor nuclear factor-κB (NF-κB), thus regulating expression of genes encoding a...
variety of proinflammatory proteins. Overexpression of cyclooxygenase-2 (COX-2) and of inducible nitric oxide synthase (iNOS) have recently emerged as important determinants of post-ischemic inflammation, which contributes to the progression of brain damage (Candelario-Jalil et al., 2003; Lerouet et al., 2002).

The peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the nuclear receptor superfamily. PPARs are ligand-dependent transcription factors that bind to specific peroxisome proliferators response elements at the enhancer sites of regulated genes (Berger and Moller, 2002). They are implicated in adipocyte differentiation, insulin sensitivity and inflammatory processes (Lemberger et al., 1996; Vamecq and Latruffe, 1999), and also down-regulate proinflammatory mediators in macrophages and microglia, mainly by inhibiting transcription of NF-κB-dependent inflammatory genes (Ricote et al., 1998; Petrova et al., 1999, Colville-Nash et al., 1998).

Synthesised PPAR-γ agonists are used as oral antihyperglycemic drugs in treating noninsulin-dependent diabetes mellitus (Yki-Jarvinen, 2004). Troglitazone was the first such drug approved for treating type 2 diabetes, but was withdrawn from the market in 1999 due to hepatic toxicity (Watkins and Whitcomb, 1998). Other drugs, such as rosiglitazone and pioglitazone, are now available and show no hepatic side effects (Gillies and Dunn, 2000; Lenhard and Funk, 2001). Of the PPAR-γ agonists tested to date, rosiglitazone binds with the highest affinity to PPAR-γ (Lehmann et al., 1995), with a Kd that parallels its in vivo anti-diabetic activity (Willson et al., 1996). The effects of PPAR-γ agonists were originally thought to be limited to controlling lipid metabolism and homeostasis. However, emerging evidence indicates that PPAR-γ activation can regulate inflammatory responses, included inflammatory disorders of the central nervous system, inhibiting expression of a variety of pro-inflammatory molecules by a mechanism termed receptor-dependent transrepression (Kielian and Drew, 2003). Furthermore, beneficial effects of PPAR-γ agonists on ischemia/reperfusion injury have been previously documented in the intestine (Nakajima et al., 2001; Ichikawa et al., 2002; Naito et al., 2002), lung (Okada et al., 2002), heart (Wayman et al., 2002; Khandoudi et al., 2002; Yue et al., 2001), kidney (Sivarajah et al., 2003) and, more recently, also in the brain (Sundararajan et al., 2005; Shimazu et al., 2005). However, the effects of these drugs on oxidative stress and inflammatory response associated with cerebral ischemia/reperfusion are still unknown. Among brain areas, the hippocampus is one of the most sensitive to ischemia/reperfusion injury (Sharma and Kumar, 1998) and PPAR-γ has been detected in this area in adult rats, with the highest levels in the CA1 pyramidal cells and the granular and polymorphic layers of the dentate gyrus (Moreno et al., 2004).

This study thus aimed to determine whether PPAR-γ activation by rosiglitazone and pioglitazone reduces cerebral ischemia/reperfusion injury in the rat hippocampus through modulation of oxidative stress and inflammation pathways.

2. Materials and methods

2.1. Animals and animal treatment

Male Wistar rats (Harlan-Italy, Udine, Italy) weighing 210 to 230 g were housed in a controlled environment at 25±2 °C with alternating 12-h light and dark cycles. They were provided with Piccione pelleted diet (n.48, Gessate Milanese, Italy) and water ad libitum. All rats were acclimatized in our animal facility for at least 1 week prior to experiments. Stressful stimuli were avoided. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116/92) as well as with EEC regulations (O.J. of E.C. L 358/1 12/8/1986).

After pretreatment as described below, anaesthetised rats were subjected to bilateral occlusion of the common carotid arteries for 30 min followed by reperfusion for 1 h, or to a sham surgical procedure. The experimental protocol was approved by the Turin University Ethics Committee.

Animals were randomly allocated into eight different pretreatment groups as described below:

- I/R group: Control group. Rats were administrated 10% (v/v) dimethyl sulphoxide (DMSO) (vehicle for rosiglitazone and pioglitazone, 1 ml/kg, i.v.) 30 min prior to ischemia (n=8).
- I/R +P group: Rats were administrated pioglitazone (1 mg/kg, i.v.) 30 min prior to ischemia (n=6).
- I/R +R1 group: Rats were administrated rosiglitazone (1 mg/kg, i.v.) 30 min prior to ischemia (n=6).
- I/R +R3 group: Rats were administrated rosiglitazone (3 mg/kg, i.v.) 30 min prior to ischemia (n=8).
- I/R +R6 group: Rats were administrated rosiglitazone (6 mg/kg, i.v.) 30 min prior to ischemia (n=6).
- Sham group: Rats were subjected to the same surgical procedures as above, except for I/R. Rats were administrated 10% (v/v) DMSO (vehicle for rosiglitazone and pioglitazone, 1 ml/kg, i.v.) 30 min prior to ischemia (n=6).
- Sham +P group: Rats were subjected to the same surgical procedures as above, except for I/R. They were administrated pioglitazone (1 mg/kg, i.v.) 30 min prior to ischemia (n=3).
- Sham +R group: Rats were subjected to the same surgical procedures as above, except for I/R. They were administrated rosiglitazone (6 mg/kg, i.v.) 30 min prior to ischemia (n=3).

2.2. Surgical procedures

Rats were anaesthetised through i.p. injection of a mixture of Ketavet 100 (Farmaceutici Gellini, Italy) and Rompun (xilazina; Bayer AG, Leverkusen, Germany) (4/1, vol/vol; 0.5 ml mixture/200 g body weight) supplemented as needed. Rosiglitazone, pioglitazone or vehicle alone were injected into the tail vein 30 min prior to ischemia. Surgery was performed in all cases between 10.00 and 12.00 a.m. Anaesthetised rats were placed onto a thermostatically controlled heating pad, a rectal temperature probe was inserted and body temperature was
monitored and maintained at 37 °C. Both common carotid arteries were exposed over a midline incision, and a dissection was made between the sternoclidomastoid and the sternohyoid muscles parallel to the trachea. Each carotid artery was freed from its adventitial sheet and vagus nerve, which was carefully separated and maintained (Ulrich et al., 1998). Ischemia was achieved by clamping the bilateral common carotid arteries for 30 min using non-traumatic artery clamps (Micro Bulldog Clamps, Harvard Apparatus Ltd., Kent, UK). During ischemia the animals were monitored for body temperature, respiration pattern, loss of righting reflex and unresponsiveness, corneal reflexes, and fixed and dilated pupils. Recirculation of blood flow was established by releasing the clips and restoration of blood flow in the carotid arteries was confirmed by careful observation. Reperfusion was allowed for 60 min. At the end of this time, the rats were killed by decapitation after aortic exsanguination. Sham-operated rats underwent identical surgical procedures except that no artery clamps were applied. After decapitation, the forebrain was rapidly dissected at 0 °C and the whole hippocampus from both hemispheres was rapidly removed and transferred to appropriate ice-chilled homogenising medium for biochemical assays.

2.3. Tissue extracts

Cytosolic and nuclear extracts were prepared by the Meldrum method (Meldrum et al., 1997). Briefly, hippocampi were homogenized at 10% (w/v) in a Potter Elvehjem homogenizer (Wheaton, Millville, NJ, USA) using a homogenization buffer containing 20 mM HEPES, pH 7.9, 1 mM MgCl2, 0.5 mM EDTA, 1% NP-40, 1 mM EGTA, 1 mM Dithiothreitol (DTT), 0.5 mM Phenylmethyl Sulphonyl Fluoride (PMSF), 5 μg/ml aprotinin, 2.5 μg/ml leupeptin. Homogenates were centrifuged at 10000×g for 5 min at 4 °C. Supernatants were removed and centrifuged at 105,000×g at 4°C for 40 min to obtain cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer containing 20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 300 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM EGTA, 1 mM DT, 0.5 mM PMSF, 5 μg/ml aprotinin, 2.5 μg/ml leupeptin. The suspensions were incubated on ice for 30 min for high-salt extraction followed by centrifugation at 15,000×g for 20 min at 4°C. The resulting supernatants containing DNA-binding proteins were carefully removed, protein content was determined using the Bradford assay (Bradford, 1976) and samples were stored at –80 °C until use.

2.4. Reactive oxygen species and GSH detection

Reactive oxygen species were measured in cytosolic fractions using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as a probe. DCFH-DA is a stable, non-fluorescent molecule that readily crosses the cell membrane and is hydrolysed by intracellular esterases to non-fluorescent 2′,7′-dichlorofluorescein (DCFH), which is rapidly oxidized in the presence of peroxides to highly fluorescent 2′,7′-dichlorofluorescein (DCF), which is then measured fluorimetrically (Ravin-dranath, 1994). Results are expressed as units of fluorescence (U.F./mg protein).

Antioxidant levels in the cytosolic fractions were evaluated in terms of reduced GSH content, by Ellman’s method (Ellman, 1959). A mixture was directly prepared in a cuvette: 2.25 ml of 0.1 M K-phosphate buffer, pH 8.0; 0.2 ml of the sample; 25 μl of Ellman’s reagent (10 mM 5,5′-dithio-bis-2-nitrobenzoic acid in methanol). After 1 min the assay absorbance was measured at 412 nm and the GSH concentration was calculated by comparison with a standard curve.

2.5. Superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured by the xanthine/xanthine oxidase mediated ferricytochrome c reduction assay as described by Flohé and Otting (1984). SOD competes for superoxide and decreases the reduction of cytochrome c. An aliquot of cytosol (50 μl) was added to reaction buffer (0.5 μmol of xanthine, 0.1 mM NaOH and 2 μmol of cytochrome c in 50 mM KH2PO4–NaPO4/0.1 mM EDTA buffer, pH 7.8). The reaction was initiated by adding 50 μl of xanthine-oxidase solution (0.2 U/ml in 0.1 mM EDTA). The absorbance change was monitored at 550 nm for 3 min at 25 °C and quantified by comparison with a blank (assay without SOD). One unit (U) of SOD activity was defined as the amount required to cause 50% inhibition of the absorbance change per minute of the blank reaction and results were normalised on the basis of total protein content (U/mg protein).

2.6. End products of lipid peroxidation

Lipid peroxidation was investigated by measurement of the main end-product of peroxidation, hydroxynonenal, in the cytosol fractions. Hydroxynonenal concentration was determined on fresh cytosol fractions by Esterbauer’s method (Esterbauer et al., 1986). An aliquot of cytosol (100 μl) was extracted in an equal volume of a solution of acetic acid/acetoniitrile (4/96, v/v). After centrifugation at 250×g for 20 min at 4 °C, 50 μl of supernatant were injected into an HPLC Symmetry C18 column (5 mm, 3.9 × 150 mm). The mobile phase used was acetonitrile/bidistilled water (42%, v/v). The hydroxynonenal concentration was calculated by comparison with a standard solution of hydroxynonenal (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) of known concentration.

2.7. Nitrite-plus-nitrate content

The nitrite-plus-nitrate concentration in cytosolic fractions was used as an indicator of nitric oxide (NO) synthesis. Nitrates in cytosol samples were stochiometrically reduced to nitrites by incubation of 250 μl of sample for 15 min at 37 °C in the presence of 1 IU/ml nitrate reductase, 500 μM NADPH and 50 μM FAD in a final volume of 400 μl. When nitrate reduction was complete, unused NADPH, which interferes with subsequent nitrite determination, was oxidised by 100 IU/ml lactate dehydrogenase and 100 mM sodium pyruvate in a final reaction volume of 500 μl and incubated for 5 min at 37 °C (Millar and
Thiemermann, 1997). Subsequently, total nitrites in the cytosol were assayed by adding 500 μl of Griess reagent (4% sulphanilamide and 0.2% naphthylendiamide in 10% phosphoric acid) to each sample (Green et al., 1981).

2.8. Western blot analysis

About 15 μg total protein were loaded. Proteins were separated on 8% or 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Blots were blocked in a blocking buffer containing phosphate-buffered saline with 5% albumin. Membranes were incubated with primary antibody (rabbit anti-iNOS, mouse anti-COX-1, rabbit anti-COX-2, mouse anti-phosphorylated p38, mouse anti-phosphorylated p42/44, rabbit anti-NF-κB p65). Blots were then incubated with secondary antibody conjugated with horseradish peroxidase (1:10,000) for 30 min at room temperature, and then developed with ECL detection system. The immunoreactive bands were visualized by autoradiography and the density of the bands were evaluated densitometrically using the program Gel Pro® Analyser 4.5, 2000 (Media Cybernetics, Silver Spring, MD, USA). The membranes were stripped and incubated with β-actin monoclonal antibody (1:5000) and subsequently with anti-mouse (1:10,000) both for 30 min at room temperature, in order to assess uniformity of gel loading. To detect ph-p38, ph-p42/44, and NF-κB p65, Western blot analysis was done on 10% SDS-PAGE; for iNOS, COX-1 and COX-2, it was done on 8% SDS-PAGE.

2.9. Materials

Unless otherwise stated, all compounds were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA).

The BCA Protein Assay kit was from Pierce (Rockford, IL, USA); Polyvinylidene difluoride (PVDF) was from Millipore Corporation (Billericia, MA, USA). Rabbit polyclonal antibodies against iNOS and against NF-κB p65 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody against COX-2 and murine monoclonal antibody against COX-1 were from Cayman Chemical Company (Ann Arbor, MI, USA). Antibodies to the phosphorylated forms of p38 and p42/44 MAPKs were from Cell-Signalling Technology (Beverly, MA, USA). Anti-mouse and anti-rabbit Ig horseradish peroxidase-linked whole antibodies and Luminol ECL detection reagents were from Amersham (Buckinghamshire, UK). The selective PPAR-γ agonists rosiglitazone and pioglitazone were from Alexis Biochemicals (San Diego, CA, USA) and were prepared in 10% DMSO (Sigma-Aldrich, MO, USA).

2.10. Statistical analysis

All values in text and figures are expressed as mean ± standard error of the mean (S.E.M.) for n observations. One-way analysis of variance with Dunnett’s post test was performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com) and a P value of less than 0.05 was considered to be significant.

3. Results

3.1. Pre-treatment with PPAR-γ agonists decreases oxidative stress and lipid peroxidation induced by ischemia/reperfusion injury

Rats that had undergone transient bilateral common carotid occlusion exhibited a 50% increase in reactive oxygen species production (141 ± 8.5 U.F./mg protein), compared with sham-operated animals (92.6 ± 4.8 U.F./mg protein) (Fig. 1A). In the group pre-treated with pioglitazone (1 mg/kg), this increase in reactive oxygen species production was reduced by almost half (119.7 ± 0.9 U.F./mg protein) compared to that occurring in the I/R group. Pre-treatment of rats with the same dose of rosiglitazone (1 mg/kg) failed to reduce reactive oxygen species levels, whereas higher doses (3 or 6 mg/kg) caused approximately 40% inhibition of the increase in reactive oxygen species production (121.3 ± 5.1 or 120.1 ± 6.2 U.F./mg protein, respectively), similarly to pioglitazone. Reactive oxygen species overproduction evoked by transient cerebral ischemia/reperfusion was associated with a massive increase in hydroxynonenal, a toxic end product of lipid peroxidation, compared to sham-
operated animals (Fig. 1A). The 10-fold increase in hydroxynonenal concentration in the I/R group (4.8±0.3 μM) versus the sham-operated group (0.4±0.1 μM) was nearly halved by pre-treatment with pioglitazone or with rosiglitazone. Similarly, SOD activity in hippocampus homogenates from rats subjected to ischemia/reperfusion (5.71±0.23 U/mg protein) was significantly higher than that of sham-operated animals (3.77±0.30 U/mg protein) (Fig. 1B). Pioglitazone pre-treatment reduced SOD activity to 4.53±0.50 U/mg protein. Rosiglitazone prevented the SOD activity enhancement induced by ischemia/reperfusion at either 3 or 6 mg/kg (4.95±0.18 and 4.53±0.22 U/mg protein, respectively), whereas 1 mg/kg provided no protection. The increased reactive oxygen species production, evoked by ischemia/reperfusion, caused a significant depletion in GSH content compared to sham-operated animals (Fig. 1B). Pre-treatment with either pioglitazone (1 mg/kg) or rosiglitazone (1, 3 or 6 mg/kg) increased antioxidant levels to values similar to the sham-operated group.

As shown in Fig. 2, ischemia/reperfusion caused a 40% increase in nitrite plus nitrate content (an indicator of NO formation) in hippocampus homogenates (42.5±3.3 μM) compared to sham-operated animals (30.2±0.8 μM). Pre-treatment with rosiglitazone (1 mg/kg) was ineffective, while higher doses (3 and 6 mg/kg) significantly reduced nitrite plus nitrate content (35.1±1.0 and 34.0±0.9 μM, respectively) and a quantitatively similar reduction was obtained with pioglitazone (1 mg/kg). However, nitrite plus nitrate levels in rats pre-treated with PPAR-γ agonists were still significantly higher than those in sham-operated animals. Hippocampal iNOS protein expression was also investigated in order to determine whether the increased nitrite-plus-nitrate content was correlated with a modulation of iNOS expression. Western blot analysis of
samples obtained from rats subjected to ischemia/reperfusion revealed a significantly increased expression of iNOS, which was down-regulated by pre-treatment with pioglitazone or rosiglitazone (data not shown).

Administration of pioglitazone (1 mg/kg) or rosiglitazone (6 mg/kg) to sham-operated rats had no significant effect on any of the markers of oxidative stress or lipid peroxidation versus non-pre-treated sham-operated rats (data not shown).

3.2. Effects of PPAR-γ agonists on COX-2 expression

COX-2 was scarcely detected in sham-operated animals, by Western blot analysis (Fig. 3); ischemia/reperfusion

Fig. 4. Effect of pre-treatment with PPAR-γ agonists on phosphorylation of p38 MAPK (panel A) and p42/p44 MAPKs (panel B) in the hippocampus of rats exposed to cerebral ischemia/reperfusion. Phosphorylated p38 and p42/p44 MAPK were detected in the cytosolic fractions of hippocampi obtained from sham-operated rats (Sham) and rats subjected to ischemia/reperfusion (I/R). Rats were administered 1 mg/kg pioglitazone (I/R+P), 1 mg/kg rosiglitazone (I/R+R1), 3 mg/kg rosiglitazone (I/R+R3) or 6 mg/kg rosiglitazone (I/R+R6) 30 min prior to ischemia/reperfusion (30/60 min). Two groups of rats received pioglitazone (1 mg/kg) or rosiglitazone (6 mg/kg) prior to sham operation (Sham+P; Sham+R). Each immunoblot of protein extracts obtained from cytosolic fractions of rat hippocampus is from a single experiment and is representative of three separate experiments. Densitometric analysis of the related bands is expressed as relative optical density (O.D.) of the bands, corrected for the corresponding β-actin contents and normalised using the related sham-operated band. Densitometry results are expressed as means±S.E.M. of three separate experiments. Statistical analysis: • P<0.05 versus Sham (one-way analysis of variance with Dunnett’s post test), ★ P<0.05 versus I/R (one-way analysis of variance with Dunnett’s post test).
significantly increased hippocampal COX-2 expression. COX-2 levels were significantly reduced by pre-treatment with 1 mg/kg pioglitazone; pre-treatment with rosiglitazone (3 or 6 mg/kg) had a similar effect, whereas 1 mg/kg had no effect. Furthermore, neither drug affected COX-2 expression when administered to sham-operated animals. In contrast, COX-1 levels showed no significant quantitative difference among different groups, not being changed by ischemia/reperfusion or by pre-treatment with either drug (data not shown).

3.3. Effects of PPAR-γ agonists on MAPK and NF-κB signalling pathways

Animals that had undergone ischemia/reperfusion displayed higher phosphorylated p38 and p42/p44 MAPKs expression than sham-operated animals (Fig. 4A and B). Pre-treatment with pioglitazone (1 mg/kg) decreased levels of the phosphorylated forms of these MAPKs compared to the I/R group. Rosiglitazone at 3 or 6 mg/kg inhibited the activation of p42/p44 and p38 MAPKs evoked by ischemia/reperfusion, but had no effect at 1 mg/kg. Both drugs showed stronger effects on p38 than on p42/p44 MAPK. Administration of either drug to sham-operated animals did not affect MAPK activation.

Similar results were obtained when NF-κB activation was evaluated in cytosol and nuclear fractions (Fig. 5). In sham-operated rats, the NF-κB p65 subunit was detected only in the cytosol and not in the nucleus. When animals underwent ischemia/reperfusion, the hippocampal fractions showed higher levels of the NF-κB p65 subunit in the nucleus than in the cytosol, thus confirming NF-κB translocation. This NF-κB signalling activation was inhibited by pre-treatment with either pioglitazone (1 mg/kg) or rosiglitazone (3 or 6 mg/kg), but not by rosiglitazone at 1 mg/kg.

4. Discussion

We report here that pre-treatment of rats with PPAR-γ agonists rosiglitazone or pioglitazone caused a substantial reduction of injury induced by transient forebrain ischemia (30 min) followed by reperfusion (60 min). In particular, in the hippocampus of rats that had undergone ischemia/reperfusion, rosiglitazone and pioglitazone attenuated: (i) the extent of oxidative stress, (ii) activation of the MAPK and NF-κB signalling cascades, (iii) the increased expression of COX-2.

The majority of in vivo models of cerebral ischemia rely on vessel occlusion predominantly affecting the forebrain (Lipton, 1999). Bilateral occlusion of the common carotid arteries in rats is a common model of incomplete global cerebral ischemia (Aragno et al., 2000; Ghoneim et al., 2002; Seif-El-Nasr and El-Fattah, 1995; Shaheen et al., 1996; Vanella et al., 1990), and results in a 50% decrease in cerebral blood flow (Eklof and Siesjo, 1972). This induced partial ischemia, without affecting the circle of Willis (collateral circulation), has been suggested to reflect the early events occurring during transitory ischemic attacks more closely (Vanella et al., 1990). The hippocampus is one of the brain areas most sensitive to ischemia/reperfusion (Sharma and Kumar, 1998). In the present study we demonstrate that transient cerebral ischemia/reperfusion causes a marked increase in reactive oxygen species production and hydroxynonenal levels, used as marker of lipid peroxidation, in the rat hippocampus. At the same time, ischemia/reperfusion reduces hippocampal levels of GSH, a significant scavenger of reactive oxygen species, and enhances SOD activity. The increase in SOD activity indicates that the brain’s antioxidant machinery is activated in response to excessive generation of reactive oxygen species. SOD catalyses the conversion of superoxide anions to molecular oxygen and hydrogen peroxide, which requires to be scavenged further by tissue thiols, such as GSH, and by catalase (Bannister et al., 1987; Fridovich, 1995). Furthermore, apart from its own toxicity, hydrogen peroxide in the presence of iron leads to the generation of toxic hydroxyl radicals (Blake et al., 1987). Collectively, the results reported here confirm the presence of a significant level of oxidative stress in our experimental model.

The presence and expression of PPAR-γ has recently been demonstrated in rat hippocampal neurons (Inestrosa et al., 2005) and emerging studies have reported the effects of PPAR-γ agonists in animal models of brain damage, including cerebral transient focal ischemia (Shimazu et al., 2005; Sundararajan et al., 2005). However, the precise neuroprotective mechanisms of
PPAR-γ agonists have not yet been fully clarified. Our results show that administration of pioglitazone or rosiglitazone 30 min prior to ischemia/reperfusion significantly decreased reactive oxygen species production and lipid peroxidation versus rats that underwent ischemia/reperfusion. Similarly, both drugs offered significant protection against GSH depletion and SOD activity induced by ischemia/reperfusion, thus confirming their ability to attenuate excessive formation of reactive oxygen species secondary to ischemia/reperfusion injury. These data suggest that the protective effects of rosiglitazone and pioglitazone against ischemia/reperfusion injury are partially due to their ability to reduce oxidative stress. These findings are supported by recent reports. Aoun et al. (2003) demonstrated that pre-treatment with PPAR-γ agonists protected an immortalized mouse hippocampal cell line against oxidative stress induced by glutamate or hydrogen peroxide. Mehta et al. (2003) reported that PPAR-γ agonists diminished reactive oxygen species generation induced by angiotensin II and tumour necrosis factor-α, in human coronary artery endothelial cells. Pioglitazone pre-treatment has also been shown to inhibit reactive oxygen species production in cardiac fibroblasts exposed to hypoxia/reoxygenation (Chen et al., 2004) and to reduce renal oxidative stress in obese rats (Dobrian et al., 2004).

Mitochondria are the major source of reactive oxygen species, which are mainly generated at complexes I and III of the respiratory chain (Kudin et al., 2005). There is now evidence indicating that rosiglitazone and pioglitazone exert direct and rapid effects on mitochondrial respiration, inhibiting complex I (Brunmair et al., 2004) and complex III (Dello Russo et al., 2003) activity. As PPAR-γ agonists partially disrupt the mitochondrial respiratory chain, both electron transport and superoxide anion generation are affected. Moreover, a novel mitochondrial target protein for PPAR-γ agonists (“mitoNEET”) has recently been identified (Colca et al., 2004). MitoNEET was found associated with components of complex III, suggesting how PPAR-γ agonists binding to mitoNEET could selectively block different mitochondrial targets. PPAR-γ agonists’ ability to influence mitochondrial function might contribute to their inhibitory effects on reactive oxygen species generation evoked by ischemia/reperfusion.

Another mechanism through which PPAR-γ agonists may provide neuroprotection is by down-regulating inflammatory response (Sundararajan and Landreth, 2004). It has been clearly shown that reactive oxygen species regulate the expression of many pro-inflammatory genes, including COX-2 (Wang et al., 2004) and iNOS (Floyd, 1999), in different brain tissues. The COX-2 gene was first established as a rapidly inducible (within 30 min) hippocampal gene in experiments with electroconvulsive seizures (Yamagata et al., 1993) and bilateral carotid artery occlusion has been reported to induce a biphasic increase in hippocampal prostaglandin E2 concentrations, with maximum at 2 and 24–48 h reperfusion (Candelario-Jalil et al., 2003). During ischemia/reperfusion, NO reacts with reactive oxygen species to produce peroxynitrates, which have deleterious effects on neuronal survival (Warner et al., 2004). We show here that, in the rat hippocampus following transient ischemia/reperfusion, nitrite-plus-nitrate content is high. The rapid increase in NO levels after the onset of ischemia is consistent with previous reports (Kumura et al., 1994; Malinski et al., 1993; Zhang et al., 1995) and apparently reflects the hippocampal increase of iNOS expression. Pre-treatment with PPAR-γ agonists markedly attenuated the hippocampal levels of both nitrite/nitrate and iNOS. COX-2 expression in rat hippocampus was also affected by ischemia/reperfusion, and administration of either rosiglitazone or pioglitazone was correlated with lower levels of COX-2. The ability of the two drugs to reduce hippocampal oxidative stress might be sufficient to prevent COX-2 over-expression. Nonetheless, we cannot, at this stage, exclude the possibility that they directly modulate expression of these pro-inflammatory genes. Most anti-inflammatory properties of PPAR-γ agonists have been suggested to arise through inhibition of NF-κB (Colville-Nash et al., 1998; Daynes and Jones, 2002; Jiang et al., 1998; Ricote et al., 1999). Inhibition of p38 MAPK activation has been reported to contribute to protection of the heart from ischemia/reperfusion injury in both normal and diabetic rats (Kanddoudi et al., 2002). In the present study, we demonstrate that the protective effects of PPAR-γ agonists against incomplete cerebral ischemia/reperfusion involve inhibition of MAPK and NF-κB signalling pathways. In particular, we found that: (i) the phosphorylated forms of p38 and p42/44 MAPKs were elevated in ischemia/reperfusion hippocampi and (ii) NF-κB translocated from cytosol to nucleus in hippocampal extracts of ischemia/reperfusion rats, thus confirming NF-κB activation. These data are in agreement with previous studies showing that p38 MAPK and NF-κB were activated in the hippocampus of rats that underwent ischemia/reperfusion (Ozawa et al., 1999; Shen et al., 2003). Moreover, the relationship between high reactive oxygen species levels and the activation of downstream signalling routes, such as MAPK and NF-κB pathways, is well accepted (Rosenberger et al., 2001; Squadrito et al., 2005). Here, for the first time, we show that MAPK and NF-κB activation by cerebral ischemia/reperfusion can be inhibited by pre-treatment with rosiglitazone or pioglitazone. PPAR-γ agonists appear to act preferentially on p38 MAPK activation rather than on p42/p44, thus confirming the pivotal role of p38 in events related to ischemia/reperfusion. MAPK and NF-κB signalling pathways may be functionally interconnected and not act independently. Inhibition of the p38 MAPK pathway has been shown to abolish NF-κB-driven activation of gene expression (Carter et al., 1999) and p38 MAPK activation has been suggested to contribute to NF-κB activation by modulating the transactivation capacity of the NF-κB p65 subunit (Vanden Berghe et al., 1998). We might thus suppose that PPAR-γ agonists affect NF-κB activation by interfering with the MAPK signalling cascade. However, in the absence of direct experimental evidence, no conclusions can be reached with regards to this suggestion.

Further considerations may be proposed on the doses of PPAR-γ agonists. It has been reported that 1 mg/kg pioglitazone reduces infarction size in transient cerebral ischemia (Sundararajan et al., 2005). Here we show that the same dose affects
oxidative stress, lipid peroxidation and COX-2 expression in the hippocampus of rats exposed to ischemia/reperfusion. Moreover, pioglitazone pre-treatment reduces MAPK and NF-κB activation. To the best of our knowledge, rosiglitazone, which is the drug with the highest affinity to PPAR-γ (Lehmann et al., 1995), has never before been tested in a model of cerebral ischemia/reperfusion injury; it is tested here in the dose range 1–6 mg/kg, and 1 mg/kg was found to have no effect, while the higher doses (3 and 6 mg/kg) reduced injury incidence and severity as well as did pioglitazone. However, rosiglitazone has a 7-times higher affinity for PPAR-γ than does pioglitazone, and we would thus have expected it to have effects at lower doses than pioglitazone. The fact that pioglitazone, which possesses a lower affinity for PPAR-γ activation, was already effective at 1 mg/kg points to the existence of other factors involved in determining the efficacy of the two drugs, such as differences in blood–brain barrier permeability. Pioglitazone has been shown to cross the blood–brain barrier (Maeshiba et al., 1997) and the protective effects of acute treatment with PPAR-γ agonists in animal models of neuroinflammation have recently been reported (Garcia-Bueno et al., 2005; Heneka et al., 2005). Nonetheless, no pharmacokinetic comparison of PPAR-γ agonists’ access to the central nervous system has been reported.

In conclusion, we show here for the first time that the PPAR-γ agonists rosiglitazone and pioglitazone exert protective effects against cerebral ischemia/reperfusion injury by reducing oxidative stress and inflammatory response. Both drugs were administered only 30 min prior to ischemia and the rapid onset of their protective effects might suggest a potential role of PPAR-γ agonists in modulating the early events occurring during transitory ischemic attacks. However, further direct evidence linking PPAR-γ agonists to their antioxidant and anti-inflammatory properties is needed. Rigorous verification of such properties might better clarify the mechanism of action of these drugs and validate their use in conditions associated with ischemia/reperfusion injury.

References


