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Neuroprotective effect of 2-hydroxy arachidonic acid in a rat model of transient middle cerebral artery occlusion☆

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ABSTRACT

Stroke modifies the composition of cell membranes by eliciting the breakdown of membrane phospholipids whose products, such as arachidonic acid (AA), are released in the cytosol. The action of enzymes such as cyclooxygenases on AA leads to inflammatory stimuli and increases the cell oxidative stress. We report here the neuroprotective effect of 2-hydroxyarachidonic acid (2OAA), a cyclooxygenase inhibitor derived from AA, as a promising neuroprotective therapy against stroke.

The effect of a single dose of 2OAA, administered intragastrically 1 h after the ischaemic insult, in a rat model of transient middle cerebral artery occlusion (tMCAO) was tested after 24 h of reperfusion. Infarct volume was measured by TTC method to evaluate the neuroprotective effect. Levels of phospholipids and neutral lipids were measured by thin-layer chromatography. The expression of cPLA2 and sPLA2 phospholipases responsible for the cleavage of membrane phospholipids, as well as the expression of antioxidant enzymes, was measured by qPCR. Lipid peroxidation was measured as the concentration of malondialdehyde and 4-hydroxynonenal.

The treatment with 2OAA reduced the infarct volume and prevented ischaemia-induced increases in transcription levels of free fatty acid (FFAs), as well as in both phospholipases A2 (cPLA2 and sPLA2). The lipid peroxidation and the transcription levels of antioxidant enzymes induced by ischaemia were also decreased by this treatment.

We conclude that 2OAA treatment results in a strong neuroprotective effect that seems to rely on a decrease in PLA2 transcriptional activity. This would reduce their action on the membrane phospholipids reducing reactive oxygen and nitrogen species generated by FFAs. Based on the transcriptional activity of the antioxidant enzymes, we conclude that the treatment prevents oxidative stress rather than promoting the antioxidant response. This article is part of a Special Issue entitled: Membrane Lipid Therapy: Drugs Targeting Biomembranes edited by Pablo Escríba-Ruíz.

1. Introduction

The understanding of biological membranes has changed dramatically over the last few decades. The simplistic view of biological membranes made of lipids that diffuse within the membrane as passive counterparts of imbued proteins representing the key molecules that regulate signal transduction has considerably evolved. Currently, membranes are viewed as a complex mosaic of functional domains, subdomains and

Abbreviations: 2OAA, 2-hydroxyarachidonic acid; 4-HNE, 4-hydroxynonenal; AA, arachidonic acid; C-Pu, caudate-putamen; CCA, common carotid artery; cPLA2, cytosolic phospholipase A2; COX, cyclooxygenase; DHA, docosahexaenoic acid; ECA, external carotid artery; FFA, free fatty acid; *gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *gclm*, glutamate-cysteine ligase modifier subunit; *hmox1*, heme oxygenase 1; ICA, internal carotid artery; LOX, lipooxygenase; MCA, middle cerebral artery; tMCAO, transient middle cerebral artery occlusion; MDA, malondialdehyde; NSAID, non-steroideal anti-inflammatory drug; *nqo1*, NAD(P)H quinone dehydrogenase 1; PBS, phosphate-buffered saline; PLA2, phospholipase A2; S1 cortex, primary somatosensory cortex S1; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT-qPCR, real-time quantitative polymerase chain reaction; sPLA2-IIA, secretory phospholipase A2; *sod2*, superoxide dismutase 2; TLC, thin-layer chromatography; TTC, 2,3,5-triphenyltetrazolium chloride.

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microdomains of lipid and protein components [1] where lipids participate directly as messengers or regulators of signal transduction.

The ability of some drugs to modulate the structure of membranes has led to a new therapeutic approach called "membrane lipid therapy" [2]. In this regard, the role of membrane lipids is particularly relevant in different neuropathologies, where many reports show the alteration of lipid membrane metabolism in cerebral ischaemia and especially in stroke [3]. In fact, the use of a membrane lipid such as docosahexaenoic acid (DHA) has been reported to present a neuroprotective role against stroke [4] and opens the pathway to using rationally designed lipids as molecules able to become therapeutic agents against stroke. This pathology is one of the leading causes of death and permanent disability [5,6]. About 30% of stroke patients suffer permanent or serious disabilities and the derived costs are estimated to be about 62.1 billion euros per year in Europe [6]. The high health and social costs, as well as the lack of effective therapies to prevent or reduce stroke-derived damage, make the development of effective therapies a pressing need [7].

Inflammatory processes and oxidative stress are well-known therapeutic targets for alleviating ischaemic damage [8]. Ischaemia-dependent inflammatory stimuli increase the action of phospholipases A2 (PLA2), which leads to the release of free fatty acids (FFAs), such as arachidonic acid (AA), derived from membrane phospholipid cleavage [9,10]. FFAs play an important role in oxidative stress. The inhibitory effect of FFAs, and particularly AA, on the oxygen consumption in mitochondria has been found to be very effective in promoting reactive oxygen species (ROS) generation [11]. Also, the action of cyclooxygenase (COX-1 and COX-2) and lipooxygenase (LOX) enzymes on AA to promote pro-inflammatory eicosanoids involves the production of free radical generation contributing to oxidative stress [12]. Arachidonic acid peroxide products derived from non-enzymatic reactions also contribute to free radical oxidation [12]. Thus, FFA release elicits increases in cell ROS and reactive nitrogen species (RNS) by different pathways and contributes to lipid peroxidation, oxidative stress and subsequent cell death [3].

COX-1 is constitutively expressed in most tissues and considered the COX isoform responsible for the physiological production of prostaglandins, while COX-2 is induced by inflammatory stimuli, which led to the concept that selective inhibition of COX-2 can reduce inflammation [13]. Deleterious side effects of blocking COX-1 have led the pharmacological industry to develop selective anti-COX-2 non-steroidal anti-inflammatory drugs (NSAIDs) rather than COX-1 blockers [14,15]. However, COX-1 has recently been described as playing a crucial role in neuroinflammation given its predominant location in microglia. Pharmacological inhibition or genetic ablation of COX-1 activity reduces the inflammatory response as well as the neuronal loss, indicating that COX-1 selective blockers have an important role in reducing neuroinflammation [13,14]. These data have led to reconsideration of the use of anti-COX-1 agents in neurodegenerative diseases with a marked inflammatory component [13]. The use of drugs acting both on COX-1 and COX-2 could result in a more effective neuroprotection, although deleterious COX-1 effects have to be prevented. In this regard, 2-hydroxy arachidonic acid (2OAA), obtained by the addition of a hydroxyl group to AA, has been reported to block both COX-1 and COX-2 activity and present a possible attenuation of the toxicity of AA [16]. These properties make this molecule a promising candidate as a neuroprotective agent.

In this study we show for the first time that treatment with the rationally designed lipid 2OAA has a strong neuroprotective effect against ischaemia/reperfusion-induced damage in a model of transient middle cerebral artery occlusion (tMCAO) in rats, preventing oxidative stress by modulating the PLA2 response.

2. Material and methods

2.1. Animals

Twenty male Swiss mice and five 8-week-old male Sprague Dawley rats were used for testing the toxicity of 2OAA. Thirty-four 8-week-old male Sprague Dawley rats, weighing 320–340 g, were used to analyse the effects of the treatment in an tMCAO model. The animals were housed at 22 ± 1 °C, in a 12 h light/dark cycle, with food and water *ad libitum*. Rats were randomly divided into three groups: 10 rats for 2,3,5-triphenyltetrazolium chloride (TTC) and mRNA assays (5 untreated rats and 5 rats treated with 2OAA); 8 rats for lipid analysis (4 untreated and 4 treated rats) and 10 rats for lipid peroxidation assays (5 untreated and 5 treated rats). Six additional rats were used for testing the effect of different doses of 2OAA.

All procedures were carried out in compliance with the ARRIVE guidelines and the Guidelines of the European Union Council (2010/63/EU), following Spanish regulation RD53/2013 for the use of laboratory animals, and were approved by the Scientific Committee of the University of León. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Surgery

tMCAO was carried out following a previously described protocol [17] with minor modifications. Briefly, anaesthesia induction of animals was performed with 3.5–4% isoflurane in O_2-enriched air with a flow of 2 L/min in an anaesthesia box. Then, anaesthesia was maintained with 2% isoflurane in O_2-enriched air with a flow of 2 L/min using a face mask adapted to rats. The animals' temperature was monitored with a rectal probe and maintained at 37 ± 0.5 °C with a heating pad. The rats were placed in a prone position and the skin of the temporal region was shaved and cleaned with iodopovidone. A deep incision in the skin, between the eye and the ear, was made by cutting through the temporalis muscle to the squamosal bone. Muscle was scraped to expose the bone over the middle cerebral artery (MCA). A Doppler probe (Perimed) was fixed with cyanocrylate in this area to evaluate MCA flow.

Carefully, the animal was placed in a supine position, the neck was shaved and cleaned with iodopovidone and a 2 cm midline incision was made to expose the right common, external and internal carotid arteries (CCA, ECA, ICA), which were carefully separated from the vagus nerve and fascia. The ECA and CCA were permanently ligated with 3–0 silk sutures (Ethicon), and the ICA was clamped (Stainless Steel Micro Serrefines, FST). A small incision was made in the CCA and a silicon-coated monofilament (0.39 mm diameter, Doccol) was introduced through it and pushed towards the ICA. The clamp was removed to allow the monofilament to go through the ICA as far as the Willis circle. When the right MCA was occluded, a striking decrease of the blood flow was detected with the Doppler probe. Then, the filament was fixed using a suture in the CCA. After suturing the skin, the Doppler probe was removed and the animals were allowed to recover from anaesthesia. After 60 min of occlusion, the animals were re-anaesthetized, the probe was fixed again, and the filament was removed allowing the reperfusion through the MCA, which was detected as an increase in the blood flow measured with the Doppler probe. Soft tissues were returned to their original place and the skin incision was sutured in a permanent way using 3–0 silk.

2.3. Toxicity and treatment assays

Toxicity assays for 2OAA were performed using the 2OAA sodium salt, 85% purity (kindly provided by Lipopharma SL). Previous assays were carried out in mice using a single daily intragastrical dose of 2OAA dissolved in 7% ethanol in soybean oil to the final concentration to test. Three groups of five mice were administered 2OAA (250 mg/kg, 500 mg/kg and 1 g/kg). None of the animals treated with 1 g/kg 2OAA survived later than five days and then one more group of five mice was administered 1 g/kg 2OAA for two days followed by 3 more days with 500 mg/kg. Since all of them survive, this protocol was tested in five rats which also survived the five days.

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The treatment in rat was then performed with 2OAA sodium salt, same batch, which was dissolved in 7% ethanol in soybean oil to a final concentration of 350 mg/ml. 1 h after ischaemia, a single dose of 1 g/kg of 2OAA (1 ml of solution) was intragastrically administered to the treated animals. The same amount of vehicle was administered to the untreated animals. Dose-response assays of 2OAA were performed by testing the effect of 500 mg/kg, 750 mg/kg and 1 g/kg in a single dose of 1 ml administered intragastrically.

2.4. Sampling

24 h after ischaemia, rats were decapitated and their brains quickly removed and placed in a cold brain matrix for rats (Rodent Brain Matrix, ASI Instruments). There, the brains were sectioned in 2 mm-thick coronal sections. The coronal 2 mm-thick section corresponding to bregma −0.8 to bregma 1.2 [18] was rapidly dissected in caudate-putamen (C-Pu) and primary somatosensory cortex (S1 cortex) areas from ischaemic and non-ischaemic hemispheres, and these tissues were rapidly frozen in dry ice for real-time quantitative PCR (RT-qPCR) and lipid analysis. The remaining sections were used for infarct volume measurement (Fig. 1).

Proper lipid peroxidation required larger amounts of tissue than those obtained in the coronal sections described above. Thus, rat brains specifically used for lipid peroxidation were sectioned in the matrix to obtain blocks between bregma 1.7 and bregma −5.3 containing the whole injured area and quickly used for lipid peroxidation assays.

2.5. Infarct volume measurement

Infarct volume was assessed using the TTC method [19]. Sections were incubated in 1% TTC (Sigma-Aldrich) in 50 mM phosphate-buffered saline (PBS), pH 7.4, for 30 min at 37 °C in darkness. Then, sections were fixed overnight in 4% paraformaldehyde in 50 mM PBS, pH 7.4, at 4 °C and digitalized at a resolution of 600 ppi with a Canoscan LIDE 200 (Canon Inc.). Infarct volume was measured using ImageJ software (NIH) and calculated with the following formula: *Percentage of infarct volume = non-stained area* (mm^2)/*total area* (mm^2) × *100.*

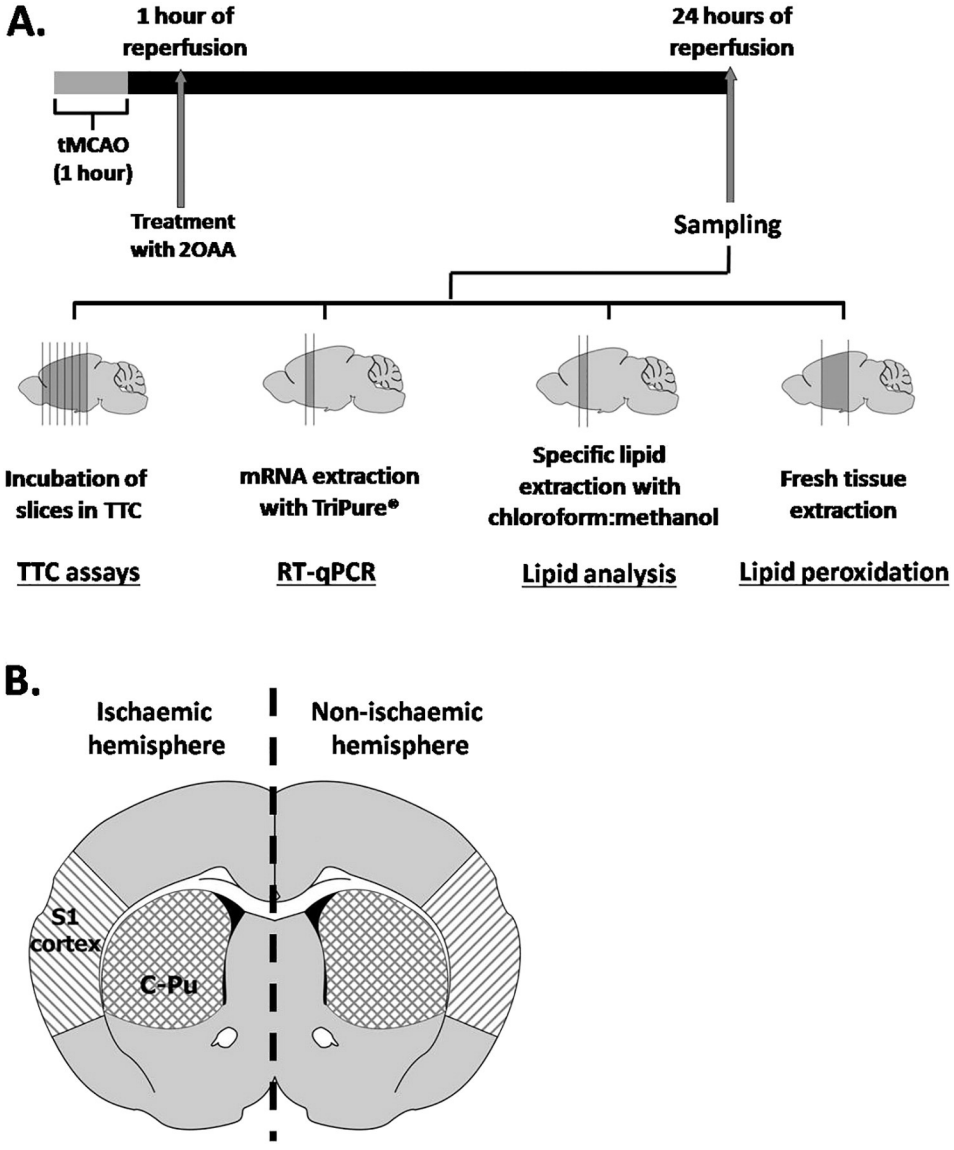

Fig. 1. Treatment and sampling. In A) the time of transient middle cerebral artery occlusion (tMCAO) and reperfusion, the times of treatment and sampling, as well as the different procedures of extraction for the different assays are shown. In B) the areas dissected for RT-qPCR and lipid analysis are shown.

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Table 1
Sequences of the primers utilized in RT-qPCR and accession number in gene databank of the NCBI, designed with Primer Express software (applied biosystems).

Gene	Forward	Reverse	NCBI reference
spla2 IIa	5′-gccaaatctcctgctctacaaac-3′	5′-acattcagcggcagctttatc-3′	NM_031598.3
cpla2	5′-ttggattgtgcgacctacgtt-3′	5′-gggtgggagtacaaggttgacat-3′	U38376.1
sod2	5′-gcacattaacgcgcagatca-3′	5′-agcgcctcgtggtacttctc-3′	NM_017051.2
gclm	5′-gcacaggtaaaacccaatagtaatca-3′	5′-cagtcaaatctggtggcatca-3′	NM_017305
nqo1	5′-gagtggcattctgcgcttct-3′	5′-caatgctgtacaccagttgaggtt-3′	NM_017000.3
hmox1	5′-ctgctgacagaggaacacaaaga-3′	5′-ggcctctggcgaagaaactc-3′	NM_012580.2
gapdh	5′-gggcagcccagaacatca-3′	5′-tgaccttgccacagcct-3′	NM_017008

2.6. RT-qPCR assays

RT-qPCR assays were performed according to MIQE guidelines [20] (Taylor et al., 2010). Total RNA was isolated with TriPure Isolation Reagent© (Roche Diagnostics) following the manufacturer's instructions. RNA integrity was checked by electrophoresis in 2% agarose gels and samples that did not present two clear bands corresponding to rRNA 28S and rRNA 18S were discarded. Concentration and purity were spectrophotometrically determined with a Nanodrop (NanoDrop Technologies).

Six hundred ng of RNA from each sample were retrotranscripted with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. RT-qPCR analyses were carried out using specific primers (Table 1) and SYBR Green PCR Master Mix (Applied Biosystems) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The optimal conditions in our assays were 2 μl of 1/10 of the retrotranscription reaction and 300 nM primers. The transcript levels of the different genes were analysed with the $2^{-\Delta\Delta Ct}$ method [21] using glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) as the reference gene. The primers used (Table 1) in these assays were designed using Primer Express software (Applied Biosystems) and secreted *PLA2-IIA* (*sPLA2-IIA*) taken from [22].

2.7. Lipid analysis

Similar coronal sections to those used in RT-qPCR assays (see 2.4 Sampling section) were obtained from rat brains, dissected as described above and assayed in lipid analysis. These samples were homogenized with a Polytron PT3100 (Kinematika) for 30 s in hypotonic buffer (1 mM EDTA in 20 mM Tris/HCl pH 7.4) at 4 °C. Homogenates were centrifuged at 800 ×*g* for 15 min at 4 °C and the supernatant was sonicated and centrifuged again (1000 ×*g*) for 10 min at 4 °C. The supernatant protein concentration was determined using a DC Protein Assay Kit (BioRad).

Lipids were extracted from the supernatant with chloroform:methanol (2:1) and centrifuged at 1000 ×*g* for 10 min at 4 °C, and phases containing protein and aqueous phase were discarded. Organic phase was purified in chloroform:hypotonic buffer (1:1), and evaporated under argon flow and resuspended in chlorophorm.

Lipid samples were resolved using thin-layer chromatography (TLC) as previously reported [23] Phospholipids were resolved with chloroform:methanol:H_2O:acetic acid (60:50:4:1) on silica G60 plates (Merck) for 90 min. Neutral lipids were resolved with heptane:diethyl ether:acetic acid 74:21:4 in the same stationary phase for 40 min to analyse neutral lipids. Proper standard lipids (phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol and phosphatidyl ethanolamine to analyse phospholipids and ceramide, cholesterol and a mix of free fatty acids to analyse neutral lipids) were also run in the plates to allow quantification. The plates were stained with 5% $CuSO_4$ in 4% H_3PO_4 at 180 °C for 10 min.

Plate digital images obtained with a GS-800 Densitometer (BioRad) were analysed with BioRad Quantity One 1D analysis software (BioRad). The lipid concentration was normalized with the protein content in each sample.

2.8. Lipid peroxidation assay

Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) concentration was used to estimate the lipid peroxidation [24]. Samples for

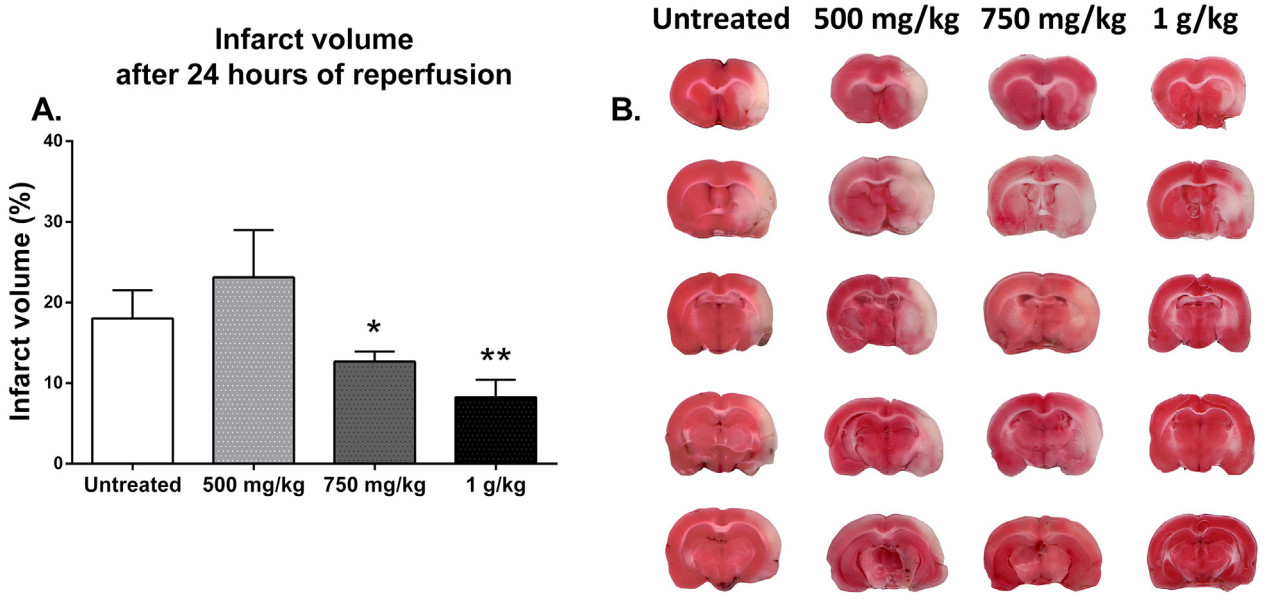

Fig. 2. Neuroprotection induced by 2OAA treatment. A) Graph of the infarct volume measured 24 h after reperfusion in animals treated with a single dose of different 2OAA concentrations. B) Representative coronal slices stained with TTC along the rat brain 24 h after reperfusion in untreated animals and animals treated with different concentrations of 2OAA ($^{*}p < 0.05$ $^{**}p < 0.01$, *t*-Student, n = 5).

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these assays were rapidly homogenized at 4 °C in 100 mM NaCl 1 in 50 mM PBS, pH 7.4, in the presence of protease inhibitors (complete protease inhibitor cocktail, EDTA-free; Roche Applied Science). Homogenates were centrifuged at 1500 ×*g* for 10 min at 4 °C. The protein concentration of supernatants was determined using a DC Protein Assay Kit (BioRad). Supernatants (100 μl), 10.3 mM N-methil-2-

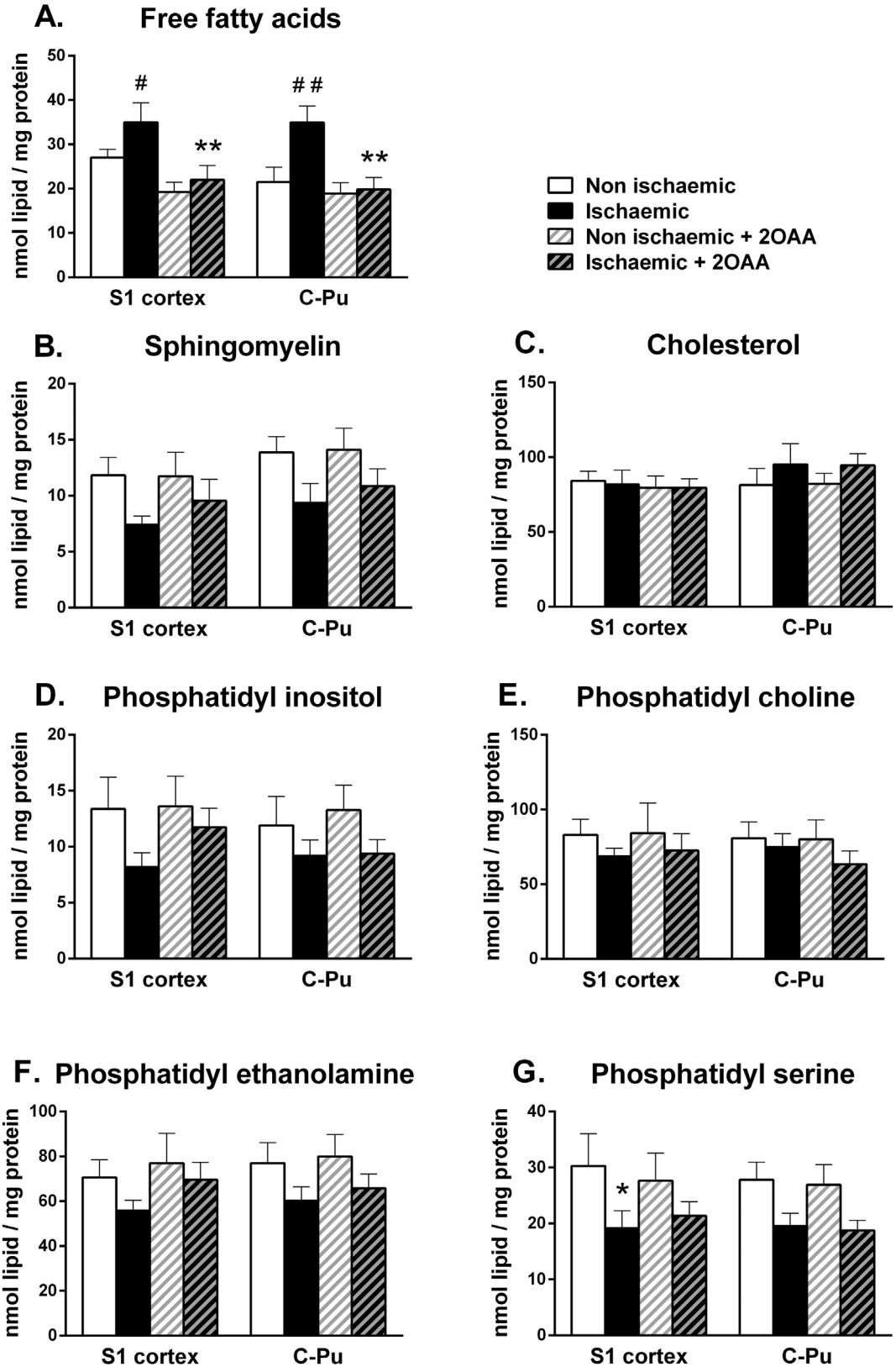

Fig. 3. Lipid modifications by ischaemia and 2OAA treatment. Concentration (mmol lipid/mg protein) of A) free fatty acids, B) sphingomyelin, C) cholesterol, D) phosphatidyl inositol, E) phosphatidyl choline, F) phosphatidyl ethanolamine and G) phosphatidyl serine after 24 h of reperfusion in two different brain areas: S1 cortex and C-Pu. * shows significant differences between treated and untreated animals and # shows significant differences as a consequence of the ischaemia ($^{*/\#}p < 0.05$ and $^{**/\#\#}p < 0.01$, two-way ANOVA, n = 4).

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phenylindole in a solution of methanol:acetonitrile (1:3) (320 μl) and 15.4 M methane sulfonic acid (75 μl), were incubated for 40 min at 45 °C. A set of MDA standard concentrations was simultaneously incubated to obtain a standard curve. This colorimetric reaction was stopped at 4 °C for 10 min, centrifuged at 10,000 × *g* for 5 min at 4 °C and absorbance measured at 586 nm in a Synergy-HT microplate reader (BioTek). MDA + 4-HNE concentration values were inferred from the MDA standard curve and normalized with the protein content of each sample.

2.9. Statistical analysis

Statistical analyses were carried out using GraphPad Prism 6 (GraphPad software). Statistics for infarct volume in the dose-response assays was performed by one-way ANOVA followed by Tukey's test. Lipid peroxidation ratios (ischaemic/non-ischaemic hemispheres) between treated and untreated animals were tested with a two-tailed Student's *t*-test. Two-way ANOVA followed by Tukey's test were performed to compare mRNA values or lipid concentrations of ischaemic and non-ischaemic hemispheres in untreated and 2OAA-treated animals. Significance was set at $p < 0.05$.

3. Results

3.1. Dose and toxicity assays

Toxicity assays for 2OAA were performed in mice and showed that doses of 1 g/kg per day resulted in mortality in all animals studied after 5 days of treatment. A 100% survival rate was observed with daily doses of 1 g/kg on the first two days followed by 500 mg/day the following days both in rats and mice.

Infarct volume measuring TTC staining was carried out 24 h after reperfusion using a single dose of 500 mg/kg, 750 mg/kg and 1 g/kg of 2OAA (Fig. 2) 1 h after tMCAO. A significant reduction (50%) in the infarct volume was observed in animals treated with the maximum dosage administered compared with untreated animals. A significant reduction in the infarct volume was also observed with a dose of 750 mg/kg and no significant changes were observed with the dose of 500 mg/kg.

3.2. Lipid analysis

Lipid analyses of areas under ischaemia (from the ipsilateral hemisphere) and non-ischaemic areas (contralateral hemisphere) in animals treated and untreated with 2OAA are shown in Fig. 3. FFA levels were significantly higher in ischaemic S1 cortex and C-Pu areas than their corresponding structures in the non-ischaemic hemisphere (Fig. 3A). At this time, the treatment with 2OAA prevented the ischaemia-induced increase of FFAs in both the S1 cortex and C-Pu.

Sphingomyelin, cholesterol, phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol and phosphatidyl ethanolamine levels were analysed (Fig. 3B–G). Sphingomyelin and phospholipid levels showed a tendency to decrease as a consequence of the ischaemia, but we could only find significant differences in phosphatidyl serine.

3.3. Phospholipase A2 expression

The effect of the ischaemia was measured in treated and untreated animals. In untreated animals, ipsilateral cytosolic *PLA2* (*cPLA2*) and *sPLA2-IIA* transcript levels in the ischaemic S1 cortex and C-Pu were significantly higher than their corresponding contralateral non-ischaemic areas, thereby indicating an ischaemia-dependent increase in the expression of both *cPLA2* and *sPLA2-IIA* (Fig. 4). In animals treated with 2OAA, we could not find significant differences between transcript levels of *cPLA2* and *sPLA2-IIA* in ischaemic areas compared with those of non-ischaemic areas, except for *cPLA2* in C-Pu (Fig. 4A and B).

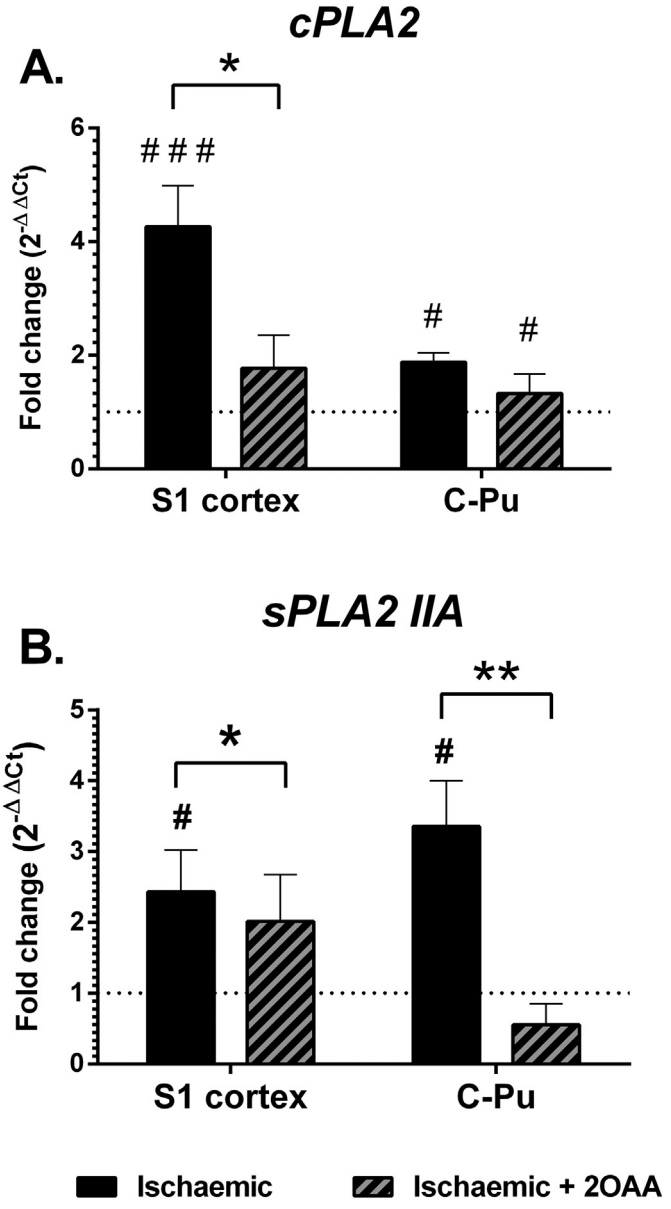

Fig. 4. Effect of ischaemia and treatment on PLA2 transcriptional level. In A) black columns show the fold change ($2^{-\Delta\Delta Ct}$) of *cPLA2* levels of ischaemic areas compared with their corresponding non-ischaemic areas (represented as a value of 1, *dotted line*) and striped columns show the fold change as a consequence of the ischaemia in *cPLA2* animals treated with 2OAA. In B) is shown the effect of ischaemia on *sPLA2-IIA*. * shows significant differences between treated and untreated animals and # shows significant differences as a consequence of the ischaemia (* or $^{\#}p < 0.05$, ** or $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.005$. Two-way ANOVA followed by Tukey test, $n = 5$).

The effect of the treatment was also analysed by comparing the PLA2 transcript levels of contralateral treated and untreated animals and comparing ischaemic structures between treated and untreated animals. We did not find significant changes in the *cPLA2* and *sPLA2-IIA* transcript levels when non-ischaemic structures of treated and untreated animals were compared. In contrast, treated animals presented significantly lower values of *cPLA2* and *sPLA2-IIA* transcripts in their ischaemic structures than those observed in the ischaemic structures of untreated animals, except for *cPLA2* in C-Pu (Fig. 4A and B).

3.4. Oxidative stress: antioxidant enzyme expression

In non-treated animals, transcript levels of the antioxidant enzymes superoxide dismutase 2 (*sod2*), heme oxygenase 1 (*hmox1*), NAD(P)H

quinone dehydrogenase 1 (*nqo1*) and glutamate-cysteine ligase modifier subunit (*gclm*) in the ischaemic S1 cortex and C-Pu were significantly higher than the corresponding non-ischaemic structures, except for *gclm* in C-Pu. These results reveal an ischaemia-dependent increase in the transcription of all these genes. In contrast, ischaemic and non-ischaemic structures of treated animals present similar values for all the enzymes studied, indicating an irrelevant effect of ischaemia in the transcript levels of these enzymes (Fig. 5) after 2OAA treatment.

The 2OAA effect on the oxidative stress is evidenced in ischaemic areas of animals treated with 2OAA, where a significant decrease in the transcript levels of all antioxidant enzymes analysed compared with those of the untreated animals except in the C-Pu of *gclm* was observed (Fig. 5).

3.5. Lipid peroxidation

In untreated animals, lipid peroxidation in the ischaemic hemisphere displayed about 1.6 times higher absorbance values than those observed in the contralateral hemisphere. In contrast, the treated animals show similar values of absorbance in both hemispheres (Fig. 6), indicating that 2OAA treatment reduces significantly the peroxidation in the damaged hemisphere.

4. Discussion

4.1. Setting up of the dose

We have analysed the effect of 2OAA, which blocks both COX-1 and COX-2. This compound has not revealed toxicity in previous studies in cultured cells and it has been suggested that it does not present a toxic effect "*in vivo*" in therapeutic doses, based on that the hydroxyl group in 2OAA would attenuate the toxicity of AA [16]. Our data show that the use of 2OAA *in vivo* provides substantial neuroprotection in terms of infarct volume, although it requires doses (1 g/kg) that prolonged for more than 48 h after tMCAO injury would lead the animals to the death. This could be due to the well-known deleterious

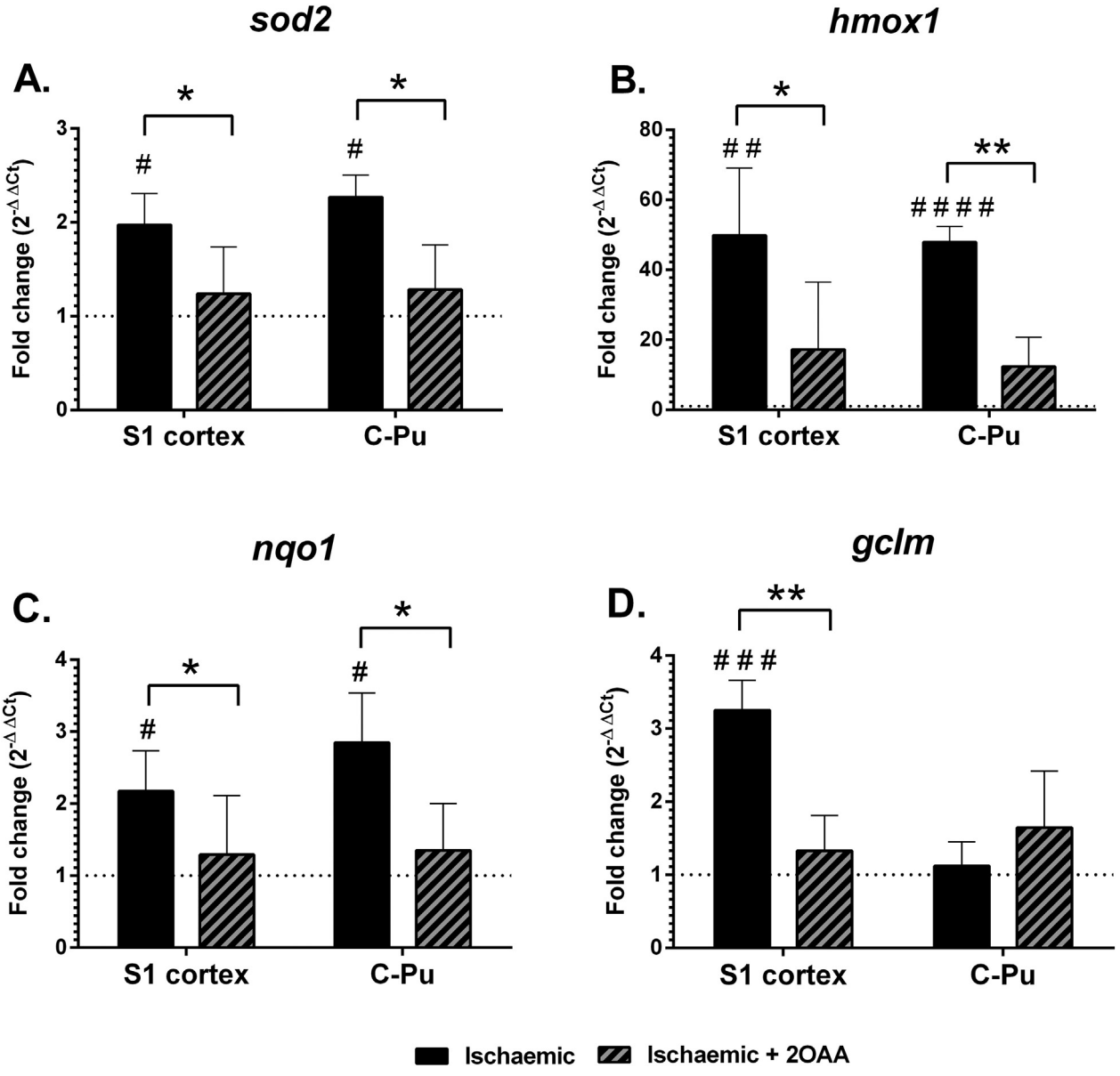

Fig. 5. Effect of ischaemia and treatment of 2OAA on antioxidant enzymes. Fold changes ($2^{-\Delta\Delta Ct}$) in the mRNA levels of A) *sod2*, B) *hmox1*, C) *nqo1* and D) *gclm* in S1 cortex and C-Pu. Ischaemic values of untreated (black columns) and treated (striped columns) animals with respect to their corresponding contralateral hemispheres (value 1, dotted horizontal line). * shows significant differences between treated and untreated animals and # shows significant differences as a consequence of the ischaemia (* or #$p < 0.05$, ** or ##$p < 0.01$, ###$p < 0.005$. Two-way ANOVA followed by Tukey test, $n = 5$).

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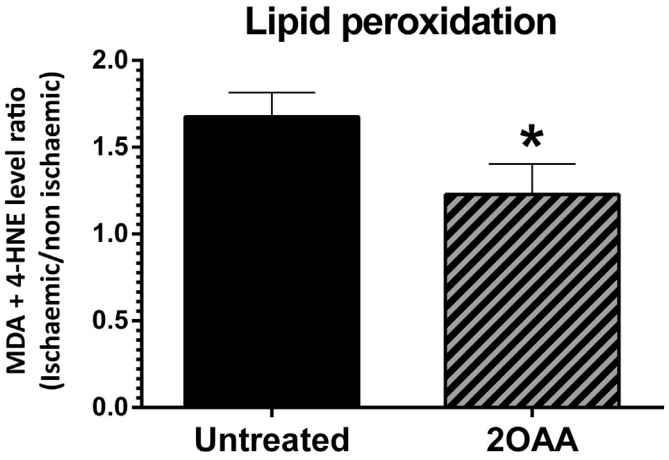

Fig. 6. Effect of treatment on lipid peroxidation levels. Normalized absorbance ratios (ischaemic/non-ischaemic) of MDA + 4HE in untreated and 2OAA treated animals. * represents significant differences as a consequence of the treatment with 2OAA (Student's *t*-test, $p < 0.05$, $n = 5$).

side effects described for COX-1 NSAIDs in peripheral tissues [25]. However, this treatment seems to be useful if it is interrupted or substantially decreased in the following days to avoid the death of the animals. In this regard, our study on the toxicity of 2OAA shows that a dose of 1 g/kg for two days did not result in mortality in any of the animals studied if doses were decreased by the third daily dose but provides substantial neuroprotection. Based on these data, we established a higher limit of 1 g/kg for a 85% purity of the product in a single dose that proved to be safe in the two first days, and chose this concentration as the most convenient dosage for studying the 2OAA neuroprotective effect since lower doses resulted in no or poor neuroprotection. Of note, additional histopathological studies in organs other than brain, beyond the scope of this study, are still required.

4.2. Neuroprotective effect of 2OAA correlates with a reduced PLA2 transcriptional activity

Cerebral ischaemia has been reported to decrease levels of phosphatidyl choline, phosphatidyl inositol, phosphatidyl serine and cardiolipin, as well as altering the fatty acid composition of phosphatidyl choline and phosphatidyl ethanolamine, in transient cerebral ischaemia in gerbils [26]. Increases in FFAs have also been detected in transient rat tMCAO [22]. Our results confirmed the increase in FFA levels 24 h after the ischaemic insult and provide additional support for the idea that this is a hallmark of the ischaemia. However, the ischaemia-dependent changes were less evident in phospholipids and neutral lipids analysed where we observed a tendency to decrease as a consequence of the ischaemia but we failed to detect statistical changes. The harmful effects of FFAs on mitochondria [27] relate directly to the increase in FFAs with the lack of staining by TTC, a substrate of dehydrogenase activity widely used to measure infarct volume. We hypothesized that treatment with 2OAA prevents the increase of FFA levels, which reduces the mitochondrial damage. This would result in a decreased infarct volume, the parameter used to measure neuroprotection.

How can the 2OAA prevent the increase in FFA levels? Since FFAs are released from the membrane by the action of PLA2 on the membrane phospholipids [9] and these enzymes have been described to present an ischaemia-dependent increase [22], we studied the effect of treatment with 2OAA on the expression of PLA2. Transcriptional activity of both cPLA2 and sPLA2 has been reported to be modulated by inflammation [28] and, consistently with the anti-inflammatory properties of 2OAA [16], we observed that treatment with this agent prevented the increase of both cPLA2 and sPLA2 transcriptional activity.

What is the meaning of differences in the transcriptional activity of cPLA2 and sPLA2? Differences in the transcriptional activity of cPLA2 and sPLA2 are observed as a consequence of the ischaemia between the S1 cortex and C-Pu and these differences are confirmed after the 2OAA treatment. Although more detailed studies are required to explain these differences, they could mirror changes in the cell populations in areas with different degrees of both neuronal demise and glial activity. In this regard, transcriptional activity of sPLA2, but not of cPLA2, has been related to GFAP-positive astrocytes [29], suggesting cell-dependent differences in the expression of these enzymes.

Thus, although we cannot discard other possible mechanisms, we can state that 2OAA treatment is able to decrease FFA levels by modulating the signalling pathways that lead to the ischaemia-dependent increase of cPLA2 and sPLA2 transcriptional activity.

4.3. 2OAA treatment prevents ischaemia-induced oxidative stress

In accordance with the effect of FFAs on mitochondria previously mentioned, PLA2 activity has been reported to increase the production of ROS [3], which increases the oxidative stress that triggers different subroutines of cell death [30]. In this regard, oxidative stress is one of the main causes of ischaemia-induced neuronal damage and leads to different types of cell death, including caspase-dependent and caspase-independent apoptosis [31]. Our results indicate that treatment with 2OAA results in a decrease of the lipid peroxidation, which mirrors a decrease in the oxidative stress. The consistent decrease in PLA2 expression and reduction in the lipid peroxidation support this decreased oxidative stress as a consequence of 2OAA treatment. We analysed the expression of a number of antioxidant enzymes (SOD2, HMOX1, NQO1 and GCLM), widely accepted as main markers of oxidative stress [32], to test whether treatment with 2OAA only decreases the ROS and RNS production or also increases the antioxidant response. Our results show that the treatment with 2OAA blocks the ischaemia-induced increases of these antioxidant enzymes. This supports the idea that the neuroprotective effect of 2OAA is based on the prevention of the oxidative stress mediated by PLA2 activity rather than increasing an antioxidant response.

Thus, the use of rational designed lipids seems to be a promising way to create molecules that can be used as pharmaceutical drugs against stroke. The use of lipids presents the advantage that in most cases they can cross the blood brain barrier, one of the limitations in brain treatment pathologies. The use of 2OAA confirms that lipids could act as therapeutic agents capable of modulating different pathways of damage, such as inflammation and oxidative stress. The action of 2OAA on the release of FFAs suggests that modifications of membrane composition play an important role in cell homeostasis.

5. Conclusions

In summary, the treatment with 2OAA results in neuroprotection against ischaemia, measured as the reduction of the infarct volume. This treatment leads to a decrease in transcriptional activity of PLA2-encoding genes, a reduction in the levels of FFAs and lipid peroxidation and the blocking of the ischaemia-induced increase in the expression of most significant antioxidant enzymes. We conclude that the 2OAA neuroprotective effect at the time here studied is based on the reduction of oxidative stress rather than on an increase of the antioxidant mechanisms. The strong neuroprotective effect of 2OAA supports the notion that a controlled dosage of blockers of both COX-1 and COX-2 could be possible therapeutic agents.

Conflict of interest

The authors have no conflict of interest to declare.

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