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ORIGINAL ARTICLE

Unfolded protein response to global ischemia following 48 h of reperfusion in the rat brain: the effect of age and meloxicam

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Abstract

The unfolded protein response (UPR) in the hippocampal regions Cornu Ammonis 1 hippocampal region, Cornu Ammonis 3 hippocampal region, and dentate gyrus, as well as in the cerebral cortex of 3-month-old and 18-month-old rats were studied in a model of 15 min of global cerebral ischemia followed by 48 h of reperfusion. UPR was measured by quantifying the protein disulfide isomerase (PDI), C/EBP-homologous protein (CHOP), GRP78 and GRP94 transcripts using qPCR and the amounts of PDI and GRP78 by western blot. The study shows how the mRNA levels of these genes were similar in 3-month-old and 18-month-old sham-operated animals, but the ischemic insult elicited a noticeable increase in the expression of these genes in young animals that was scarcely appreciable in older animals. The striking increase in the mRNA levels of these genes in 3-month-old animals was abolished or even reverted by treatment with meloxicam, an anti-inflammatory agent. Western blot assays showed that the UPR was still detectable 48 h after ischemia in some of the studied areas, and provided evidence that the UPR is different between young and older animals. Western blot assays carried out in young animals also showed that meloxicam elicited different effects on the levels of PDI and GRP78 in the cerebral cortex and the hippocampus. We conclude that the UPR response to ischemic/reperfusion insult is age- and probably inflammation-dependent and could play an important role in ischemic vulnerability. The UPR appears to be strongly decreased in aged animals, suggesting a reduced ability for cell survival.

Keywords: age, brain global ischemia, inflammation, meloxicam, UPR.

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The endoplasmic reticulum (ER) is a multifunctional organelle that co-ordinates protein folding, lipid biosynthesis, calcium storage, and release. Perturbations that disrupt ER homeostasis lead to ER stress and up-regulation of a signaling pathway called the unfolded protein response (UPR) (Naidoo 2009a). The transcriptional response of a number of genes, such as *PDI, GRP78*, and *GRP94*, is widely used to measure the UPR (Harding *et al.* 2002; Schröder and Kaufman 2005; Zhang and Kaufman 2006; Naidoo 2009b), as is C/EBP-homologous protein (CHOP), a

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Abbreviations used: 18MI/R, Ischemia 18 months old; 18MS, Sham-operated 18 months old; 3MI/R, Ischemia 3 months old; 3MS, Sham-operated 3 months old; AMPA, Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CA1, Cornu Ammonis 1 hippocampal region; CA3, Cornu Ammonis 3 hippocampal region; CD11b (Mac-1), Integrin alpha-M; cDNA, Complementary deoxyribonucleid acid; CHOP, C/EBP-homologous protein; COX, Cyclooxygenase; CX, Cerebral cortex; DG, Dentate gyrus; EAAC1, Excitatory amino acid carrier 1; ER, Endoplasmic reticulum; GABA, Gamma-aminobutyric acid; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GFAP, Glial fibrillary acidic protein; GLAST, Glutamate/aspartate transporter; GLT-1, Glutamate transporter-1; GluR1, AMPA receptor subunit GluR1; GluR2, AMPA receptor subunit GluR2; GRP78, Glucose-related protein 78 also binding immunoglobulin protein (BIP); GRP94, Glucose-related protein 94; Herp, Homocysteine-induced endoplasmic reticulum protein; Hsp70, Heat shock protein 70; I/R, Ischemia/Reperfusion; Mx, Meloxicam; NMDA, *N*-methyl-D-aspartate; NR1, NMDA receptor subunit NR1; NR2A, NMDA receptor subunit NR2A; NR2B, NMDA receptor subunit NR2B; PDI, Protein disulfide isomerase; RT, Reverse transcription; UPR, Unfolded protein response.

stress-inducible transcription factor that activates a set of target genes that leads to apoptosis (Wang *et al.* 1998). The UPR is elicited by different types of cellular damage, including ischemia (Hu *et al.* 2000, 2001; DeGracia and Montie 2004; Tajiri *et al.* 2004; Paschen and Mengesdorf 2005; Truettner *et al.* 2009; Nakka *et al.* 2010), and increases in the expression of a number of UPR-related genes have been reported following different ischemic insults (Hu *et al.* 2000, 2009; Truettner *et al.* 2009).

Age has been reported to be involved in a reduced capacity for calcium buffering by the ER, greater susceptibility of ER chaperones to oxidative damage and modifications to the UPR in response to neuronal injury and neurodegeneration (Verkhratsky and Shmigol 1996; Tsai *et al.* 1998; Rabek *et al.* 2003; Van der Vlies *et al.* 2003; Erickson *et al.* 2006; Zhang *et al.* 2006; Hoozemans *et al.* 2009; Naidoo 2009a, b, 2011; Saxena *et al.* 2009; Scheper and Hoozemans 2009). The expression of UPR-related genes such as *GRP78* and *PDI* has been shown to decrease with age in rodents (Gavilán *et al.* 2006; Hussain and Ramaiah 2007; Naidoo *et al.* 2008; Nuss *et al.* 2008). We report here a comparative study on the effect of ischemic damage on the UPR in young and aged animals.

Since the ischemic insult elicits both the UPR and a striking inflammatory response (Feuerstein *et al.* 1997; Barone and Feuerstein 1999; Chamorro and Hallenbeck 2006; Rodriguez-Yanez and Castillo 2008; Amantea *et al.* 2009; Montori *et al.* 2010a, b), a correlation between inflammation and ischemia-induced UPR seems plausible. However, there is a scarce literature addressing this point, so we also report here how the use of the anti-inflammatory agent meloxicam modifies the ischemia-induced UPR in both the hippocampus and cerebral cortex, two areas of the brain with different ischemic vulnerability (Ordy *et al.* 1993; Yang *et al.* 2000; Jiang *et al.* 2004 Gee *et al.* 2006; Ferriero and Miller 2010).

Materials and methods

Animals

Young (3 months old) and old (18 months old) male Sprague–Dawley rats [Charles River reference Crl:OFA(SD)] weighting 350–450 g and 600–800 g, respectively, were housed at 22 ± 1°C in a 12 h light/dark controlled environment with free access to food and water. Rats were divided randomly into ischemic and sham groups. Experiments were performed in accordance with the Guidelines of the Council of the European Union (86/609/EU), following Spanish regulations (RD 1201/2005, BOE 252/34367-91, 2005) for the use of laboratory animals. Experimental procedures were also 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.

Transient global ischemia

Animals were placed in the anesthesia induction box, supplied with 4% halothane (Sigma-Aldrich, Madrid, Spain) at 3 L/min in 100% oxygen. After induction, anesthesia was maintained with 1.5 to 2.5% halothane at 800 mL/min in 100% oxygen using a rat face mask. Both common carotid arteries were then exposed and transient global ischemia was induced by bilateral occlusion for 15 min with atraumatic aneurysm clips and moderate hypotension (40–50 mm Hg) using trimetaphan (kindly provided by Roche Applied Science, Mannheim, Germany) as a hypotensor agent (15 mg/mL, 0.3 mg/min). Small changes in the halothane concentration were used to modulate arterial tension, given its intrinsic hypotensive effect (Bendel *et al.* 2005). Rectal temperature was maintained at 36 ± 1°C during surgery with a feedback-regulated heating pad. The femoral artery was exposed and catheterized to allow continuous recording of arterial blood pressure and the administration of trimetaphan. When animal arterial blood pressure recovered, the catheter was removed and the animals were sutured. After recovering consciousness, they were maintained in an air-conditioned room at 22 ± 1°C during 48 h (time of reperfusion). For sham-operated rats, all procedures were performed exactly as for ischemic animals except that the carotid arteries were not clamped.

Anti-inflammatory treatment

To determine whether inflammation is involved in the modification of the UPR transcriptional response to ischemia, we used meloxicam, a non-steroidal anti-inflammatory agent, reported to inhibit 53.3% cyclooxygenase (COX)-1 and 77.5% COX-2 activity (Fleischmann *et al.* 2002). Five sham-operated and five I/R-injured 3-month-old rats were treated subcutaneously with meloxicam (0.5 mg/kg) (Boehringer Ingelheim, Madrid, Spain) 1 and 24 h after surgery. Five sham-operated and five I/R-injured 18-month-old rats were also treated with meloxicam, but none of the I/R-injured animals survived for more than 36 h.

RNA extraction and reverse transcription (RT)

Forty-eight hours after the ischemic insult, animals were decapitated and their brains were quickly removed. Sagittal slices of approximately 1 mm were obtained with a surgical blade and Cornu Ammonis 1 hippocampal region (CA1), Cornu Ammonis 3 hippocampal region (CA3) and the dentate gyrus (DG) were dissected from each slice using a dissecting microscope. The different samples as well as the entire cerebral cortex were then frozen and kept at −80°C until use. Total RNA was extracted using Tripure™ isolation reagent (Roche Applied Science, Mannheim, Germany), according to the instructions of the manufacturer. The contaminating DNA were removed by incubation with DNase (Sigma-Aldrich, Madrid, Spain) and confirmed by PCR. After isolation, RNA integrity was assessed using the Experion RNA HighSens Analysis Kit (Biorad Laboratories, Wilmington, DE, USA) following the manufacturer's instructions. The yield of total RNA was determined by measuring the absorbance (260/280 nm) of ethanol-precipitated aliquots of the samples. Total RNA concentrations were determined using a NanoDrop ND-3300 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RT was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and 600 ng of total RNA as a template following the recommendations of the manufacturer.

Real-time PCR

After retrotranscription, the cDNA was diluted in sterile water and used as template in the real-time PCR assays. GenBank was used as

Table 1 Primers used in real-time PCR assays and the GenBank accession numbers

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Genbank
GAPDH	GGGCAGCCCAGAACATCA	TGACCTTGCCCACAGCCT	NM_017008
PDI	CTGCTGTTCCTGCCCAAGAGTGT	TGGCTCATCAGGTGGGGCTTG	NM_012998
GRP78	CGTCCAACCCGGAGAACA	ATTCCAAGTGCGTCCGATG	NM_013083
GRP94	GTGGGTGCTGGGCCTCT	GACTTCATCGTCAGCTCTCACA	NM_001012197
CHOP	GCATCCCTAGCTTGGCTGACT	ATCTGGAGAGCGAGGGCTTT	NM_001109986

a source for all mRNA sequences. Primers were designed using Primer Express software (Applied Biosystems). The forward and reverse primers used in this study are shown in Table 1. Real-time PCR was performed using a StepOnePlus™ Real-Time PCR System and the SYBR®Green PCR Master Mix (Applied Biosystems). The optimal cDNA quantity and primer concentration was determined using a standard curve constructed from the qPCR results of increasing amounts of cDNA and different concentrations of primers. The optimal PCR conditions were obtained using 2 μL of 1/10 cDNA dilution as template and 300 nM of each primer. The normalization was done using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference gene. The mRNA levels of each gene *GAPDH*-normalized were expressed as $2^{-\Delta Ct}$, while the relative change in the mRNA levels of the genes studied following I/R with respect to the sham-operated group was determined by the equation:

Fold change = $2^{-\Delta\Delta Ct}$

ΔCt = (Ct target − Ct GAPDH);

$\Delta\Delta Ct$ = (Ct target − Ct GAPDH)ischemic animals − (Ct target − Ct GAPDH) sham-operated animals (Livak and Schmittgen 2001).

Western blot

Total protein was extracted using Tripure™ isolation reagent (Roche Applied Science) following the manufacturer's instructions. Protein concentrations were determined using the Bradford method (BioRad, Hercules, CA, USA) and stored in 8 M urea and 4% SDS in the presence of a protease inhibitor (Complete protease inhibitor cocktail, EDTA free; Roche Applied Science). Protein samples (30 μg per lane) were loaded and resolved on a 10% polyacrylamide gel (SDS PAGE; BioRad) at 110 V for 120 min. Then, proteins were transferred to a nitrocellulose membrane using a dry transfer system (Invitrogen, Carlsbad, CA, USA) at 20 V for 7 min. Nitrocellulose membranes were blocked in 5% non-fat milk, 0.2% Tween-20 in Tris-buffered saline (TBS-T) for at least 60 min at 25°C and incubated overnight at 4°C, with the following primary antibodies: a rabbit polyclonal antibody against GRP78 (1 : 1000) (Abcam, Cambridge, UK), a mouse monoclonal antibody against protein disulfide isomerase (PDI) (1 : 1500) (Abcam) and a mouse monoclonal antibody against β-actin clone AC-74 (1 : 5000) (Sigma-Aldrich). Primary antibodies were labeled with appropriate secondary anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase (Dako, Glostrup, Denmark), using a 1 : 3000 dilution, and were developed using the Chemiluminescence Luminol Reagent (Santa Cruz Biotech, Madrid, Spain). Densitometry analysis of the different lanes was performed with ImageJ 1.46r (ImageJ software; NIH, Bethesda, MD, USA).

Statistical analysis

All results are expressed as mean ± SEM. Two-way ANOVA tests were conducted to look for interactions between age and ischemia or between meloxicam treatment and ischemia. This test was followed by the post hoc non-parametric Bonferroni test. The significance was set at the 95% confidence level. The statistical analysis was carried out using Graph Pad Prism 5 (Graph Pad software, San Diego, CA, USA).

Results

PDI

The ischemic insult resulted in increased PDI mRNA levels in all structures (except in CA3 in 18M animals), and the response was significantly higher in young animals than in older ones (Fig. 1a).

The intrinsic effect of age was measured comparing sham-operated 18 months old (18MS) animal PDI levels with those of the sham-operated 3-month-old (3MS) animals. In aged animals, significantly higher transcript levels were observed in CA1, but no differences were detected for CA3, dentate gyrus (DG) or cerebral cortex (CX) (Fig. 2a). The age-dependent effect of ischemic injury was studied by comparing the transcript levels of ischemia 18 months old (18MI/R) and ischemia 3 months old (3MI/R) animals and revealed significantly lower PDI mRNA levels in all studied structures in aged animals (Fig. 2a).

PDI protein amounts in the cerebral cortex and dentate gyrus significantly increased as a consequence of the ischemic insult in young animals; however, this challenge induced significant decreases in CX and CA3 and significant increases in CA1 and DG of old animals (Fig. 3). Similar amounts of PDI were observed in young and aged sham-operated animals, except in the cerebral cortex, where levels were higher in aged animals. Significant decreases in the amount of PDI in CA3 and CX were observed in insulted aged animals compared with those of the insulted young aged animals, but we failed to detect any significant differences in the remaining structures studied.

CHOP

The widely used marker of pre-apoptosis, CHOP, was used for an estimation of delayed cell death in the cerebral cortex and the different hippocampal areas (Fig. 1b). I/R induced a

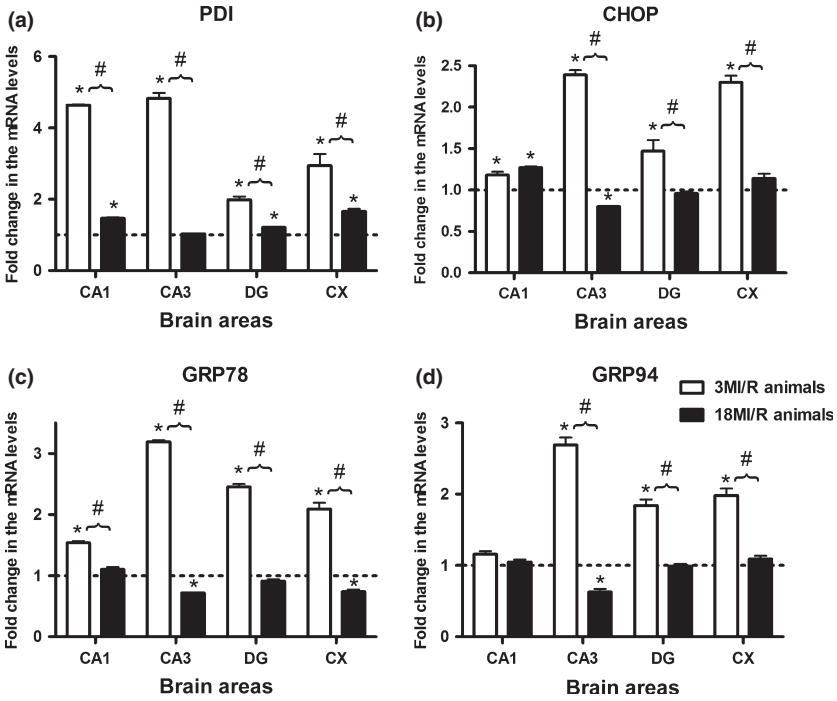

Fig. 1 Effect of ischemia on mRNA levels. Fold change ($2^{-\Delta\Delta Ct}$) in (a) protein disulfide isomerase (PDI), (b) C/EBP-homologous protein (CHOP), (c) GRP78, and (d) GRP94 mRNA levels between I/R injured animals and their sham-operated animals (indicated a value 1 by the dotted horizontal lines) in the hippocampal Cornu Ammonis 1 hippocampal region (CA1), Cornu Ammonis 3 hippocampal region (CA3), dentate gyrus (DG), and cerebral cortex (CX) for each age group. Significant differences with respect to sham-operated animals are indicated by $^{*}p < 0.05$. Significant differences between 3MI/R and 18MI/R animals are indicated by $^{\#}p < 0.05$. Two-way ANOVA test ($n = 5$).

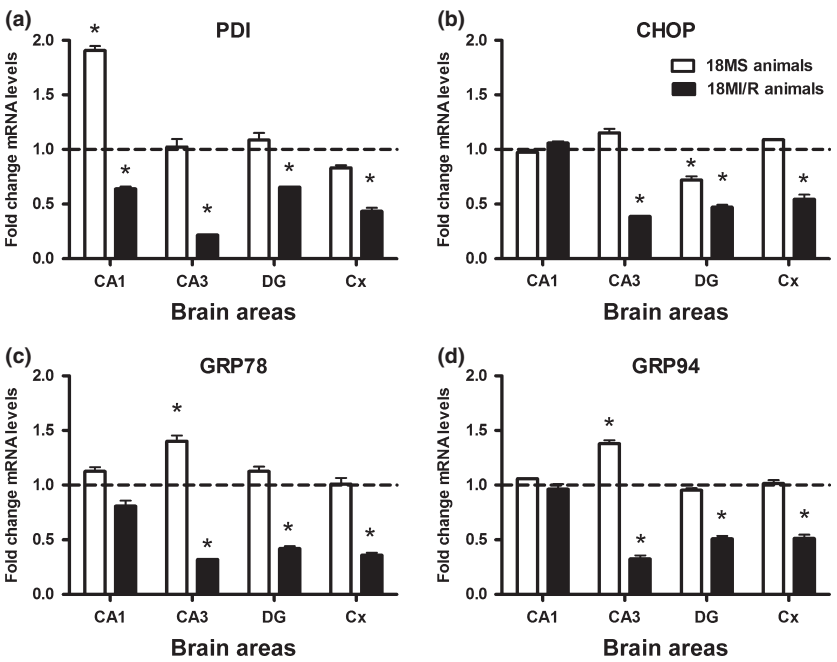

Fig. 2 Age effect on mRNA levels. Fold change ($2^{-\Delta\Delta Ct}$) in (a) protein disulfide isomerase (PDI), (b) C/EBP-homologous protein (CHOP), (c) GRP78, and (d) GRP94 mRNA levels in aged animals compared with young animals (a value of 1, indicated by the dotted horizontal lines) in the hippocampal Cornu Ammonis 1 hippocampal region (CA1), Cornu Ammonis 3 hippocampal region (CA3), dentate gyrus (DG) and cerebral cortex (CX) for each age group. Significant differences with respect to young animals are indicated by $^{*}p < 0.05$. Two-way ANOVA test ($n = 5$).

significant increase in the CHOP mRNA level in young animals (Fig. 1b), while in old animals, we failed to detect any significant changes (except in CA1 and CA3) as a consequence of the challenge. Interestingly, the analysis in young animals showed that the ischemic-dependent increased CHOP response in CX, CA3, and DG was significantly higher than that observed in the CA1 hippocampal area (Fig. 1b). These differences were not found in aged animals.

Regarding the age effect, we failed to detect differences in the mRNA levels when aged and young sham-operated animals were compared, except in DG (Fig. 2b). In contrast, 18MI/R animals presented significantly stronger decreases in the mRNA levels in most of structures studied than those

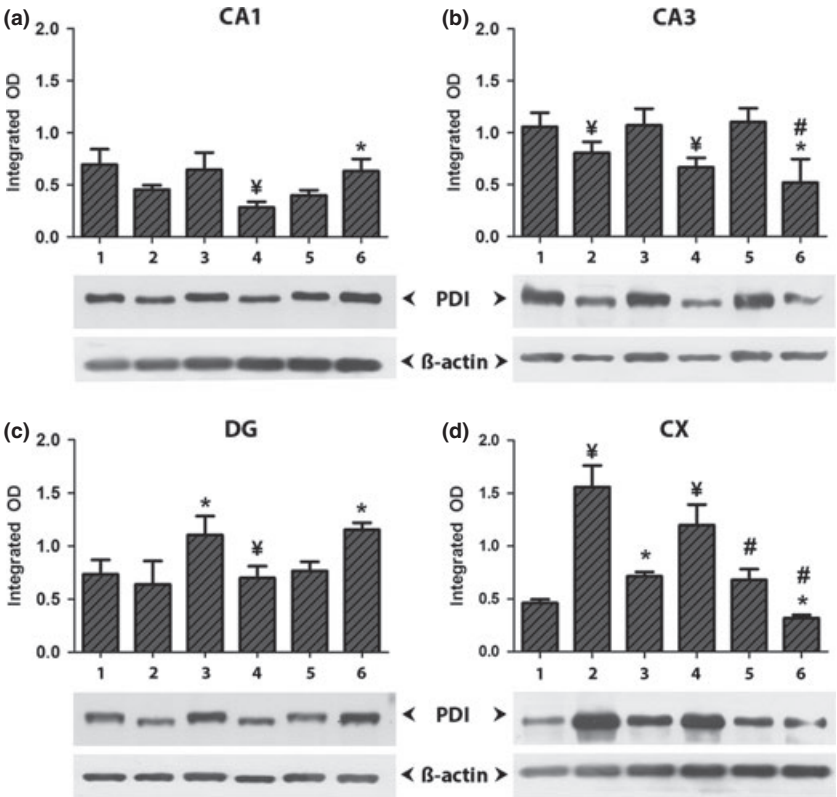

Fig. 3 Protein disulfide isomerase (PDI) modifications. Effect of I/R, age and meloxicam on PDI (55 kDa) amounts in Cornu Ammonis 1 hippocampal region (CA1) (a), Cornu Ammonis 3 hippocampal region (CA3) (b), dentate gyrus (DG) (c), and cerebral cortex (CX) (d) after 48 h of reperfusion. PDI protein bands from western blot assays and the averages of the densitometric analysis using five rats (mean ± SEM) normalized to β-actin (40 kDa) are shown. Representative bands and average values are shown in lane 1 (3MS), lane 3 (3MI/R), lane 5 (18MS) and lane 6 (18MI/R). The effect of meloxicam treatment in sham and injured animals is shown in lane 2 (3MS + Mx) and lane 4 (3MI/R + Mx). * shows significant differences of I/R compared to sham-operated animals, # indicates an age-dependent significant effect, and ¥ shows significant differences as a consequence of meloxicam treatment ($p < 0.05$). Two-way ANOVA ($n = 5$).

observed in 3MI/R, except in CA1 where no significant changes were observed (Fig. 2b).

GRP78 and GRP94

The chaperone response to ischemic insult was measured in GRP78, located in the lumen of the ER, and in GRP94, located in the cytosol. Both behaved in a similar way and young insulted animals showed significant higher mRNA levels in most structures studied when compared to those of the young sham-operated animals. The only exception was CA1, which presented lower increases in GRP78 compared with the other structures and similar values for injured and sham-operated young animals. In contrast, the insulted aged animals presented similar mRNA levels compared to their correspondent sham-operated animals in most structures, except CA3, where significant decreases were observed in both GRP78 and GRP94. The cerebral cortex also showed decreased GRP78 mRNA levels in aged injured animals (Fig. 1c and d).

Similar GRP78 and GRP94 mRNA levels were found in young and aged sham-operated animals, except in CA3 where the mRNA levels appeared increased in an age-dependent way (Fig. 2c and d). The comparative effect of ischemia between young and old animals also showed a similar response in both GRP78 and GRP94 and demonstrated an age-dependent significant decrease in their mRNA levels in the hippocampus, while the cerebral cortex remained unchanged (Fig. 2c and d).

Western blot assays of GRP78 are shown in Fig. 4. In the cerebral cortex, ischemic insult resulted in increased GRP78 protein expression in young animals, but decreases in this protein in old animals. Ischemia-dependent increases in GRP78 were observed in the hippocampal areas in both young and old animals, except in young CA1, where increases were not significant.

GRP78 levels in 18MS compared with 3MS were similar in CA1, higher in DG and lower in the cerebral cortex and CA3. In contrast, the levels of GRP78 in aged injured animals were significant lower in cerebral cortex and CA3, similar in CA1 and higher in GD compared with those of young injured animals.

Meloxicam treatment

The treatment with meloxicam resulted in a general inhibition of the UPR to I/R injury. Figure 5 shows a significant increase in transcript levels, in almost all of the genes and structures studied, in non-treated injured animals compared to non-treated sham-operated ones (open columns and dotted lines, respectively). This effect is completely reversed by meloxicam treatment, which resulted in no significant difference in any of the gene transcripts studied (compare the filled columns with the dotted lines).

Western blot assays showing the effect of meloxicam treatment on PDI expression are shown in Fig. 3 (lanes 2 and 4). An outstanding increase in this protein was observed in the cerebral cortex in both sham and ischemic animals as a

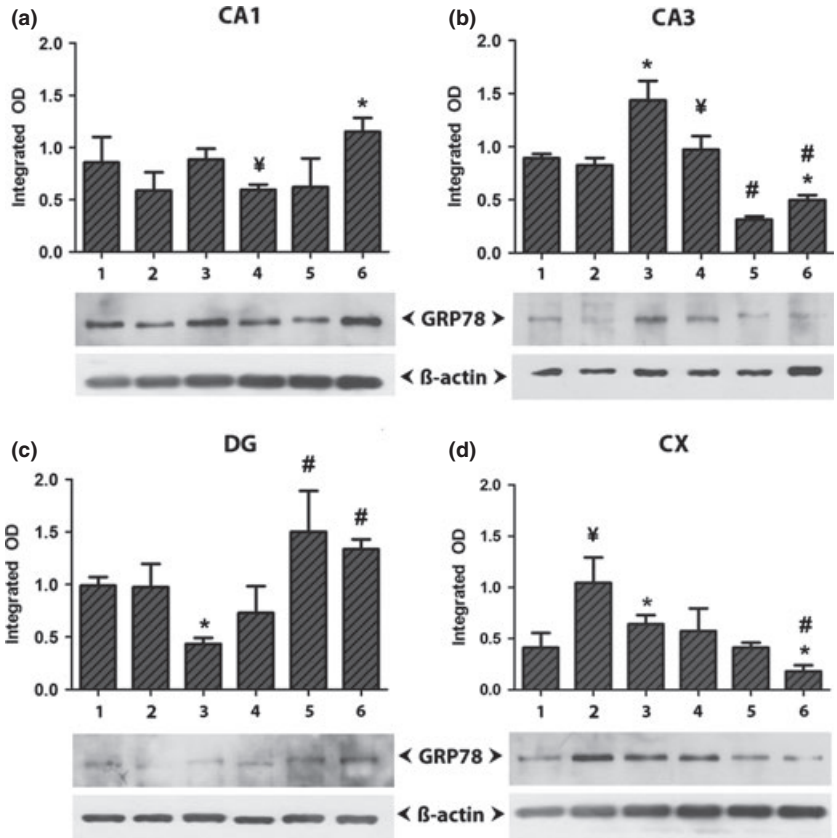

Fig. 4 GRP-78 modifications. Effect of I/R, age, and meloxicam on GRP78 (78 kDa) amounts in Cornu Ammonis 1 hippocampal region (CA1) (a), Cornu Ammonis 3 hippocampal region (CA3) (b), dentate gyrus (DG) (c), and cerebral cortex (CX) (d) after 48 h of reperfusion. GRP78 protein bands from western blot assays and the averages of the densitometric analysis using five rats (mean ± SEM) normalized to β-actin (40 kDa) are shown. Representative bands and average values are shown in lane 1 (3MS), lane 3 (3MI/R), lane 5 (18MS), and lane 6 (18MI/R). The effect of meloxicam treatment in sham and injured animals is shown in lane 2 (3MS + Mx) and lane 4 (3MI/R + Mx). * Shows significant differences of I/R compared to sham-operated animals, # indicates an age-dependent significant effect, and ¥ shows significant differences as a consequence of meloxicam treatment ($p < 0.05$). Two-way ANOVA ($n = 5$).

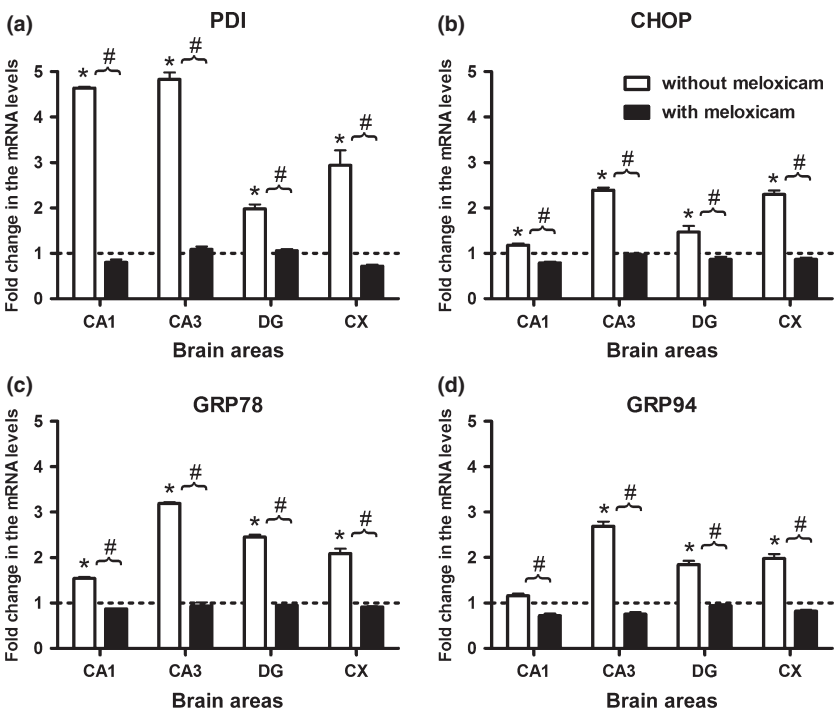

Fig. 5 Meloxicam effect. Fold change ($2^{-\Delta\Delta Ct}$) in the (a) protein disulfide isomerase (PDI), (b) C/EBP-homologous protein (CHOP), (c) GRP78, and (d) GRP94 mRNA levels of meloxicam treated and non-treated I/R-injured animals with respect to their respective sham-operated animals (a value of 1, indicated by the dotted horizontal line) in Cornu Ammonis 1 hippocampal region (CA1), Cornu Ammonis 3 hippocampal region (CA3), dentate gyrus (DG), and cerebral cortex (CX). Significant differences with respect to sham-operated animals are indicated by $*p < 0.05$. Significant differences between treated and untreated I/R-injuried animals are indicated by $^{\#}p < 0.05$. Two-way ANOVA test ($n = 5$).

consequence of treatment with meloxicam. However, in the hippocampus, a decrease occurred in both sham and ischemic animals treated with meloxicam.

In the sham-operated animals, treatment with meloxicam resulted in increases in GRP78 protein expression in the cerebral cortex, but no changes were observed in the

hippocampal areas (Fig. 4, lanes 2 and 4). Significant decreases in GRP78 were observed in CA1 and CA3 in injured animals treated with meloxicam when compared with non-treated injured animals.

Discussion

The protein response

ER-stress induced neuronal cell death has been suggested to play an important role in stroke (DeGracia and Montie 2004; Tajiri *et al.* 2004). In agreement, agents that counteract the ER stress, such as salubrinal, have been reported to decrease the damage after I/R injury in focal cerebral ischemia (Nakka *et al.* 2010). The presence of protein aggregates, indicating the presence of ER stress in a similar model to that used in this study, has been reported to appear 1 h after reperfusion (Hu *et al.* 2000; Truettner *et al.* 2009). However, the molecules related with the unfolded protein response (UPR) present different responses. Thus, some chaperone molecules (Hsp70, GRP78, Hsp60, GRP94) have been reported to increase progressively in the hippocampus in the first 24 h, while PDI levels remain unchanged (Truettner *et al.* 2009). Our data on PDI expression in the hippocampus of young animals are consistent with those of Truettner *et al.* (2009), and suggest that PDI levels are not modified by ischemic challenge. In contrast, PDI level modifications in the cerebral cortex indicated a different UPR in the hippocampus and cerebral cortex, fitting with the differential responses to the ischemia widely described for these structures, as mentioned below.

Our data on GRP78 expression in some structures of young animals are also consistent with the return to normal values at 24 h reported by Truettner *et al.* (2009). However, we still detected structures with injury-dependent increases in GRP78, indicating that the UPR is still detectable at the protein level 48 h after challenge. The different behavior of both PDI and GRP78 following the insult in young and aged animals reveals age-dependent differences in the I/R-induced UPR and confirms that this response is also detectable in aged animals 48 h after the ischemic insult.

Additional support for the different behavior of PDI and GRP78 expression was provided by treatment with the anti-inflammatory agent meloxicam. This treatment resulted in decreases in the levels of these molecules in the hippocampus and increases in the cerebral cortex. This suggests that the differential vulnerability reported for these structures (Vallet and Charpiot 1994; Jiang *et al.* 2004; Gee *et al.* 2006; Kumari Naga *et al.* 2007; Stanika *et al.* 2010) could be largely dependent on inflammation.

The mRNA response

Protein and mRNA responses do not necessarily behave in the same way; in fact, discrepancies are usually observed. Different mechanisms have been indicated to explain the relative utilization of a given mRNA, including translational inhibition or activation (Mitchell and Tollervey 2001; Wilusz *et al.* 2001; Gorospe *et al.* 2011). Discrepancies between protein and mRNA levels during hypoxia seem to depend mainly on mRNA turnover and translational control (Gorospe *et al.* 2011). Therefore, mRNA and protein behaviors mirror different processes happening in the cell, explaining many of their discrepancies. In addition, the possibility of amplifying mRNA molecules allows the quantification of molecular changes that are not detected at the protein level.

One of the first effects of the UPR is to block the translation of proteins; however, some mRNAs become preferentially translated (for reviews see Walter and Ron 2011; Korennykh and Walter 2012). Consistently, this report shows increases in the mRNA of some UPR-related gene mRNAs contrasting with the noticeable decrease described in a number of glutamatergic and GABAergic system genes following a similar 48 h I/R insult (Naidoo *et al.* 2008; Dos-Anjos *et al.* 2009; Montori *et al.* 2010a, b, c, 2012; Llorente *et al.* 2013). These data suggest that I/R results in a decrease in neurotransmission in both excitatory (glutamatergic) and inhibitory (GABAergic) systems, while it activates the UPR to reduce the cellular damage resulting from the insult. In this regards, assays with salubrinal in focal cerebral ischemia, that is, promoting the UPR by inhibiting the dephosphrylation of the eukaryotic translation initiation factor 2 subunit α (eIF2α), significantly decreases I/R-induced damage (Nakka *et al.* 2010).

The correlation between the UPR and delayed cell death has been previously indicated. In this regard, ischemic preconditioning has been reported to be a very effective way of preventing delayed neuronal death (Kato *et al.* 1994; Shamloo *et al.* 1999; Kirino 2002), and this has been reported to decrease protein aggregation (Liu *et al.* 2005). A post-conditioning treatment, with a series of mechanical interruptions of the reperfusion, has also been reported to reduce ischemic/reperfusion damage, with decreases in caspase 12 and CHOP expression, but increased expression of GRP78 (Yuan *et al.* 2011). These data support the notion of the UPR is a crucial factor in neuronal vulnerability. In this regard, our mRNA results suggest that the UPR and delayed cell death in hippocampus are greater than in cerebral cortex 48 h after challenge. These data suggest that the UPR may overcome the reticulum stress in some areas but not in others, which might be a clue to understanding the differential vulnerability of different brain regions to ischemia.

Previous in situ hybridization studies in young animals following global cerebral ischemia have shown increased mRNA transcripts in a number of chaperone proteins (GRP78, HERP and GRP94) following ischemic damage. The peak of these increases appears at 24 h and return to similar values to sham-operated animals at 48 h (Truettner *et al.* 2009). However, our results in young animals indicate

that 48 h after challenge, there is still a strong increase in the mRNA levels of all genes analyzed, including CHOP, a widely used marker of apoptosis (Tajiri *et al.* 2004; Chan *et al.* 2011).

The role of CHOP has been analyzed in some ischemia studies. Thus, CHOP-knockout mice have been reported to present a decreased loss of neurons following ischemia compared to wild-type mice in bilateral common carotid occlusion. Primary hippocampal neurons from $CHOP^{-/-}$ mice also showed greater resistance to hypoxia/reoxygenation-induced cell death (Tajiri *et al.* 2004). Ischemia-associated ER stress has been hypothesized to be induced predominantly through the CHOP-dependent signaling pathway in neurons of the hippocampus (Osada *et al.* 2010). Our data are in agreement with this idea, and also support the hypothesis that *CHOP* gene expression and ER stress are involved in differences in neuronal vulnerability. In this regard, the CHOP response observed in this study is parallel to that observed for GRP78 and GRP94, corroborating the differential response between the cerebral cortex and hippocampus in young ischemic animals.

What is the effect of anti-inflammatory agents? CD11b and GFAP mRNA levels are used as microglial and astroglial markers, respectively, as well as indicators of inflammation (Massaro *et al.* 1990; McGeer and McGeer 1995; Kim and de Vellis 2005; Yatsiv *et al.* 2005; Giovannoni 2006; Hamby *et al.* 2007). Meloxicam treatment, following the ischemic insult, has been reported to result in a different response in the hippocampus and the CX when mRNA levels for CD11b and GFAP are analyzed. Thus, treatment with meloxicam results in a normalization of the levels of these inflammatory markers in the hippocampus (i.e., they are similar to those in sham-operated animals), although in the CX, they are still significantly higher than in sham-operated controls (Montori *et al.* 2010a). Meloxicam treatment in this model has also been shown to lessen the ischemia-induced decreases in mRNA levels in a number of glutamatergic and GABAergic genes (Montori *et al.* 2010a, b, c, 2012; Llorente *et al.* 2013). Although more studies are needed using different anti-inflammatory agents, different time points of analysis and different doses, our data show that meloxicam treatment decreases CHOP and chaperone increases following ischemic insult, suggesting that reducing inflammation could result in a decrease in mortality. In our hands, treatment with meloxicam revealed that *UPR* gene transcripts decreased in a similar way in the hippocampus and the cerebral cortex. These data suggest that the differential vulnerability to ischemic damage correlates with the extent of inflammation, but it seems that *UPR* gene response is independent of this different vulnerability.

Age-dependent response

Our study also reveals that *UPR* gene transcripts levels were similar in young and aged sham-operated animals; however, the ischemic insult elicited noticeable age-dependent differences in the UPR. In addition, differences observed between structures in young animals were less noticeable in aged animals, and the response to I/R insult was lower. Thus, our CHOP data support an age-dependent decrease in delayed cell death, and this response correlates with a dampened UPR. This idea is also supported by our data on the chaperone protein levels. In fact, our results support the idea that aged animals have a noticeable decrease in their ability to activate the ischemia-induced UPR.

Conclusions

In summary, our data support the idea that, in young animals, *UPR* gene transcription increases following ischemic insult, which contrasts with the general decrease in transcriptional activity previously reported for glutamatergic and GABAergic gene systems. Thus, the UPR could play an important role in ischemic vulnerability. A role for inflammation in the UPR was shown by the use of meloxicam. Finally, the UPR appeared to be strongly decreased in aged animals.

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Conflicts of interest

None.

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