Conjugated linoleic acid prevents age-dependent neurodegeneration in a mouse model of neuropsychiatric lupus via the activation of an adaptive response[S]

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Abstract **Oxidative stress is a key mediator of autoimmune/neurodegenerative disorders. The antioxidant/anti-inflammatory effect of a synthetic conjugated linoleic acid (CLA) mixture in MRL/MpJ-Fas^{lpr} mice (MRL/*lpr*), an animal model of neuropsychiatric lupus, was previously associated with the improvement of nuclear factor-E2-related factor 2 (Nrf2) defenses in the spleen and liver. However, little is known about the neuroprotective ability of a CLA mixture. This study investigated the age-dependent progression of oxidative stress and the hyperactivation of redox-sensitive compensatory pathways (macroautophagy, Nrf2) in old/diseased MRL/*lpr* mice brains and examines the effect produced by dietary CLA supplementation. Disrupted redox homeostasis was evidenced in the blood, liver, and brain of 21- to 22-week-old MRL/*lpr* (Old) mice compared with 8- to 10-week-old MRL/*lpr* (Young) animals. This alteration was associated with significant hyperactivation of compensatory mechanisms (macroautophagy, Nrf2, and astrocyte activation) in the brains of Old mice. Five-week daily supplementation with CLA (650 mg/kg^{-1} body weight) of 16-week-old (CLA+Old) mice diminished all the pathological hallmarks at a level comparable to Young mice or healthy controls (BALB/c). Such data demonstrated that MRL/*lpr* mice can serve as a valuable model for the evaluation of the effectiveness of neuroprotective drugs. Notably, the preventive effect provided by CLA supplementation against age-associated neuronal damage and hyperactivation of compensatory mechanisms suggests that the activation of an adaptive response is at least in part accountable for its neuroprotective ability.**—Monaco, A. I. Ferrandino, F. Boscaino, E. Cocca, L. Cigliano, F. Maurano, D. Luongo, M. S. Spagnuolo, M. Rossi, and P. Bergamo. **Conjugated linoleic acid prevents age-dependent neurodegeneration in a mouse model of neuropsychiatric lupus via the activation of an adaptive response.** ***J. Lipid Res.*** **2018.** 59: **48–57.**

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The MRL/MpJ-Fas^{lpr} (MRL/*lpr*) mouse is a prototypical model for human systemic lupus erythematosus in which age-dependent manifestations of pathological signs (e.g., splenomegaly) are associated with high levels of autoantibodies and oxidative stress markers (1). In particular, MRL/*lpr* and its congenic strain MRL/MpJ (MRL/+) spontaneously develop several lupus-like manifestations (e.g., high serum levels of autoantibodies, skin lesions, lymph node and spleen enlargement), but in MRL/+ mice, similar symptoms become visible much later (2). Notably, MRL/*lpr* mice exhibit an accelerated form of lupus-like disease associated with behavioral manifestations/neurological signs (3, 4) and a marked increase of degenerating neurons (5), while no data indicating the occurrence of such pathological symptoms in age-matched MRL/+ mice are available. For these reasons, MRL/*lpr* mice have been indicated as a useful animal model for the evaluation of neuroprotective drugs (6), but their utilization in such studies has so far been limited.

Abbreviations: CLA, conjugated linoleic acid; CLA+Old, CLA-treated Old; FJB, Fluoro-Jade B; FJB+, FJB-positive; γGCL, γ-glutamylcysteine ligase; GFAP, glial fibrillary acidic protein; GFAP+, GFAP-positive; G6PD, glucose 6 phosphate dehydrogenase; GSR, glutathione-S-reductase; LC3, microtubule-associated protein light chain 3; MRL/+, MRL/MpJ; MRL/*lpr*, MRL/MpJ-Fas^{lpr}; Nrf2, nuclear factor-E2-related factor 2; N-Tyr, nitrosylated protein; Old, 21- to 22-week-old MRL/*lpr*; PC, carbonylated protein; p62, p62/sequestosome-1; ROS, reactive oxygen species; TEAC, trolox equivalent antioxidant capacity; Young, 8- to 10-week-old MRL/*lpr*.

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The brain, likely owing to its high oxygen requirements, seems to be more susceptible to peroxidation compared with other organs, and the neurodegenerative diseases associated with perturbation of redox balance are frequently accompanied by the activation of defensive mechanisms such as astrogliosis (7), macroautophagy (8), and nuclear factor-E2-related factor 2 (Nrf2) activation (9). Interestingly, although oxidative stress signaling has a relevant role in the etiology of neurological diseases (10), the alteration of such redox-sensitive pathways in the brain tissue of this animal model has not been investigated yet.

Astrocytes are the major cell population within the central nervous system and the main regulator of oxidative homeostasis. In reaction to injury, these cells leave their quiescent state and become reactive. This process (astrogliosis) includes several grades of severity, varying from cell and astrocytic process hypertrophy to functional changes [increased expression of glial fibrillary acidic protein (GFAP), Nrf2 hyperactivation] and gross morphological alterations (glial scar formation) (11). Macroautophagy (hereafter referred to as autophagy) is a catabolic mechanism that involves the sequestration and transport of organelles and macromolecules to the lysosomes for degradation. As such, autophagy plays a key role in maintaining cellular homeostasis. Interestingly, autophagy is a regulator of the oxidative stress response (12), and a feedback loop between autophagy and Nrf2 has been demonstrated (13).

Nrf2 is the main transcriptional activator of genes responsible for the maintenance of cell redox homeostasis through the production/recycling of the main intracellular antioxidant, GSH. Among these genes are phase 2 enzymes, glutathione reductase (GSR), glucose 6 phosphate dehydrogenase (G6PD), and γ-glutamylcysteine ligase (γGCL), that play fundamental roles in the direct elimination of reactive oxygen species (ROS) and in maintaining redox homeostasis. Therefore, the Nrf2 pathway has been implicated as a potential drug target in the prevention/therapy of neurodegenerative conditions (14).

Most Nrf2 activators are vegetable/spice constituents (15), whereas only two types of fatty acids contained in foods of animal origin exhibited such ability: the n-3 PUFAs (DHA and EPA) (16) and the *cis*$_9$, trans$_{11}$ isomer of conjugated linoleic acid (CLA) (17, 18). CLA is the collective name used for indicating two geometric and positional isomers of linoleic acid (C18:2), namely, *cis*$_9$, trans$_{11}$ and trans$_{10}$, *cis*$_{12}$ representing approximately 90% and 10% (respectively) of the total amount of CLA naturally found in ruminant foods (19). Notably, dietary supplementation with a synthetic CLA mixture containing an equimolar amount (50:50) of these two isomers produced several beneficial effects (anti-carcinogenic, anti-diabetic, anti-inflammatory) in animal models (20), although its neuroprotective potential in vivo has not been investigated yet. In this context, previous studies indicating the ability of synthetic CLA to improve Nrf2-mediated defenses in nonbrain organs of MRL/*lpr* mice (21, 22) led us to hypothesize that its dietary supplementation might alleviate neurodegenerative signs in old/diseased MRL/*lpr* mice. The infrequent use of MRL/*lpr* mice in the evaluation of neuroprotective drugs and the lack of data on the age-dependent alteration of redox-sensitive pathways (Nrf2, autophagy, astrogliosis) in their brain tissue prompted us to evaluate the neuroprotective ability of synthetic CLA.

The first aim of the present study was to investigate the association of age-dependent decline of redox homeostasis with the progression of neurodegeneration in MRL/*lpr* mice. A second set of experiments addressed the question of whether dietary supplementation with a synthetic CLA mixture exhibits neuroprotective effects.

MATERIALS AND METHODS

Materials

Reagents were from Sigma-Aldrich (St. Louis, MO) or Serva (Serva electrophoresis GmbH, Heidelberg, Germany). Synthetic mixture containing 76% CLA (composed by a 50:50 ratio of trans$_{10}$, *cis*$_{12}$ and *cis*$_9$, trans$_{11}$ isomers; 38.5 and 37.4%, respectively), was purchased from Tonalin (Natural Inc.). The list of primary antibodies used is shown in supplemental Table S1.

Animals and CLA treatment

MRL/*lpr*, MRL/+, and BALB/c mice, originally purchased from the Jackson Laboratory (Bar Harbor, ME), were bred under standard conditions of temperature and light in pathogen-free conditions at our animal facility (Accreditation no. DM.161/99). Animals were maintained in a temperature-controlled room (24°C), under a 12 h light/dark cycle in pathogen-free conditions with free access to drinking water and standard chow (Mucedola 4RF21-GLP; composition is shown in supplemental Tables S2, S3). All experimental procedures were carried out in accordance with national and international policies (EU Directive 2010/63/EU for animal experiments); the experiments were approved by the Institutional Review Committee and authorized by the Italian Ministry of Health (n. 833/2015-PR).

Two groups (n = 10, each; 5 females and 5 males), consisting of sex-matched 8- to 10-week-old MRL/*lpr* (Young) or 20- to 22-week-old MRL/*lpr* (Old) mice, were used to evaluate the age-dependent deterioration of brain redox status. A third group (n = 12), consisting of sex- and body weight-matched mice, (6 females and 6 males ranging from 16 to 17 weeks of age; average body weight 37.1 ± 3.6 g) was used to investigate the neuroprotective ability of CLA. MRL/*lpr* mice were administered with an oral dose of a 76% synthetic CLA (30 mg; 650 mg·kg^{-1}) daily for 5 weeks (CLA+Old). Old mice (diseased/positive control) and age-matched BALB/c mice (18–21 weeks old; n = 6 each) were used as healthy/negative controls. In the present study, the dose of synthetic CLA used, upon normalization to the body surface (23), was similar to that of our previous studies (21, 22), and when converted to a human equivalent dose (3.1 g/day), its amount was comparable to that utilized in clinical studies (24).

Sample preparation

After euthanasia, blood, brain, liver, and spleen samples were collected from each mouse. Serum was prepared and stored at −80°C until the evaluation of autoantibody and redox status. Spleen was blotted on filter paper and weighed. Liver was cut into pieces and stored at −80°C for subsequent analyses of markers of redox status/oxidative stress.

Brains were quickly removed, thoroughly washed in an ice-cold saline solution, blotted on filter paper, and placed on ice. Brain hemispheres were then separated and dissected into two main

sections, containing the cerebral cortex, the hippocampus, and the subicular cortex. The prefrontal cortex (the anterior first third) from both hemispheres was used for RT-PCR analysis and the evaluation of redox status/oxidative stress. The posterior brain section from one hemisphere, containing the remaining two-thirds of cerebral cortex (subicular cortex, hippocampus, corpus callosum, and lateral ventricle) was immediately fixed in Bouin's solution (48 h at room temperature), dehydrated, embedded in paraffin, and used for immunohistochemical analysis. Brain cortex from the other hemisphere was cut into small slices, snap frozen in liquid nitrogen and stored at −80°C until its use in enzymatic, immunoenzymatic (Western blotting), and fatty acid analyses.

Protein extraction

Prefrontal cortex extracts prepared with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 8, supplemented with protease inhibitor cocktail 1:200, v/v) were used to determine the level of oxidative stress markers [nitrosylated proteins, N-Tyrs; carbonylated proteins, PCs). Cytosolic and nuclear protein extracts from either anterior or posterior brain sections were prepared according to previously published protocols (25). Cytosolic extracts were used for the analysis of markers of redox status (total GSH, GSH, GSSG), the activity of the enzymes GSR and G6PD, and the immunoenzymatic evaluation (Western blotting) of proteins involved in autophagy [Beclin 1, microtubule-associated protein light chain 3 (LC3), p62-sequestrosome-1 (p62)] and in the Nrf2 pathway (GSR, G6PD). Nuclear extracts were used for detecting Nrf2 translocation into the nucleus. Cytosolic extracts prepared from liver tissue were used to evaluate GSH and GSSG concentration and the extent of oxidative stress (PC levels). Protein concentration in the different extracts was measured using the Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

Evaluation of systemic autoimmunity progression

Splenomegaly and autoantibody production have been widely used as markers to monitor murine systemic autoimmunity progression. The spleen weight of the individual animals was expressed as a percentage of total body weight [spleen weight (g) × 100/body weight (g)]. The titres of anti-double stranded DNA and anti-tissue transglutaminase (anti-tTG) IgGs in mouse blood sera were quantified by ELISA according to published methods (26).

Evaluation of antioxidant potential and redox status

GSH and GSSG concentrations in blood plasma, brain and liver were quantified using the 5,5′-dithionitrobenzoic acid (DTNB)-GSSG reductase recycling assay. Brain and liver sample aliquots supplemented with 1-methyl-2-vinylpyridinium trifluoromethanesulfonate were used for the evaluation of GSSG. Total GSH (GSH+GSSG) and GSH and GSSG concentrations, upon normalization to the protein content, were expressed as nmol·mg^{-1} min^{-1}. Total GSH and GSH:GSSG ratio were used to indicate antioxidant potential and redox status modification, respectively (27). Blood serum antioxidant potential was also evaluated by using the Antioxidant Assay Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. A calibration curve was prepared with a known Trolox amount, and antioxidant capacity was expressed as Trolox-equivalent antioxidant capacity (TEAC; mM).

Measurement of oxidative stress markers

PC and N-Tyr concentration was measured to evaluate the extent of oxidative modifications to proteins. PC concentration was evaluated in serum, brain, and liver extracts by using a standard procedure (28) and it was expressed as nmol of PC mg^{-1} protein. N-Tyr content in brain homogenates was quantified by ELISA according to a published protocol (29). Brain samples were diluted (1:1500, 1:3000, 1:6000), and data were expressed as Optical Density mg^{-1} of protein.

Activity of phase 2 enzymes

GSR and G6PD belong to the group of Nrf2-activated antioxidant/detoxifying enzymes (phase 2) responsible for the maintenance of GSH homeostasis. Their activities were spectrophotometrically assayed in cytoplasmic extracts using standard protocols (30, 31) and, upon normalization to the protein content, they were expressed as nmol of NADPH mg^{-1} min^{-1} or as IU/mg^{-1} min^{-1}, respectively.

Immunoenzymatic analysis

Western blot analysis was performed on posterior brain sections. Briefly, cytoplasmic or nuclear protein extracts (15 μg) were fractionated by 10% or 12.5% SDS-PAGE and then electroblotted onto PVDF membranes (Immobilon™, Merck-Millipore, Darmstadt, Germany). The membranes were then incubated with primary antibody dilutions (see supplementary Table S1), followed by incubation with the appropriate peroxidase-conjugated goat anti-rabbit IgGs (1:3000 Santa Cruz Biotechnology, Dallas, TX, or Cell Signaling Technology, Danvers, MA) (1 h at room temperature). Immunocomplexes were visualized by enhanced chemiluminescence and autoradiography according to the manufacturer's instructions (Western Blotting Luminol Reagent, Santa Cruz Biotechnology) and quantified by densitometric analysis using the ChemiDoc XRS system (Bio-Rad). Protein expression data were quantified with Quantity One Software (Bio-Rad) and normalized using either β-actin or Lamin B1.

Evaluation of neuronal degeneration

Fluoro-Jade B (FJB) staining, a selective and highly sensitive procedure for identification of degenerating nerve cells (32), was carried out to compare the degree of neurodegeneration in Old mice and healthy control mice (BALB/c) to examine the potential beneficial effects resulting from CLA supplementation (CLA+Old group). Brain sections were stained according to Ballok et al. (4) and then mounted with DPX (Sigma-Aldrich, St. Louis, MO). The results were observed using epifluorescence with blue excitation light and an FITC filter (λ_{ex}480 nm; λ_{em} 525 nm). Serial brain sections prepared from BALB/c, Old, and CLA+Old mice were processed for detecting glial fibrillary acidic protein (GFAP) expression, used as marker of gliosis (33). GFAP-immunostaining was carried out according to a previously published protocol (34). Briefly, rehydrated sections were incubated (overnight at 4°C) with anti-GFAP IgG followed by 2 h (at room temperature) exposure to biotinylated anti-rabbit IgG. Immunocomplexes were visualized using the VECTASTAIN Elite ABC Kit (Vector Laboratories) according to the manufacturer's instructions. Slices were counterstained with hematoxylin, dehydrated, clarified, and mounted. Images were observed and acquired by Kontron Elektronik Imaging System Ks300 (Zeiss). Images were acquired at ×200 magnification using AxioVision 4.8 software, and the number of GFAP-positive (GFAP+) cells was expressed as n = GFAP+ cells /0.1 mm^2. At least 10 sections were imaged for each mouse. The number of hypertrophic astrocyte processes was counted using ImageJ software and expressed as the number of (GFAP+ processes/cells/0.1 mm^2).

Brain fatty acid profile

Fatty acids were extracted and trans-methylated according to standard methods (35, 36). Fatty acid methyl esters were separated

on an HP-Innowax column (30 m×0.25 mm×0.25 μm Agilent J&W) on an Agilent 7890A/5975C GS-MS instrument. Fatty acid identification was achieved by the comparison of their mass spectra with those contained in the Wiley7, NIST05 Library and by injection of authentic standards (Sigma-Aldrich).

Quantitative real-time PCR analysis

Phase 2 enzyme (GCL and GSR) mRNA level in brain cortex of Young, Old, and CLA+Old mice was measured as previously described (18). The raw cycle threshold values (Ct values) obtained for target genes were subtracted from the Ct value obtained for the reference gene. The final graphical data were derived from the $R = (E_{target})^{\Delta Ct\_target\ (control\ -\ sample)} / (E_{ref})^{\Delta Ct\_ref\ (control\ -\ sample)}$ formula. The Universal Probe Library Assay Design Center (Life Science, Roche) was used for designing primers (supplementary Table S4).

Graphic and statistical analyses

All the graphic and statistical analyses were conducted using the GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA) and one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$).

RESULTS

CLA pretreatment inhibits systemic autoimmunity and oxidative stress signs in the blood of Old mice

In preliminary experiments, systemic autoimmunity (splenomegaly, anti-double stranded DNA, double stranded DNA, and anti-tissue transglutaminase IgG titer) and redox status markers (TEAC, total GSH, PC) were evaluated in the blood serum of MRL/*lpr* mice to confirm the late onset of autoimmune signs in MRL/+ mice (2), the beneficial effect of synthetic CLA (20, 21) and to investigate the similarities between MRL/+ and BALB/c mice.

The level of autoimmunity markers found in BALB/c and MRL/+ was similar to that measured in Young animals. They were significantly increased in Old mice in comparison with Young samples ($P < 0.01$) and these modifications were almost completely inhibited by CLA pretreatment (supplemental Fig. 1A, B). Interestingly, significantly higher TEAC and total GSH values were measured in the blood serum of BALB/c animals compared with those found in MRL/+ mice ($P < 0.05$). Total GSH, TEAC, and PC concentration were markedly altered in Old animals in comparison with Young mice ($P < 0.05$), while in CLA+Old animals, their values were similar to those measured in Young mice (supplemental Fig. 1C–E).

In addition, as liver plays a central role in the maintenance of redox homeostasis and detoxification, GSH, GSSG, and PC concentration were measured in liver homogenates of MRL/*lpr* mice to corroborate the antioxidant ability of CLA (21). As shown in Supplemental Fig. S2, the alteration of the redox status in Old animals was indicated by the significant decline of total GSH content ($P = 0.028$), GSH/GSSG ratio ($P = 0.02$), and the marked increase of PC concentration ($P < 0.001$) in comparison with Young animals. Interestingly, redox status deterioration was significantly inhibited by CLA pretreatment ($P < 0.01$).

Hyperactivation of Nrf2 pathway in the brain of Old mice

Because redox status perturbation in neurodegenerative disorders is frequently accompanied by the activation of the Nrf2 pathway (9), we preliminarily evaluated its hyperactivation in the brain tissue of Young and Old mice. The brains of Old mice exhibited significantly higher activity of phase 2 enzymes (GSR and G6PD) compared with that of Young mice ($P < 0.005$) (**Fig. 1A**, B). Moreover, samples from Old mice exhibited markedly higher GSR, G6PD, and Nrf2 protein levels, in cytoplasmic and nuclear extracts, respectively, compared with Young animals ($P < 0.001$) (Fig. 1C).

CLA pretreatment prevents age-associated disruption of brain redox homeostasis

GSH and GSSG concentrations in the different animal groups (Young, Old, and CLA+Old) were then measured to determine the age-dependent deterioration of brain redox status and the beneficial effect of CLA supplementation. Total GSH (GSH+GSSG) and GSH:GSSG ratio were used to describe both its antioxidant potential and redox environment, respectively. Significantly lower concentrations of total GSH were found in the brains of Old animals (64.6 ± 10.0) in comparison with Young mice (81.5 ± 11 nmol/mg protein) ($P = 0.006$), and this age-associated decline was almost completely inhibited by CLA pretreatment (CLA+Old) (**Fig. 2A**). Similarly, the significant decrease of the GSH:GSSG ratio ($P < 0.001$) in Old animals was effectively rescued in CLA-treated animals (Fig. 2B). PC and N-Tyr concentrations were measured to determine the age-dependent progression of oxidative modification of proteins in the brain tissue and the protective effect of CLA in Old mice. Significantly higher PC and N-Tyr amounts were measured in Old (15.3 ± 6.4 nmol mg^{-1} or 22.6 ± 1.8 OD mg^{-1}, respectively) compared with Young mice (5.0 ± 2.8 nmol mg^{-1} or 10.1 ± 0.8 OD mg^{-1}) ($P < 0.05$), and their accumulation in the brain tissue was significantly reduced by CLA treatment (9.6 ± 2.4 nmol mg^{-1}, 7.4 ± 0.7 OD mg^{-1}, $P < 0.01$) (Fig. 2C, 2D).

CLA pretreatment down-regulates the compensatory hyperactivation of Nrf2 pathway in the brains of Old mice

On the basis of preliminary findings showing the association between oxidative stress and the hyperactivation of the Nrf2 pathway in the brains of Old mice, the ability of CLA to downregulate such compensatory mechanisms was investigated next. **Figure 3** shows data indicating activity, mRNA, and protein levels of phase 2 enzymes measured in the brains of Young, Old, and CLA+Old mice. The G6PD and GSR activity found was approximately twice as high (70.9 ± 11.7 IU/mg/min and 78.8 ± 22.2 nmol NADPH mg^{-1} min^{-1}) in Old animals as that measured in Young mice (44.2 ± 16.3 and 51.2 ± 12.0, respectively) ($P < 0.01$), while the activity found in CLA+Old homogenates was comparable to that found in Young animals (Fig. 3A). The significantly higher GCL mRNA and G6PD protein levels measured in the Old group ($P < 0.001$) was inhibited by CLA pretreatment (Fig. 3B, 3C). In addition, although the GSR

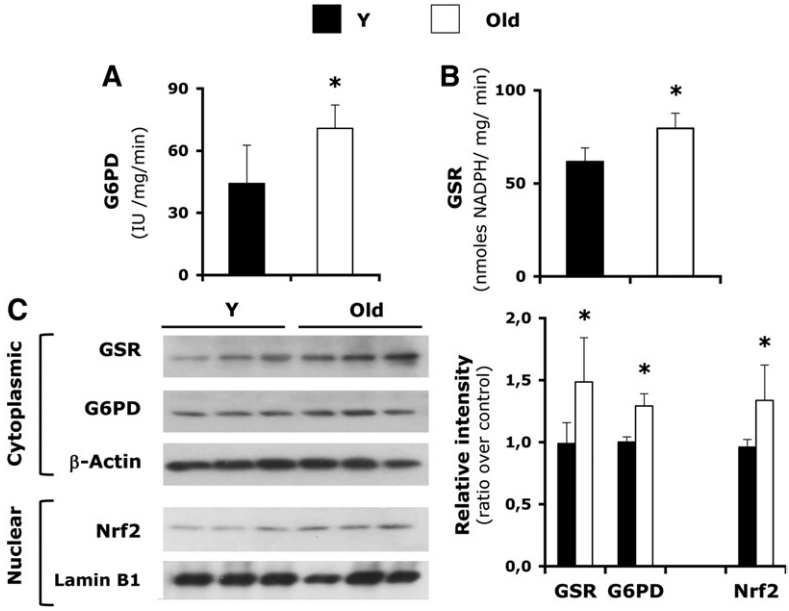

Fig. 1. Age-dependent hyperactivation of Nrf2 pathway in MRL/*lpr* brain. GSR (A) and G6PD activity (B) measured in brain tissue samples of Young (Y) and Old mice. The data are expressed as mean ± SD from triplicate analyses (n = 6 animals/group). Representative immunoblot of GSR, G6PD in cytoplasmic protein extracts, and Nrf2 expression in both cytoplasmic and nuclear protein extracts (C, left panel). The bands were quantified using densitometric analysis and normalized to β-actin and Lamin B1, respectively. Values are expressed as average fold increase (± SD) compared with Young (C, right panel). * $P < 0.05$.

content in the brains of Old mice did not differ significantly from that measured in Young animals (at both the protein and mRNA level), its level was still significantly decreased in CLA+Old animals (Fig. 3B, 3C) ($P < 0.01$).

The neuroprotective activity of CLA is not associated with its incorporation in brain lipids

The fatty acid profile was examined in Old and CLA+Old brain cortex to investigate whether the ability of CLA to modulate the Nrf2 pathway is associated with its bioaccumulation in the brain membrane. Our data indicate that CLA content in brain tissue is not altered by its dietary supplementation (supplemental Table 5).

CLA pretreatment inhibits age-associated brain activation of autophagy pathway

As autophagy hyperactivation, similarly to Nrf2, occurs during neurodegenerative disorders to compensate for increased protein damage, the levels of genes and proteins involved in the autophagic pathway (Beclin 1, p62, LC3) were determined in the brain tissue of Young, Old, and CLA+Old mice. **Figure 4A** shows that the levels of p62 and Beclin 1 mRNA tend to increase in Old when compared with Young mice, but do not reach statistical significance between the two groups. After the densitometric analysis of Western blotting film, the analysis of proteins, indicated that autophagy-related markers in Old mice were markedly higher (Fig. 4B) compared with those found in Young mice. In particular, Beclin 1 and p62 expression in the brain homogenates of the Old group was 46% and 36% higher, respectively, than that found in the Young group. Interestingly, p62 content in Old brain tissue was markedly higher compared with Young mice ($P < 0.05$) but the great variability of Beclin 1 protein in different brain samples did not allow the achievement of statistical significance between the two groups (Fig. 4B). Notably, mRNA and protein levels of the considered autophagy markers in CLA+Old animals were significantly reduced, and their levels were comparable to those measured in Young mice (Fig. 4A, 4B) ($P < 0.001$).

CLA pretreatment inhibits neuronal degeneration in Old mice

Our goal in the second part of the study was to examine the preventive effects of synthetic CLA in Old mice. In these experiments, the extent of neurodegeneration markers (FJB staining, GFAP expression) measured in the brain

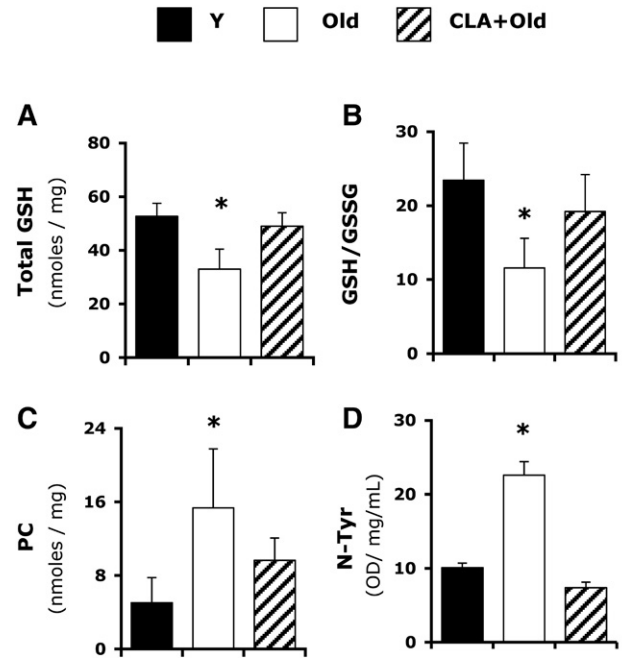

Fig. 2. CLA pretreatment inhibits age-dependent oxidative stress in MRL/*lpr* mice brain. Brain total GSG (GSH+GSH) (A), redox status (GSH/GSSG ratio) (B), and PC (C) or N-Tyr (D) levels were measured from brain cortex samples of the different groups (Young, Old, CLA+Old). The data are expressed as mean ± SD from triplicate analyses (n = 6 animals/group). * $P < 0.05$. Y, Young.

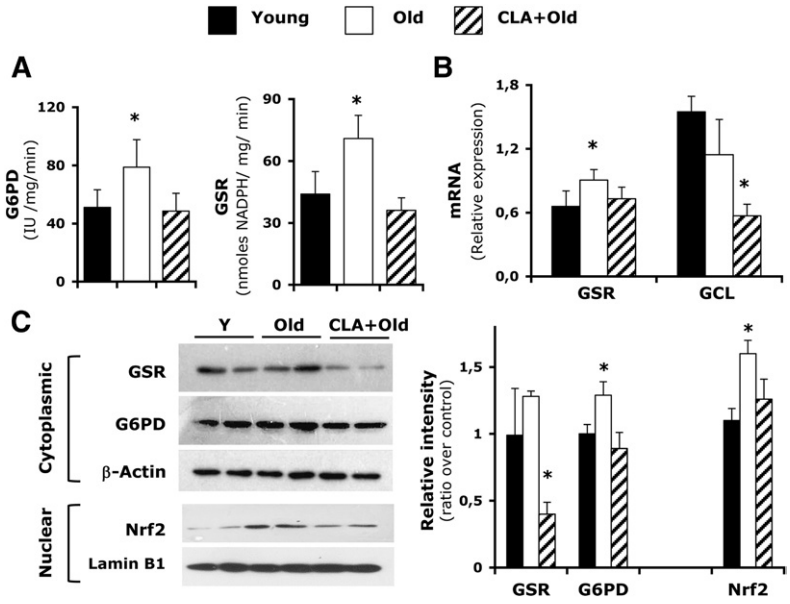

Fig. 3. CLA pretreatment prevents Nrf2-hyperactivation in brain of MRL/*lpr* mice. GSR and G6PD activity (A), GSR and GCL mRNA levels (B), and GSR and G6PD protein levels (C) were measured in brain cortex samples of three experimental groups (Young, Old, CLA+Old). The data are expressed as mean ± SD from triplicate analyses (n = 6 animals/group). * Significantly different ($P < 0.05$) from Young samples. Representative immunoblot of GSR, G6PD, and Nrf2 expression in cytoplasmic and nuclear protein extracts, respectively (C, left panel). The bands were quantified using densitometric analysis and normalized to β-actin and Lamin B1, respectively. Values are expressed as average fold increase (± SD) compared with Young (C, right panel). * $P < 0.05$. Y, Young.

cortex of CLA+Old was compared with that found in Old and BALB/c mice (which were used as diseased and healthy controls, respectively). Interestingly, the suitability of BALB/c mice utilization as healthy controls was supported by results showing the significantly lower number of FJB+ (FJB-positive) cells in their brain cortex compared with Young mice ($P = 0.02$) (supplemental Fig. S3).

As expected, stronger FJB staining was evidenced in the cortical sections of Old brain samples (**Fig. 5B**) compared with healthy animals (Fig. 5A), while the fluorescence signal was completely inhibited in CLA+Old mice (Fig. 5C). In detail, the FJB+ cell number in Old animals was approximately 20 times higher (61.5 ± 12.3 cells/ 0.1 mm^2) than that found in a healthy brain control (BALB/c mouse) (3.0 ± 1.8 cells/0.1 mm^2), and its level in CLA+Old mice was sharply lower in comparison to Old mice ($P < 0.005$). The number of FJB+ cells in CLA+Old was found to be significantly higher than that measured in BALB/c mice (12.2 ± 3 cells/0.1 mm^2) ($P < 0.05$) (Fig. 5D).

CLA prevents severe reactive astrogliosis in Old mice

GFAP immunostaining was carried out to evaluate astrogliosis severity in Old animals and the possible healing effect resulting from CLA pretreatment. As shown in **Fig. 6A–**C, the presence of GFAP+ cells (active astrocytes) was observed in different areas of the mouse brain in all experimental groups (in particular, the corpus callosum, cerebral ventricle hippocampus, and the subicular cortex). In addition, the occurrence of severe astrogliosis was indicated by the presence of glial scars in different brain areas of Old animals (Fig. 6D) but not in CLA+Old or healthy animals (Fig. 6A, C, right panels). In particular, the panels in Fig. 6D denote the presence of glial scars in the corpus callosum of Old mice (at two different magnifications). Moreover, the number of GFAP+ cells in Old mice (85 ± 8) was significantly higher than that measured in BALB/c or CLA+Old mice (67 ± 6 or 71 ± 3 cells/ 0.1 mm^2, respectively) ($P < 0.05$) (Fig. 6E). In addition, the differences between the brains of healthy (BALB/c) and pathological mice (Old) also consisted of increased staining density and resulted in an altered astrocyte shape (higher number of hypertrophic GFAP+ processes/cell). In fact, the number of GFAP+ processes in Old animals was approximately twice as high (6.0 ± 0.5) compared with BALB/c mice (3.5 ± 0.2) ($P < 0.001$), while the number in CLA+Old brain was comparable to that of healthy controls (3.3 ± 0.3) (Fig. 6F). Finally, Western blotting experiments were carried out to confirm immunohistochemical data. As shown in Fig. 6G, Old brain samples contained significantly higher GFAP protein levels compared with healthy/BALB/c animals (6-fold higher $P < 0.01$).

DISCUSSION

Neurodegenerative disorders are among the most important health problems of contemporary society and animal models of such diseases are gaining increasing importance as tools for improving the knowledge of the underlying pathogenic mechanisms and the evaluation of therapeutic treatment. Overall, the results presented herein show, for the first time, that MRL/*lpr* mouse is a valuable animal model for studying age-dependent biochemical alteration because it exhibits early brain oxidative stress, already evident at 20 weeks of age. Also, this is the first study indicating that the association of the neuroprotective effect of CLA and the inhibition of oxidative stress-activated compensatory mechanisms (astrogliosis, macroautophagy, and Nrf2) might be caused by the activation of an adaptive response.

The accumulation of degenerating neurons in the cortex of Old mice, as visualized by FJB staining, is consistent with literature reports (4) and its positive association with systemic autoimmunity marker levels (splenomegaly and autoantibody titer) in Old mice is consistent with a causal link between immunological alterations and neurodegenerative processes (3). This is the first time that an astrocyte activation

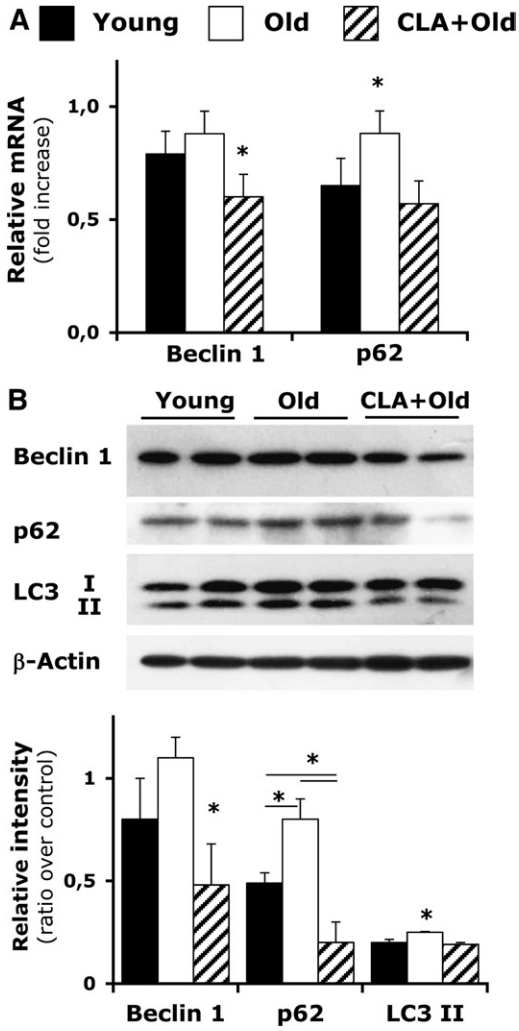

Fig. 4. CLA pretreatment prevents autophagy-hyperactivation in brain of MRL/*lpr* mice. Beclin 1 and p62 mRNA level in brain tissue samples of the different experimental groups (Young, Old, CLA+Old) (A). Results are expressed as mean ± SD (n = 6 animals/group). B, top panel: Representative immunoblots of Beclin 1, p62, and LC3 protein levels in brain tissue samples of Young, Old, and CLA+Old group. The bands were quantified using densitometric analysis and normalized to β-actin. Values are expressed as average fold increase (± SD) compared with Young (B, bottom panel). $^*P < 0.05$.

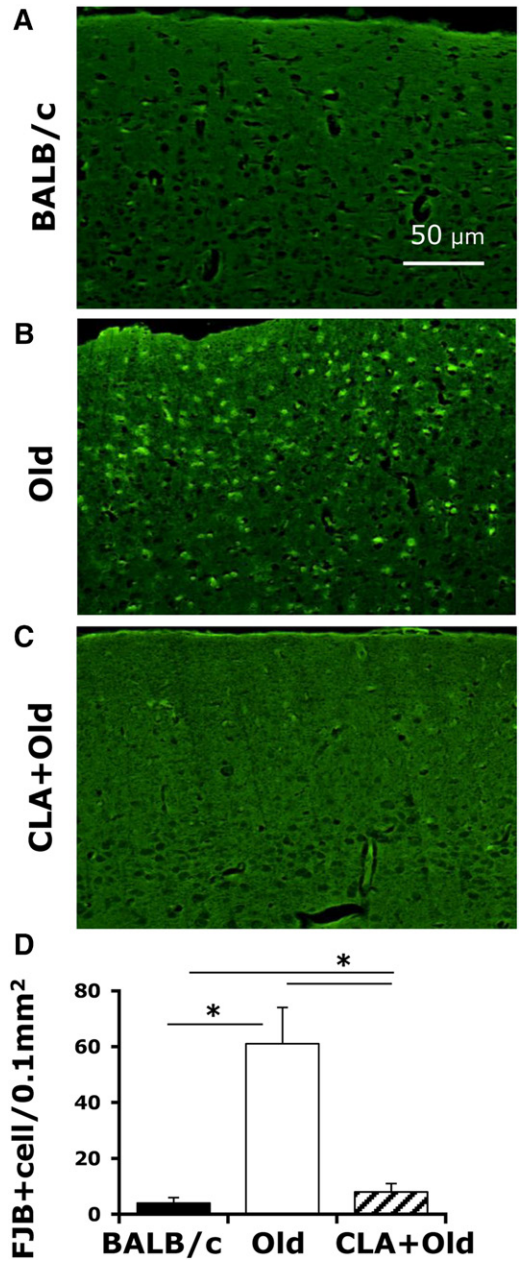

Fig. 5. CLA pretreatment prevents neuronal degeneration in Old mice. Representative photomicrographs showing FJB staining in the cortical region of BALB/c (healthy control) (A), Old (B), and CLA-pretreated mice (CLA+Old) (C). D: The number of FJB+ cells/0.1 mm^2 was counted and expressed as mean ± SD (n = 5 animals/group). $^*P < 0.05$.

has been demonstrated in aged MRL/*lpr* mice, as indicated by intense GFAP immune staining, cell hypertrophy, and scar formation (37).

Oxidative stress plays a relevant role in the progression of neurodegenerative disorders (1), but its causal link with neuronal degeneration in MRL/*lpr* mice had been never evaluated. GSH plays a central role against neuroinflammation (38), and data showing age-associated decline of the antioxidant potential (total GSH, TEAC) and redox status alteration (GSH:GSSG ratio) in the blood, liver and brain of MRL/*lpr* mice are consistent with previous results (22) and with studies showing its preventive role in autoimmune and neurodegenerative diseases (39). In addition, an increased PC concentration in the brain and liver, along with PC and N-Tyr accumulation in the blood, support the role of oxidative stress signaling in the etiology of neurodegenerative disorders (10, 40).

There is considerable evidence showing that neuroprotective effect displayed by several food constituents (e.g., curcumin, diallyl trisulfide) may result from the activation of an adaptive response (9). This is the phenomenon whereby the exposure to a subtoxic level of stress elicits preconditioning and enhances cells protection against further toxic insults by the stimulation of distinct yet interconnected defensive mechanisms (Nrf2, autophagy, Ubiquitin-proteasome) (9, 41). Autophagy and Nrf2 hyperactivation in the brain of Old mice indicate the brain effort

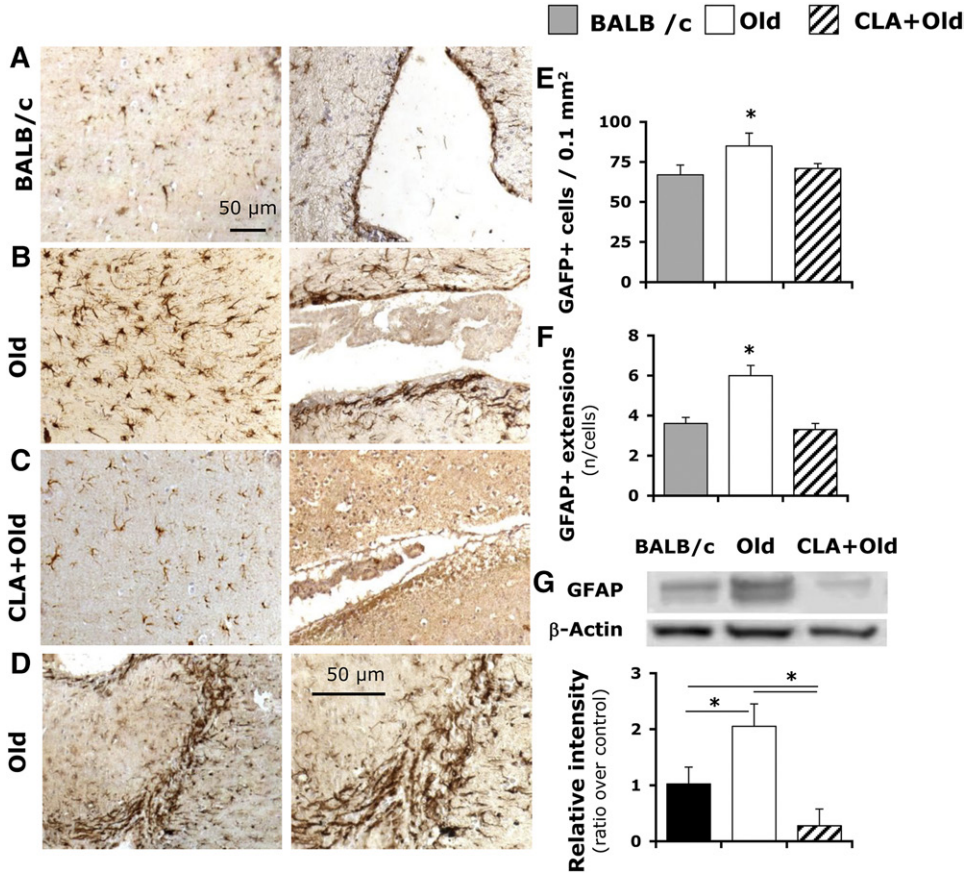

Fig. 6. CLA pretreatment prevents severe astrogliosis in Old mice. Representative photomicrographs of GFAP staining of the hippocampal (left) and lateral ventricle (right) regions of BALB/c (A), Old (B), and CLA+Old (C) animals are shown. Glial scar presence in the corpus callosum of Old mice at two different magnifications (D). The number of GFAP+ cells/0.1 mm^2 was counted and its number was expressed as mean ± SD (n = 5 per group) (E). The number of GFAP+ hypertrophic processes/cell/0.1 mm^2 was counted and its number was expressed as mean ± SD (n = 5 per group) (F). Typical immunoblot of GFAP protein expression in brain extracts is shown (G, upper panel). *Significantly different ($P < 0.05$) from BALB/c samples. The bands were quantified by densitometric analysis (from at least duplicate experiments) and normalized using β-actin. The values are expressed as average fold increase (± SD) compared with control (G, lower panel). * $P < 0.05$.

to balance excessive ROS yield or cell aggregates/dead organelles (13, 42). The concomitant increase of oxidative stress markers in brain samples of Old mice points out that the deterioration of the brain redox status might not be efficiently compensated by the upregulation of such defense mechanisms. Here, we show the ability of CLA to improve the redox status in the brain of Old mice as well (20, 21) while providing evidence of a possible biochemical mechanism underlying its neuroprotective ability. Indeed, the negative association between brain antioxidant defenses and the activation of compensatory mechanisms (Nrf2, autophagy) in different MRL/*lpr* groups shows that CLA acts as an indirect antioxidant by decreasing tissue vulnerability to oxidative challenge through the activation of an adaptive response. Nrf2, under mild stress conditions, translocates into the nucleus and triggers the transcription of a wide array of antioxidant/detoxifying enzymes, which protect against exogenous stressing agents (43) or against the age-related decline of redox homeostasis (44). Accordingly, the preventive effect of dietary CLA against oxidative-stress and hyperactivation of Nrf2 pathway in the brain of CLA+Old mice is likely the result of its ability to improve antioxidant/detoxifying defenses via the activation of an Nrf2-mediated adaptive response. Such activation of this defensive mechanism by CLA, characterized by a hormetic/biphasic dose-response pattern (i.e., low-dose stimulation and high-dose inhibition), is not completely unexpected, and it is shared by n-3 PUFAs (DHA and EPA) (16, 45). Moreover, the ability of CLA and PUFA to trigger Nrf2 activity (16–18, 46), as well as their pro-oxidant (23, 47) and antioxidant effects (45, 48), are consistent with the hormetic effects of ROS on Nrf2 pathways (9).

On the other hand, the lack of association between CLA accumulation in the brain membrane of treated mice and its biological effects is consistent with a recent review reporting that the neuroprotective effects of EPA are not associated with its incorporation in brain tissue (49) and with research data showing low isomer bioavailability in the brain of rats receiving an acute dose (2 g) of CLA mixture (50). As the absence of measurable CLA amounts in the brain of CLA+Old mice can be the outcome of the low CLA dose used in our experimental conditions, we hypothesize that its

neuroprotective ability can be consequential to a systemic effect. The neuroprotective ability elicited by CLA supplementation is clearly multifactorial and the contribution of its entry/metabolism in MRL/*lpr* brain cannot be excluded.

In conclusion, we show for the first time the neuroprotective activity of synthetic CLA in an animal model of neuropsychiatric disease (the MRL/*lpr* mouse). The presented data suggest that the beneficial activity of CLA is mediated at least in part by its ability to improve systemic antioxidant and detoxifying defenses via the activation of the Nrf2 pathway. Moreover, on the basis of our previous studies indicating the ability of the cis_9 $trans_{11}$ CLA to activate this pathway (17, 18), it can be inferred that this isomer may be responsible for the neuroprotective activity of the CLA mixture. Further research is currently in progress to confirm this hypothesis.

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