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Oxidative stress and inflammatory response evoked by transient cerebral ischemia/reperfusion: effects of the PPAR-alpha agonist WY14643

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Original Citation:

Availability:

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Elsevier Editorial System(tm) for Free Radical Biology and Medicine

Manuscript Draft

Manuscript Number: FRBM-D-06-00262R1

Title: Oxidative stress and inflammatory response evoked by transient cerebral ischemia/reperfusion: effects of the PPAR- α agonist WY14643.

Article Type: Original Contribution

Section/Category:

Keywords: PPAR- α ; hippocampus; ischemia/reperfusion; oxidative stress; inflammation.

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Manuscript Region of Origin:

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of p38 mitogen-activated protein kinase and nuclear factor- κ B. The PPAR- α antagonist, MK886, abolished the beneficial effects of WY14643. The levels of S100B protein, a marker of cerebral injury used in stroke trials to monitor injury, were high in the hippocampus of rats exposed to I/R, but markedly reduced by WY14643. We propose that WY14643 protects the brain against excessive oxidative stress and inflammation and may thus be useful in treating stroke.

Turin, 27th April 2006

Dr. Harry Ischiropoulos
Associate Editor,
Free Radical Biology & Medicine

Dear Dr. Ischiropoulos,

Please find enclosed our revised manuscript entitled “**Oxidative stress and inflammatory response evoked by transient cerebral ischemia/reperfusion: effects of the PPAR- α agonist WY 14643**” (Manuscript no. FRBM-D-06-00262). The manuscript has been modified according to the reviewers's comments, which are included as separate document.

Yours sincerely,

Massimo Collino

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Reply to Reviewer 1:

We would like to thank the reviewer for her/his kind comments relating to our paper and for his/her global good assessment.

Reply to Reviewer 2:

We thank the reviewer for her/his suggestions because they have allowed us to improve the quality of our manuscript.

We agree with the reviewer that the paper contains too many references (point 1). We delete useless sentences and references. Now the total number of references is 60.

According to the reviewer's suggestion (point 2), in the Discussion section (pg. 17-18) we added comments on the comparison with our previous work on cerebral I/R and PPAR- γ . We compared the extent of oxidative stress and inflammatory response, which are quite similar in the two studies. However, we pointed out that in the present work we have performed time-course experiments, while in the previous one we have chosen a single reperfusion time, thus, not allowing a proper comparison between the two studies.

Oxidative stress and inflammatory response evoked by transient cerebral ischemia/reperfusion: effects of the PPAR- α agonist WY14643.

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Running title

The PPAR- α agonist WY14643 prevents cerebral I/R injury

Oxidative stress and inflammatory response evoked by transient cerebral ischemia/reperfusion: effects of the PPAR- α agonist WY14643.

Abstract

The study investigated the effect of the selective peroxisome proliferator-activated receptor- α (PPAR- α) agonist, WY14643, on ischemia/reperfusion (I/R) injury in the rat hippocampus. Transient cerebral ischemia (30 min), followed by 1-24 h reperfusion, significantly increased the generation of reactive oxygen species, nitric oxide (NO) and lipid-peroxidation end-products, as well as markedly reduced levels of the endogenous antioxidant glutathione. Reperfusion for 3-6 h led to increased expression of the proteins of heme-oxygenase-1 (HO-1), cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1). Pre-treatment with WY14643 suppressed oxidative stress and expression of HO-1, iNOS and ICAM-1, but had no effect on COX-2. These effects are due to suppression of the activation of p38 mitogen-activated protein kinase and nuclear factor- κ B. The PPAR- α antagonist, MK886, abolished the beneficial effects of WY14643. The levels of S100B protein, a marker of cerebral injury used in stroke trials to monitor injury, were high in the hippocampus of rats exposed to I/R, but markedly reduced by WY14643. We propose that WY14643 protects the brain against excessive oxidative stress and inflammation and may thus be useful in treating stroke.

Keywords: *PPAR- α hippocampus, ischemia/reperfusion, oxidative stress, inflammation.*

Introduction

Recent studies have confirmed the pivotal role of oxidative stress and inflammatory response in the pathogenesis of acute ischemic stroke [1,2]. Reactive oxygen species (ROS) have been indicated as one of the earliest and most important components of tissue injury after reperfusion of the ischemic organ [3]. The brain is very susceptible to the damage caused by oxidative stress, due to its rapid oxidative metabolic activity, high polyunsaturated fatty acid contents, relatively low antioxidant capacity and inadequate neuronal cell repair activity [4]. ROS and end-products of lipid peroxidation have been shown to up-regulate a number of cytoprotective genes, including heme oxygenase-1 (HO-1), which is an important endogenous anti-oxidative defence mechanism against post-ischemic tissue damage [5]. Furthermore, ROS can activate diverse downstream signalling pathways, such as mitogen-activated protein kinases (MAPKs) and the transcription factor nuclear factor- κ B (NF- κ B), thus regulating expression of genes encoding a variety of pro-inflammatory proteins [6]. Over-expression 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 [7,8]. In several animal models, the intercellular adhesion molecule-1 (ICAM-1) has been shown to play an important role in the recruitment of leukocytes after cerebral ischemia/reperfusion (I/R) injury, and its expression has been associated with cerebral infarct size [9]. Recent clinical studies have shown that protein S100B levels are increased in the cerebrospinal fluid and serum of patients with acute stroke [10]. S100B is a member of the S100 family of acidic, calcium-binding proteins, mainly expressed in the brain [11], and its levels have been demonstrated to reflect the extent of brain damage following cerebral I/R [12].

Peroxisome proliferator-activated receptor- α (PPAR- α) is one of the three subtypes of the nuclear receptor PPAR family [13]. Activation of PPAR- α , by either natural ligands, such as

polyunsaturated fatty acids and eicosanoids, or synthetic ligands, such as hypolipidemic fibrates, stimulates target-gene transcription via the formation of heterodimeric transcription factor complexes with the retinoid x receptor [14]. A growing body of evidence suggests that PPAR- α activation can regulate inflammatory responses, including inflammatory disorders of the central nervous system, inhibiting the expression of many pro-inflammatory molecules, by a mechanism termed receptor-dependent transrepression [15]. PPAR- α agonists induce a negative transcriptional regulation of the NF- κ B signalling pathway [16], thus modulating iNOS expression in human astrocytes [17] and COX-2 expression in smooth muscle cells [18]. PPAR- α activation also reduces the levels of ICAM-1 in the rat heart after I/R injury [19]. In addition, PPAR- α activation stimulates the expression of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase [20], both of which are involved in brain ischemic tolerance [21]. The selective PPAR- α agonists WY14643 and fenofibrate attenuate infarct volume in a mouse stroke model [22], but the molecular mechanisms of their neuroprotective effects have not been investigated. Moreover, beneficial effects of fenofibrate have been reported in the cortex of adult mice exposed to cerebral I/R [23]. Among brain areas, the hippocampus is one of the most sensitive to I/R injury [24] and PPAR- α has been clearly 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 [25]. In the present study, we determined whether PPAR- α activation by the selective agonist WY14643 may reduce transient cerebral I/R injury in the rat hippocampus through modulation of oxidative stress and inflammatory response. Additionally, to improve understanding of the mechanisms of action of WY14643, we also investigated its effects on the intracellular signalling pathways MAPK and NF- κ B. Finally, we examined whether MK886, a specific PPAR- α antagonist, attenuates the protective effects of WY14643.

Materials and Methods

Animals and surgery.

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 Piccioni pellet diet (n.48, Gessate Milanese, Italy) and water *ad libitum*. All rats were acclimatised 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 the protection of animals used for experimental and other scientific purposes (D.M. 116/92) as well as with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the U.S. National Institutes of Health. The experimental protocol, approved by the Turin University Ethics Committee, was performed as described elsewhere [26]. Briefly, rats were anaesthetised through i.p. injection (30 mg/kg) of Zoletil 100 (mixture of tiletamine and zolazepam, Laboratoires Virbac, France), supplemented as needed. 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 sternocleidomastoid and the sternohyoid muscles, parallel to the trachea. Each carotid artery was freed from its adventitial sheath and vagus nerve, which was carefully separated and maintained. 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, U.K.). 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 1, 3, 6 or 24 h. Post-surgery, the animals were kept for at least 3 h in a 37°C incubator to ensure that postoperative recovery was satisfactory. Thereafter, they were group-housed under temperature- and light-controlled conditions with food and water *ad libitum*. At the end of the reperfusion, the anaesthetised 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 an appropriate ice-chilled homogenising medium for biochemical assays.

Drugs and Treatments.

Animals were randomly allocated into different groups: I/R groups (they received i.v. bolus injection of the vehicle, 2 ml/kg 10% [v/v] DMSO, $n = 6$ per group); WY14643 groups (the drug was administered in the dose-range 0.1 – 6 mg/kg, $n = 6$ per group); WY14643 + MK886 groups (both drugs were administered at the dose of 6 mg/kg, $n = 6$ per group). In all the groups ischemia lasted 30 min, while reperfusion was allowed for 1, 3, 6 or 24 hours. Rats in which the common carotid arteries were exposed but not occluded served as sham-operated controls (Sham, $n = 8$). Two additional groups of rats received WY14643 (6 mg/kg) or MK886 (6 mg/kg) prior to the sham operation ($n = 4$ per group). WY14643 and MK886 were prepared in 10% DMSO and injected i.v. 30 and 60 min prior to ischemia, respectively. For all experiments, concentrations of drugs were calculated such that all animals received equal volumes of DMSO.

Tissue extracts.

Cytosolic and nuclear extracts were prepared by the Meldrum method [27]. Briefly, hippocampi were homogenized at 10% (w/v) in a Potter Elvehjem homogeniser (Wheaton, Millville, NJ, USA) using a homogenisation 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 μ g/ml aprotinin, 2.5 μ g/ml leupeptin. Homogenates were centrifuged at 1,000 g for 5 min at 4°C. Supernatants were removed and centrifuged at 105,000 g at 4°C for 40 min to obtain the 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 DTT, 0.5 mM PMSF, 5 μ g/ml aprotinin, 2.5 μ g/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 nuclear proteins were carefully removed and protein content was determined using a BCA protein assay following the manufacturers' directions. Samples were stored at -80°C until use.

ROS and GSSG/GSH detection.

ROS 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 oxidised, in the presence of peroxides, to highly

fluorescent 2',7'-dichlorofluorescein (DCF). The DCF is then measured fluorimetrically [28].

Results are expressed as units of fluorescence (UF)/mg protein.

Antioxidant levels in the cytosolic fractions were evaluated in terms of glutathione disulphide (GSSG) / glutathione (GSH) ratio, by the Owens and Belcher method [29]. A mixture was directly prepared in a cuvette: 0.05 M Na-phosphate buffer, pH 7.0; 1 mM EDTA, pH 7.0; and 10 mM DTNB plus an aliquot of the sample. GSH content was evaluated after 2 min at 412 nm and expressed as $\mu\text{g}/\text{mg}$ protein. Suitable volumes of diluted GSH reductase and of NADPH were then added to evaluate the total GSH level. The difference between total GSH and GSH content indicates the GSSG content (expressed as $\mu\text{g}/\text{mg}$ prot); the ratio between GSSG and GSH content is considered a good measure of antioxidant status.

End products of lipid peroxidation.

Lipid peroxidation was investigated by measuring the main end-product of peroxidation, hydroxynonenal (HNE), in the cytosolic fractions. HNE concentration was determined on fresh cytosolic fractions by Esterbauer's method [30]. An aliquot of cytosol (100 μl) was extracted in an equal volume of a solution of acetic acid/acetonitrile (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, 3.9x150 mm). The mobile phase used was acetonitrile:bidistilled water (42%, v/v). The HNE concentration was calculated by comparison with a standard solution of HNE (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) of known concentration.

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 stoichiometrically reduced to nitrites by incubating 25 μ l of sample for 15 min at 37°C, in the presence of 1 I.U./ml nitrate reductase, 500 μ M NADPH and 50 μ M FAD in a final volume of 40 μ l. When nitrate reduction was complete, unused NADPH, which interferes with subsequent nitrite determination, was oxidised by 100 I.U./ml lactate dehydrogenase and 100 mM sodium pyruvate in a final reaction volume of 50 μ l, and incubated for 5 min at 37°C. Subsequently, total nitrites in the cytosol were assayed by adding 50 μ l of Griess reagent (4% sulphanilamide and 0.2% naphthylendiamide in 10% phosphoric acid) to each sample [31].

Western blot analysis.

About 15 μ g total protein were loaded. Proteins were separated on 8% (iNOS, COX-1, COX-2, ICAM-1, S100B) or 10% (HO-1, ph-p38 MAPK, NF- κ B p65) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidenedifluoride (PVDF) membrane, which was then incubated with SuperBlock blocking buffer. Membranes were incubated with primary antibody (rabbit anti-iNOS, mouse anti-COX-1, rabbit anti-COX-2, mouse anti-phosphorylated p38, rabbit anti-NF- κ B p65, rabbit anti-HO-1, goat anti-S100B, goat anti-ICAM-1). Blots were then incubated with secondary antibody conjugated with horseradish peroxidase for 30 min at room temperature and developed with the ECL detection system. The immunoreactive bands were visualised by autoradiography and the density of the bands were evaluated densitometrically using the Gel Pro[®] Analyser 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA). The membranes were stripped and

incubated with β -actin monoclonal antibody and subsequently with anti-mouse antibody, both for 30 min at room temperature, in order to assess gel-loading homogeneity.

Materials.

Unless otherwise stated, all compounds used in this study were purchased from the Sigma-Aldrich Company Ltd. (St. Louis, Missouri, USA). WY14643 and MK886 were purchased by Alexis Biochemicals (San Diego, CA, USA). The BCA Protein Assay kit and SuperBlock blocking buffer were from Pierce Biotechnology Inc. (Rockford, IL, USA); PVDF was obtained from the Millipore Corporation (Bedford, Massachusetts, USA). Goat polyclonal antibody against ICAM-1 and S100B, horseradish peroxidase-conjugated donkey anti-goat IgG and rabbit polyclonal antibodies against iNOS, HO-1 and NF- κ B p65 were from Santa Cruz Biotechnology (Santa Cruz, California, USA). Rabbit polyclonal antibody against COX-2 and murine monoclonal antibody against COX-1 were from the Cayman Chemical Company (Ann Arbor, MI, USA). Antibodies against the phosphorylated form of p38 MAPK was obtained from Cell-Signalling Technology (Beverly, MA, USA). The anti-mouse and anti-rabbit Ig horseradish peroxidase-linked whole antibodies and Luminol ECL detection reagents were from Amersham (Buckinghamshire, UK).

Statistical analysis.

All values in both text and figures are expressed as means \pm 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,

California, USA, www.graphpad.com) and p values below 0.05 was considered to be significant.

Results

Pre-treatment with the PPAR- α agonist WY14643 decreases oxidative stress and lipid peroxidation induced by I/R injury

Rats that had undergone transient bilateral common carotid artery occlusion for 30 min, followed by 1h reperfusion, exhibited a 50% increase in ROS production (154.6 ± 10.5 U.F./mg protein) compared to sham-operated animals (93.6 ± 3.7 U.F./mg protein) (Fig. 1A). This increase was reduced by pre-treatment with the PPAR- α agonist WY14643, in a dose-dependent manner, with maximum effect at 6 mg/kg (85.4 ± 4.7 U.F./mg protein). I/R-induced ROS overproduction was associated with a significant depletion in GSH content and a massive increase in HNE, a toxic end-product of lipid peroxidation, compared to sham-operated animals (Fig. 1B and 1C, respectively). As described for ROS production, the protective effects of WY14643 on GSH levels and lipid peroxidation were dose-dependent in the range 1 – 6 mg/kg. Thus 6 mg/kg, the most effective dose, was taken as reference dose for all subsequent experiments. When the time-course of WY14643 effects was evaluated, a slight difference among the measured markers was recorded. ROS production peaked at 1 h reperfusion and then decreased continually over 24 h, with values always significantly higher than those recorded in sham-operated animals (Fig. 1a). WY14643 inhibited the increase in ROS production totally at 1 h reperfusion, and by half at 3 h, with no effect at longer reperfusion times. The robust increase in GSSG/GSH ratio and HNE concentration at 1 h reperfusion was drastically reduced at longer reperfusion times, reaching the same levels recorded in sham-operated animals at 6-24 h reperfusion (Fig. 1b and 1c, respectively). WY14643 raised the GSH level and lowered the GSSG level until 3 h reperfusion, thus

matching the time interval in which the GSSG/GSH ratio measured in I/R group was statistically different from that occurring in the sham-operated group. A similar time-course curve was found for the effect of WY14643 pre-treatment on lipid peroxidation. Interestingly, when rats were pre-treated with the selective PPAR- α antagonist MK886 (6 mg/kg) the protective action of WY14643 on ROS production, GSH content and HNE levels were consistently abolished (Fig. 1a, 1b and 1c, respectively). Administration of WY14643 (6 mg/kg) or MK886 (6 mg/kg) to sham-operated rats had no significant effect on any of the measured markers compared to rats that were only sham-operated (data not shown).

I/R-induced increased expression of HO-1 is prevented by WY14643 in the rat hippocampus

Western blot analysis of hippocampus specimens from rats subjected to I/R revealed a significantly increased expression of HO-1 at 1 and 3 h reperfusion, and the expression level of this enzyme remained statistically above that recorded in sham-operated animals up to 6 h reperfusion (Fig. 2). WY14643 pre-treatment caused a massive decrease in the protein level only at 1 h reperfusion, with no effect at longer reperfusion times.

Effects of WY14643 on MAPK and NF- κ B signalling pathways

To evaluate the intracellular signalling pathways that might be involved in the protective mechanisms evoked by WY14643 pre-treatment, we ran tests to measure MAPK and NF- κ B activation. Transient cerebral I/R resulted in the phosphorylation of p38 MAPK in a time-dependent manner: it was maximally phosphorylated at 1 h reperfusion, activation lasting up to 3 h and then decreasing (Fig. 3A). In sham-operated animals there was no detectable

phosphorylation of p38 MAPK. WY14643 (6 mg/kg) inhibited the p38 MAPK activation evoked by I/R at both 1 and 3 h reperfusion.

Similar results were obtained when NF- κ B activation was evaluated in both cytosolic and nuclear fractions (Fig. 3B). In sham-operated rats, the NF- κ B p65 subunit was detected only in the cytosol and not in the nucleus. When animals underwent I/R, Western blot analysis of the hippocampal fractions showed higher levels of the NF- κ B p65 subunit in the nucleus than in the cytosol, thus confirming NF- κ B translocation. This NF- κ B signalling activation was recorded at 1, 3 and 6 h reperfusion, but not at 24 h. Pre-treatment of rats with WY14643 produced significant attenuation of the I/R-mediated NF- κ B translocation up to 3 h reperfusion. The preservation of p38 MAPK and NF- κ B activity afforded by WY14643 was abolished by MK886 pre-treatment. Administration of WY14643 or MK886 to sham-operated animals had no effect on activation of MAPK and NF- κ B signalling pathways (data not shown).

Effects of WY14643 on COX-2, iNOS and ICAM-1 expression

Western blot analysis detected very little COX-2 in sham-operated animals (Fig. 4). Transient cerebral I/R produced a significant increase in COX-2 expression in the hippocampus. Time-course experiments showed that COX-2 induction was already detectable at 1 h reperfusion and maximal at 3-6 h. Pre-treatment with WY14643 did not alter COX-2 overexpression evoked by I/R at any of the experimental times. COX-1 protein levels showed no significant quantitative differences among different groups, not being changed either by I/R or by WY14643 pre-treatment.

Like COX-2 protein, both iNOS and ICAM-1 levels were low in sham-operated animals, and were induced following cerebral I/R (Fig. 5A and 5B, respectively). Densitometric analysis of

the autoradiograms showed that, in the hippocampus of rats that had undergone I/R, iNOS and ICAM-1 protein levels were clearly visible and increased in comparison with sham-operated rats after at least 3 h reperfusion. iNOS protein levels were still significantly elevated after 24 h reperfusion, whereas, at the same reperfusion time, ICAM-1 expression was partially attenuated. WY14643 administration did not affect basal iNOS or ICAM-1 protein levels, but prevented their induction evoked by I/R at 3 or 6 h reperfusion. The drug did not modify protein expression either at 1 or at 24 h reperfusion. The attenuation in iNOS and ICAM-1 expression obtained after WY14643 pre-treatment was reversed by administration of the specific PPAR- α antagonist MK886.

The nitrite-plus-nitrate content in hippocampus homogenates was also measured in order to determine whether the inhibition of iNOS expression evoked by WY14643 was correlated with a modulation of NO concentration. The nitrite-plus-nitrate levels, measured at 3 h reperfusion, were almost doubled in I/R rats ($2.11 \pm 0.07 \mu\text{M}$) compared to sham-operated animals ($1.12 \pm 0.09 \mu\text{M}$) and were decreased by WY14643 pre-treatment, in a dose-dependent manner, with maximal effect at the highest dose, 6 mg/kg ($1.21 \pm 0.11 \mu\text{M}$). Administration of MK886 in WY14643-pre-treated rats produced an increase in hippocampal nitrite-plus-nitrate concentration ($2.01 \pm 0.15 \mu\text{M}$), similar to that recorded in I/R rats.

WY14643 is protective against S100B overexpression evoked by I/R in the rat hippocampus

Rats that had undergone cerebral I/R exhibited a significant increase in S100B protein content compared to sham-operated rats (Fig. 6). Time-course experiments showed maximum protein expression at 3 h reperfusion, it being already clearly detectable at 1 h reperfusion. S100B protein levels at 24 h reperfusion were not quantitatively different from those measured in sham-operated animals. Pre-treatment of rats with WY14643 almost completely prevented

this rise in S100B levels at all reperfusion times, but 24 h. MK886 administration brought the hippocampal S100B content back to values similar to those measured in I/R rats, thus abolishing the protective effect mediated by WY14643.

Discussion

The study provides compelling evidence that the selective PPAR- α agonist WY14643 protects the rat hippocampus from transient cerebral I/R injury. This neuroprotective effect is manifested by reduced protein S100B levels and is associated with an inhibition of oxidative stress and inflammatory response. An increase in S100B levels has been suggested to be a reliable indicator of both impaired hippocampus function and infarct size in patients with cerebral I/R [10]. Moreover, elevated levels of S100B have been positively correlated with the extent of injury in rat hippocampus slices exposed to I/R [32]. Our results demonstrate that WY14643 causes a marked reduction of S100B levels in the rat hippocampus. This protective effect was maintained until 6 h of reperfusion and was abolished by the PPAR- α antagonist, MK886, thus confirming the key role of PPAR- α activation in neuroprotection.

Cerebral I/R is known to induce generation of ROS, as well as expression of cytokines, adhesion molecules and enzymes involved in the inflammatory response, and is known to be regulated by oxygen- or redox-sensitive mechanisms [33].

Bilateral occlusion of the common carotid arteries in rats is a common model of incomplete global cerebral ischemia and results in a 50% decrease in cerebral blood flow [34]. The induced ischemia is partial, as the blood flow through the circle of Willis is not affected, and this model has thus been described as clinically relevant in that it reflects the early pathophysiological events occurring during transient ischemic attacks [35]. Using this model, we recently demonstrated [26] that I/R injury causes a robust increase in typical markers of inflammation and oxidative stress in the rat hippocampus and that agonists to the subtype γ of the PPAR family exert protection against cerebral I/R injury, with results similar to those here reported. However, in our previous work [26] the extent of oxidative stress and inflammatory response was investigated only at 1 h of reperfusion, thus not allowing proper comparison

with the present study, where time-course experiments were performed. Here we confirm that transient cerebral I/R causes excessive formation of ROS, associated with GSH depletion and lipid peroxidation. Furthermore, we extend our findings, showing that the presence of a significant level of oxidative stress and lipid peroxidation is confined to the early stage of reperfusion, up to 3 h, while the development of the inflammatory response is partially delayed. Administration of WY14643, 30 min prior to I/R, decreased ROS production and lipid peroxidation in rats subjected to I/R and, at the same time, offered protection against GSH depletion. The effects of WY14643 were evident for up to 3 h reperfusion. Similar results on oxidative stress modulation have been reported when another PPAR- α agonist, fenofibrate, was tested in a mouse model of middle cerebral artery occlusion [23]. We also show here that pre-treatment with the PPAR- α antagonist MK886 abolished the protection afforded by WY14643, thus suggesting that WY14643 does not exert direct antioxidant effects, but rather that its beneficial effects are due to activation of PPAR- α . This finding is in agreement with other reports showing that WY14643 enhances expression of antioxidant enzymes such as SOD and catalase in the rat liver [20]. Interestingly, PPAR- α deficient mice have been found to exhibit significant increases in oxidative stress and lipid peroxidation much earlier in their life than wild-type mice [36].

Overall, our data suggest that the mechanism underlying the protective action of WY14643 against mild forebrain I/R is secondary to the reduced formation of ROS, which occurs in the early stage of reperfusion. There are different possible ways in which decreased ROS formation may account for the neuroprotective effect, including by decreasing oxidative damage, blocking the apoptotic processes, or inhibiting expression of oxidative stress-inducible enzymes and proteins. Recent reports suggest that HO-1, a stress-related protein that catalyses the rate-limiting step in heme degradation, represents an important endogenous anti-oxidative defence mechanism against post-ischemic tissue damage [37]. HO-1 has been shown to be important for attenuating overall ROS production through its ability to degrade

heme and to produce carbon monoxide (CO), biliverdin/bilirubin, and to release free iron.

Biliverdin and bilirubin, produced by HO-1, may act as physiological antioxidants and potent scavengers of oxygen radicals [38]. On the other hand, HO-1 also produces iron, which might lead to increased oxidative stress and CO, which might be dangerous. Despite this potential toxicity, there is increasing evidence for a beneficial role of HO-1 in ischemic diseases [37]. Moreover, recent findings have led to a re-definition of the HO pathway as not only an anti-oxidative mechanism but also a more complex and better co-ordinated cytoprotective system, with effects on several signal transduction pathways [39].

We report here that the HO-1 protein level is increased in the rat hippocampus following I/R and that pre-treatment with WY14643 causes reduced HO-1 expression. However, since induction of HO-1 is mediated by ROS production [5] it is unclear whether the drop in HO-1 protein expression is a direct effect of WY14643 or a consequence of lower oxidative stress levels in rats to which WY14643 was administered before transient cerebral I/R. Collectively, this first set of our experimental data clearly shows that the neuroprotective effect of WY14643 is parallel with oxidative stress reduction.

PPAR- α agonists have also been shown to be beneficial in other ischemic models, and this benefit has been attributed to the anti-inflammatory action of these drugs. Cuzzocrea et al. [40] found that administration of WY14643 30 min before the onset of gut ischemia significantly reduced intestinal I/R injury in rats. The improved outcome was accompanied by reductions in neutrophil infiltration, proinflammatory cytokine expression, nitrotyrosine formation, and ICAM-1 and P-selectin expression. Similarly, renal and liver I/R injury was reduced after WY14643 pre-treatment, partially due to its anti-inflammatory effects [41,42]. Several groups have also demonstrated that activation of PPAR- α by different exogenous and endogenous agonists protects the heart from I/R injury, by attenuating release of NO and proinflammatory cytokines and inhibiting neutrophil accumulation [19,43,44]. Significant information regarding the role of PPAR- α activators in the control of the inflammatory response also

comes from clinical trials. Administration of fibrates to hyperlipidemic and atherosclerotic patients decreased plasma concentrations of fibrinogen, interleukin-6, C-reactive protein, interferon- γ and TNF- α [18,45,46].

In the present study, we found that two important enzymes involved in the ischemic inflammatory cascade, iNOS and COX-2, were up-regulated in a time-dependent manner by I/R, with maximal levels between 3 and 6 h reperfusion. Similarly, ICAM-1, an important mediator of inflammatory cell transmigration across the activated endothelium, was present in low amounts in the hippocampus of sham-operated rats and was induced by I/R. The time-course of the expression of these proteins confirms that the inflammatory response is partially delayed after development of oxidative stress, which is already massive at 1 h reperfusion. We show that a single injection of WY14643 protects the rat hippocampus from I/R-induced iNOS and ICAM-1 over-expression, for up to 6 h reperfusion, and that the protective effect is reverted by administration of the PPAR- α antagonist MK886. iNOS up-regulation was paralleled by an increase in hippocampus NO content, which was attenuated by WY14643 pre-treatment. NO may react with ROS to produce peroxynitrites, with deleterious effects on neuronal survival. Thus its reduction represents a further mechanism for neuroprotection by PPAR- α agonists.

Surprisingly, the anti-inflammatory effects of WY14643 did not involve modulation of COX-2 protein expression at any reperfusion time. Reports in this connection are controversial: whereas inhibition of COX-2 expression by PPAR- α ligands has been demonstrated [47], other reports find that PPAR- α activation does not affect [48] or possibly increases the expression of COX-2 [49]. Recently, evidence has been accumulating to show that there is continual cross-talk between NO and the prostaglandin biosynthetic pathways [50].

Nevertheless, the possibility of WY14643 inhibiting iNOS in a manner uncoupled from that of COX-2 has been reported in human astrocytes [51]. We might speculate that WY14643 can

differentially affect transcriptional factors involved in iNOS and COX-2 induction; however, further investigation is required to determine the mechanism(s) more precisely.

While our data document dramatic effects of PPAR- α agonists on inflammation and oxidative injury following cerebral I/R in rat hippocampus, the mechanisms underlying these actions are not clear. As most anti-inflammatory properties of PPAR- α agonists are mediated by inhibition of the transcription factor NF- κ B [52], we investigated the effects of WY14643 pre-treatment on this signalling pathway. At the same time, we evaluated the effects of WY14643 on MAPK activation. Among members of the MAPK family, we focused on the p38 isoform, because it is most easily activated by ischemia [53] and its inhibition has been reported to contribute to neuroprotection [54]. We found that: (i) the phosphorylated form of p38 MAPK was elevated in I/R hippocampi and (ii) NF- κ B translocated from cytosol to nucleus in hippocampus extracts of I/R rats, thus confirming NF- κ B activation. These data are in agreement with reports that p38 MAPK and NF- κ B were activated in the hippocampus of rats undergoing I/R [55,56]. Moreover, the relationship between high ROS levels, HO-1 up-regulation and activation of downstream signalling routes, such as MAPK and NF- κ B pathways, is well accepted [5]. Here, for the first time, we show that MAPK and NF- κ B activation by cerebral I/R can be inhibited by pre-treatment with WY14643, and that this effect is reverted by the PPAR- α antagonist MK886, indicating that WY14643 efficiently inhibits these signalling pathways. Our results are consistent with reports that PPAR- α activators may inhibit p38 MAPK phosphorylation [57] and directly interfere with the p65 subunit of NF- κ B [52]. As has been reported [16], WY14643 might also inhibit NF- κ B nuclear translocation by inducing expression of the inhibitory protein inhibition of kappaB (IkB)- α , which sequesters the NF- κ B subunits in the cytoplasm and consequently reduces their DNA binding activity. In addition, IkB- α is one of the NF- κ B target genes, and its high expression allows IkB- α to enter the nucleus where it binds to NF- κ B, enhancing its

dissociation from DNA and causing its exportation to the cytoplasm [58]. Nonetheless, our studies cannot conclusively determine whether PPAR- α -mediated inhibition of p38 MAPK and of NF- κ B activity represents a direct mechanism by which WY14643 prevents inflammation during transient cerebral I/R. We cannot rule out the possibility that suppression of MAPK activation and NF- κ B translocation by WY14643 might be a secondary event through its modulation of oxidative stress parameters.

The hypothesis that MAPK and NF- κ B signalling pathways may be functionally interconnected and not act independently must also be taken into account. Inhibition of the p38 MAPK pathway has been shown to abolish NF- κ B-driven activation of gene expression [59] and p38 MAPK activation has been suggested to contribute to NF- κ B activation by modulating the transactivation capacity of the NF- κ B p65 subunit [60]. We might thus suppose that PPAR- α agonists affect NF- κ B 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.

In summary, our results show that the PPAR- α agonist WY14643 causes a substantial reduction of cerebral I/R injury by reducing oxidative stress and inflammatory response. Its protective effects are dependent on the activation of PPAR- α , since they are abolished by pre-treatment with the PPAR- α antagonist MK886. Further studies are needed to determinate additional molecular mechanisms by which PPAR- α agonists downregulate the oxidative stress and inflammatory response implicated in the pathogenesis of cerebral I/R injury. The finding that protection is provided when rats are pre-treated 30 min before the onset of ischemia might provide support for future clinical applications of PPAR- α agonists in acute stroke. However, more investigation is warranted to determine whether administration can be delayed long enough to be clinically useful in the management of pathological conditions associated with I/R injury.

Acknowledgements:

This research was supported by Turin University funding (ex-60%).

Figure Legends

Fig. 1 Dose-response (A, B, C) and time-course (a, b, c) curves of WY14643 effects on oxidative stress and lipid peroxidation in the hippocampus of rats exposed to cerebral I/R. ROS production, GSSG/GSH ratio and HNE levels were measured subsequent to sham-operation (Sham) or I/R (30 min/1 h) on fresh cytosolic fractions of hippocampus from rats pre-treated with WY14643 (WY) in the range 0.1 – 6 mg/kg (panels A, B and C). ROS production, GSSG/GSH ratio and HNE levels were detected at 1, 3, 6 and 24 h reperfusion (panels a, b and c). WY14643 (6 mg/kg) was administered 30 min prior to ischemia, MK886 (6 mg/kg) was administered 60 min prior to ischemia. Data are expressed as means \pm S.E.M. ($n = 6$ in each group). 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).

Fig. 2 The PPAR- α agonist, WY14643, exerts protective effect on the induction of HO-1 expression evoked by I/R in the rat hippocampus. HO-1 levels were measured subsequent to sham-operation (Sham) or I/R. Ischemia lasted 30 min, while reperfusion was allowed for 1, 3, 6 and 24 h. Rats were administered 6 mg/kg WY14643 (I/R + WY14643) 30 min prior to I/R, as described in the Materials and Methods section. 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. Results are expressed as means \pm 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).

Fig. 3 Effect of WY14643 pre-treatment on phosphorylation of p38 MAPK (A) and nuclear translocation of p65 NF- κ B (B) evoked by cerebral I/R injury. Phosphorylated p38 MAPK was detected at 1, 3, 6 and 24 h reperfusion in the hippocampus homogenates from sham-operated rats (Sham) and rats subjected to I/R (Panel A). NF- κ B translocation from the cytosol to the nucleus was evaluated at the same reperfusion time, measuring NF- κ B p65 subunit levels in both cytosol and nuclear fractions and expressing the results as nucleus/cytosol ratio (Panel B). Rats were administered 6 mg/kg WY14643 (I/R+WY14643) or 6 mg/kg MK866 and 6 mg/kg WY14643 (I/R+WY14643+MK886) before I/R, as described in the Materials and Methods section. 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 \pm 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).

Fig. 4 The PPAR- α agonist, WY14643, has no effect on COX-1 and COX-2 protein expression. Western blot analysis of protein extracts from the hippocampi of sham-operated rats (Sham) or animals that underwent I/R. Reperfusion was allowed for 1, 3, 6 and 24 h. Rats were administered 6 mg/kg WY14643 (I/R + WY14643) 30 min prior to I/R, as described in the Materials and Methods section. Each immunoblot is from a single experiment and is representative of three separate experiments.

Fig. 5 WY14643 protects against the induction of iNOS (A) and of ICAM-1 (B) expression evoked by cerebral I/R. iNOS and ICAM-1 levels were measured subsequent to sham-operation (Sham) or cerebral I/R. Rats were administered 6 mg/kg WY14643 (I/R +

WY14643) or 6 mg/kg MK886 and 6 mg/kg WY14643 (I/R + WY14643 + MK886) before I/R, as described in the Materials and Methods section. 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. 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).

Fig. 6 WY14643 protects against the S100B overexpression evoked by cerebral I/R. S100B levels were measured subsequent to sham-operation (Sham) or cerebral I/R. Rats were administered 6 mg/kg WY 14643 (I/R + WY14643) 30 min prior to I/R. Protein levels were detected at 1, 3, 6 and 24 h reperfusion (Panel A). 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 \pm 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). Western blot analysis of protein extracts obtained from the hippocampi of rats that underwent I/R (30 min/3h) and sham-operated animals (Sham) (Panel B). Two groups of rats received 6 mg/kg WY14643 (I/R + WY14643) or 6 mg/kg MK886 and 6 mg/kg WY14643 (I/R + WY14643 + MK886) before I/R, as described in the Materials and Methods section. The immunoblot is representative of three separate experiments.

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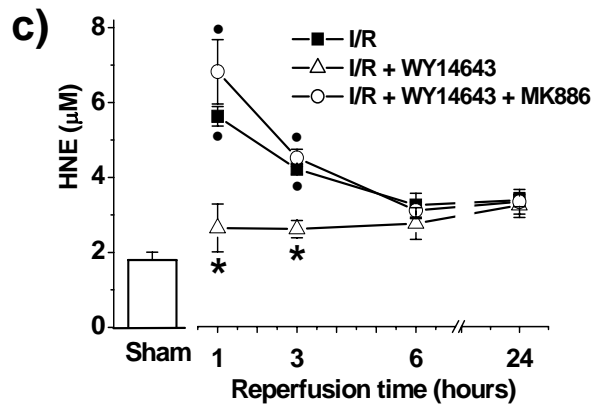
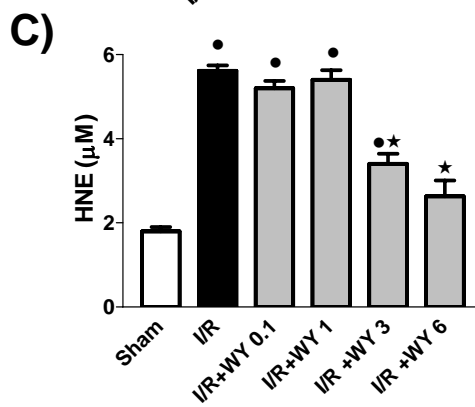
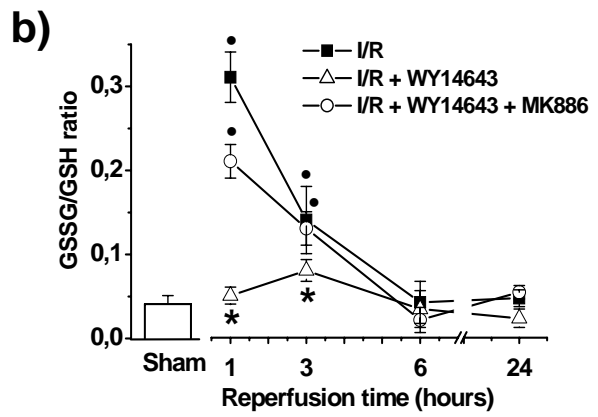
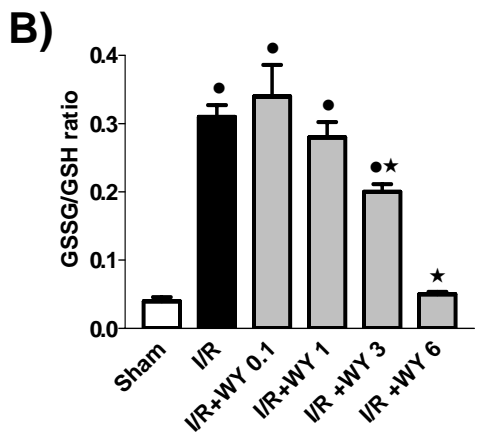
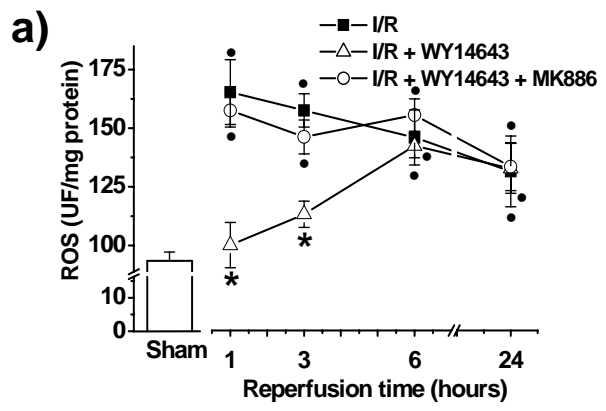
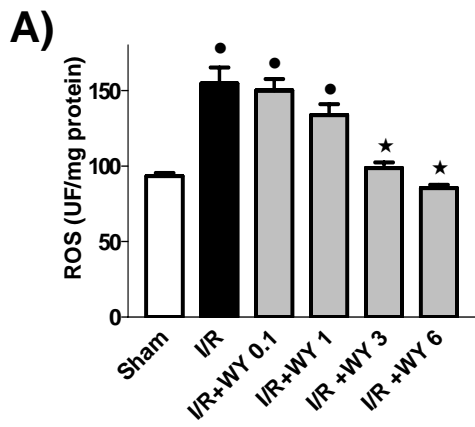
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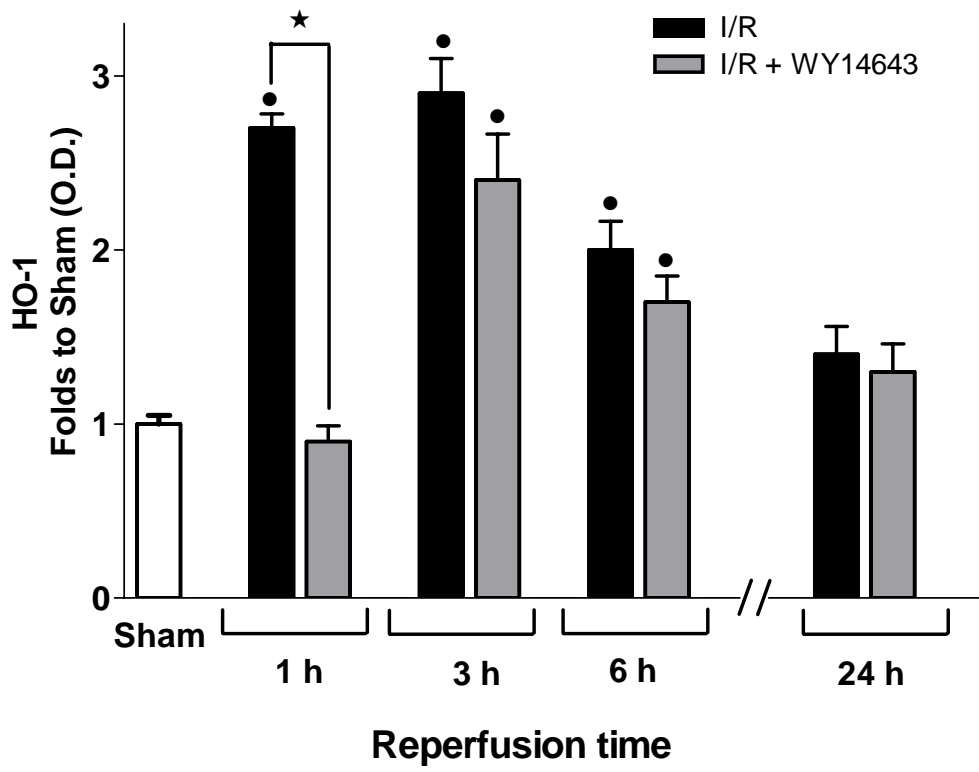
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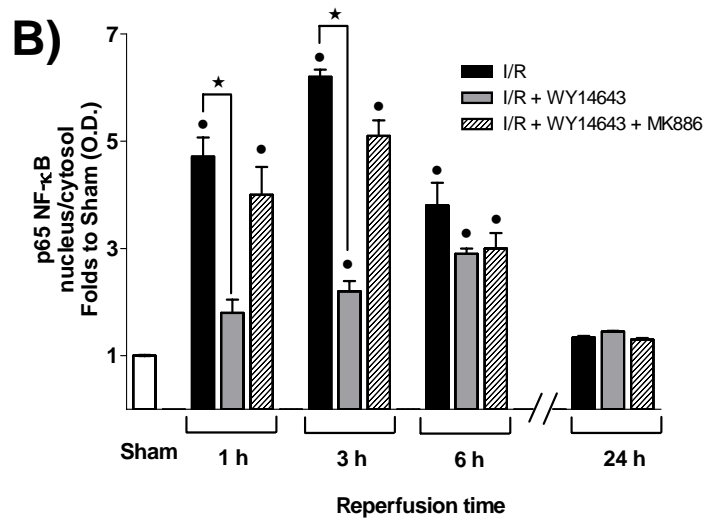
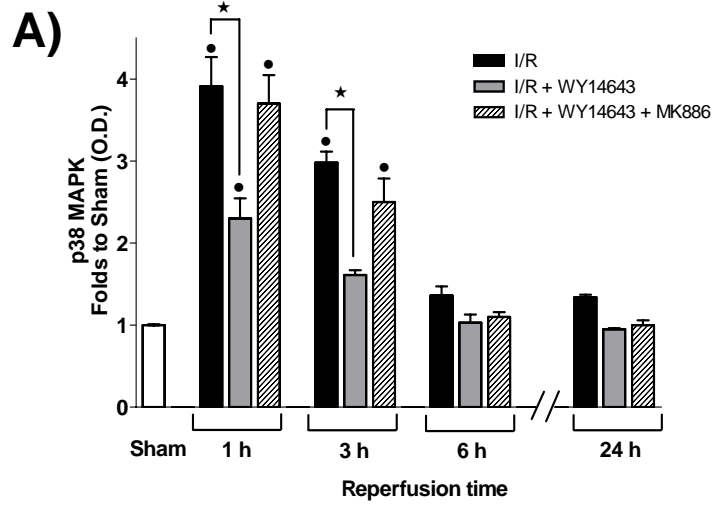
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Figure(s)

