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The oxidative damage and inflammation caused by pesticides are reverted by lipoic acid in rat brain

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ABSTRACT

We have previously demonstrated that the administration of low doses of dimethoate, glyphosate and zineb to rats (i.p. 1/250 LD50, three times a week for 5 weeks) provokes severe oxidative stress (OS) in specific brain regions: *substantia nigra*, cortex and hippocampus. These effects were also observed in plasma. Lipoic acid (LA) is considered an "ideal antioxidant" due to its ability to scavenge reactive species, reset antioxidant levels and cross the blood–brain barrier. To investigate its protective effect we administered LA (i.p. 25, 50 and 100 mg/kg) simultaneously with the pesticide mixture (PM) for 5 weeks. After suppression of PM administration, we evaluated the restorative effect of LA for a further 5 weeks. LA prevented OS and the production of nitrites + nitrates [NOx] caused by PM in a dose-dependent manner. The PM-induced decrease in reduced glutathione and α-tocopherol levels in all brain regions was completely restored by LA at both high doses. PM administration also caused an increase in prostaglandins E_2 and $F_{2\alpha}$ in brain that was reduced by LA in a dose-dependent fashion. Taking into account the relationship between OS, inflammation and apoptosis, we measured caspase and calpain activity. Only milli- and micro-calpain isoforms were increased in the PM-treated group and LA reduced the activities to basal levels. We also demonstrated that interrupting PM administration is not enough to restore the levels of all the parameters measured and that LA is necessary to achieve basal status. In our experimental model LA displayed a protective role against pesticide-induced damage, suggesting that LA administration is a promising therapeutic strategy to cope with disorders suspected to be caused by OS generators, especially in brain.

1. Introduction

The incidence of neurodegenerative diseases has shown a significant increase over the past few decades. Parkinson's disease (PD) is characterized by a gradual loss of dopaminergic neurons in the midbrain *substantia nigra* (SN) and is the second most common neurodegenerative disease disorder after Alzheimer's disease. A wide range of variables such as age, heredity and environmental factors has long been thought to be involved in the etiology of PD (Paolini et al., 2004; Dauer and Przedborski, 2003). Epidemiological studies have linked pesticide exposure to the incidence of many neurodegenerative disorders (Le Couteur et al., 1999; Kirby et al., 2001; Barlow et al., 2005; Patel et al., 2006). Inflammation and overproduction of free radicals – two factors undoubtedly involved in glial activation and programmed cell death – are characteristics found during the clinical progression of these neurodegenerative disorders.

Humans are exposed to complex mixtures of toxic compounds every day in their residential and occupational environments (Di Monte, 2003; Liu et al., 2003). In particular, agrochemicals are continuously used on a massive scale for global food production and persist as residues in food of both vegetal and animal origin, as well as in the air and water (Bolognesi and Morasso, 2000). Many of the most commonly used pesticides in the world lead to the generation of oxygenated and/or nitrogenated reactive species (ROS/RNS) which affects both the antioxidant levels in mammalian cells and the activity of the scavenging enzyme system (Banerjee et al., 1999; Barlow et al., 2005).

The central nervous system is highly sensitive to free radical damage for many reasons: it has high oxidative metabolic rate, high ratio of membrane surface area to cytoplasmic volume, enriched content of unsaturated lipids (mainly polyunsaturated fatty

Abbreviations: CASP, caspase-3; CALP, calpain; CC, cerebral cortex; DHLA, dihydrolipoic acid; D, dimethoate; G, glyphosate; HYP, hippocampus; LA, α-lipoic acid; MDA, malondialdehyde; [NOx], nitrate plus nitrite; OS, oxidative stress; PCs, protein carbonyls; PD, Parkinson's disease; PM, pesticide mixture; PUFAs, polyunsaturated fatty acids; RNS, reactive nitrogenated species; ROS, reactive oxygenated species; SN, *substantia nigra*.

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acids or PUFAs), elevated rate of free radical generation derived from neurotransmitters metabolism, and poor radical scavenging mechanisms (Chong et al., 2005). Brain is characterized by extreme sensibility to pro-oxidative noxa as well as a high density of resting microglia (especially in the SN) and high iron levels compared to other tissues, These are other possible reasons why dopamine-containing neurons are particularly vulnerable to oxidative stress (OS) (Gao et al., 2003). We have previously demonstrated that simultaneous intoxication with various agrochemicals in low doses produced systemic oxidative stress (OS) affecting the brain, liver and kidney, and that this condition is clearly reflected in peripheral plasma (Astiz et al., 2009a,b,c). Our laboratory studied three of the most commonly used pesticides worldwide: zineb (Z), glyphosate (G), and dimethoate (D), either alone or in combination. Zineb (zinc ethylene-bis-dithiocarbamate) is a widely used contact fungicide (Heikkila et al., 1976; Fitsanakis et al., 2002), glyphosate (N-phosphonomethyl-glycine) is a systemic herbicide (Daruich et al., 2001; Beuret et al., 2005) and dimethoate (O,O-dimethyl-S-methyl-carbamoyl-methyl phosphorodithioate) is the most extensively used organophosphorus insecticide of systemic action (Sharma et al., 2005a,b).

α-Lipoic acid (LA) is a well-known cofactor of multi-enzymatic complexes which catalyzes the oxidative decarboxylation of α-ketoacids. LA satisfies almost all the criteria of an "ideal antioxidant", being readily absorbed from the diet and rapidly converted to its redox couple, dihydrolipoic acid (DHLA) in many tissues. Both LA and DHLA effectively quench a number of free radicals ($RO_2^{\cdot}$, HOCl, $OH^{\cdot}$, ONOOH, $O_2^{\cdot -}$, singlet oxygen) and this antioxidant activity can be displayed in both lipid and aqueous-cell compartments. They can also chelate redox active transition metals (Fe, Cu), avoiding the production of ROS by the Fenton and Häber-Weiss reactions (Maczurek et al., 2008). Moreover, it is well-known that DHLA interacts with other antioxidants such as glutathione, ascorbate, ubiquinol and indirectly with α-tocopherol, preventing their oxidation (Marangon et al., 1999; Holmquist et al., 2007).

Since exposure to agrochemicals is able to induce OS and there is a clear connection among this condition, inflammation (glial activation) and programmed cell death, we investigated: (i) whether the OS condition induced by the administration of low-doses of pesticides could be mitigated (or even reverted) by different doses of LA; (ii) whether after suppression of pesticide exposure (wash-out period), LA is necessary to revert the oxidative damage previously caused to lipids and proteins; and (iii) the effect of LA administration on inflammatory and programmed cell death biomarkers in brain.

LA may be a good candidate as an antioxidant agent for using in neurodegenerative diseases in view of the proven experimental evidence of its broad scavenger properties (Bilska and Włodek, 2005), its ability to efficiently cross the blood–brain barrier (Gilgun-Sherki et al., 2001) and its long-term biosafety (Cremer et al., 2006; Petersen Shay et al., 2009). The present findings add current knowledge on the protective use of LA to prevent the neurotoxic effects caused by environmental toxins.

2. Materials and methods

2.1. Chemicals

All chemicals used were of reagent grade and were obtained from Sigma Chemical Co. (CA, or Buenos Aires, Argentina) or Merck Laboratories (Darmstadt, Germany). The pesticides employed – zineb (Z), dimethoate (D), and glyphosate (G) – were of analytical grade and a gift from INTA (Instituto Nacional de Tecnología Agropecuaria, Castelar, Argentina).

2.2. Animals and treatments

Male Wistar rats weighing 190 ± 20 g (2 months old) with pathogen-free certified status were used. They were allowed to acclimatize for a week prior to commencement of the experiment. The rats were maintained under controlled temperature conditions

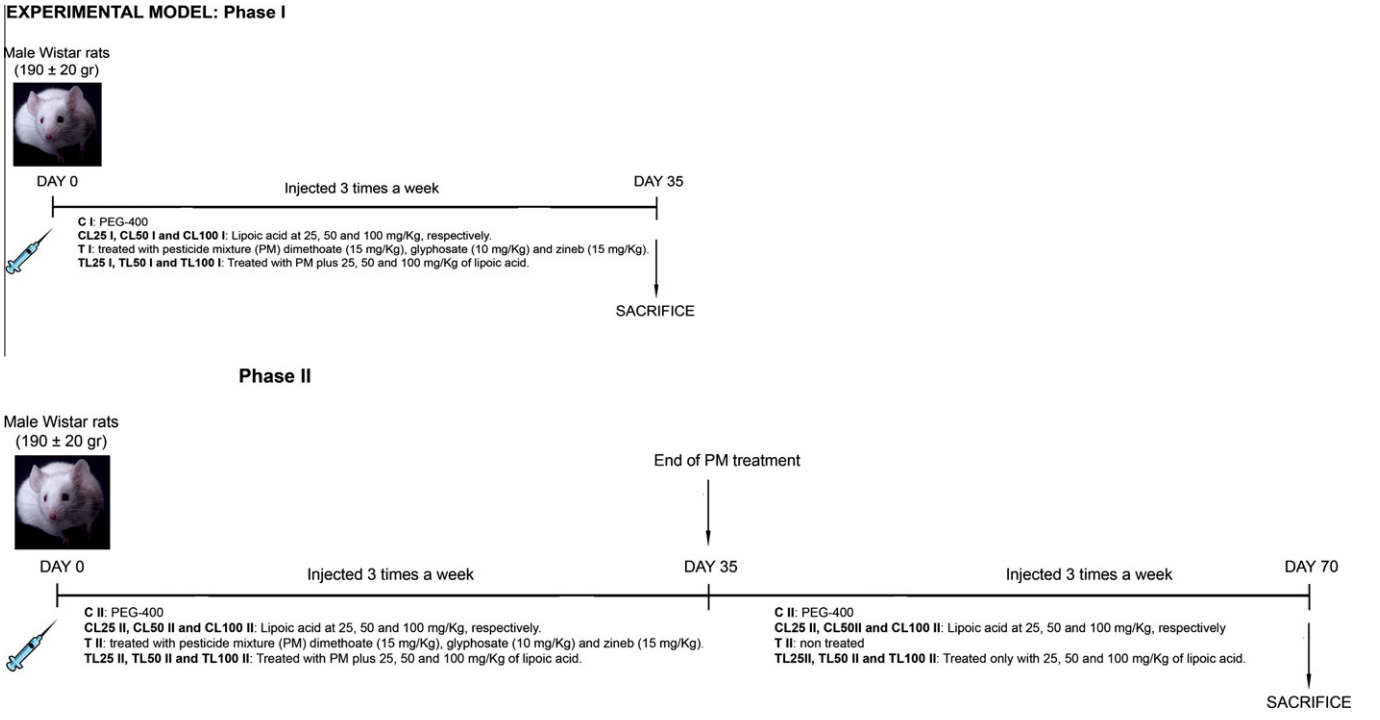

Fig. 1. Experimental design. The scheme shows the main parameters that characterized the experimental treatments for phases I and II (drugs, doses schedule drug, and treatment periods). Also frequencies of the treatments (i.p. injections), and the duration of the wash out period were indicated.

(25 ± 2 °C) and a normal photoperiod of 12 h dark/12 h light. They were fed with standard Purina chow from Ganave Co. (Santa Fe, Argentina) and given water *ad libitum*. Clinical examination and body weight evaluation were performed every week during the experiment. The animals were randomly divided into groups of six rats each.

The experiment was divided in two phases (I and II) schematized in Fig. 1. In phase I we evaluated the possible protective effect of LA administered intraperitoneally (i.p.) in three different doses (25, 50 and 100 mg/kg body weight (bw)) simultaneously with the sub-chronic administration of a pesticide mixture (PM) consisting of Z (15 mg/kg bw) plus G (10 mg/kg bw) plus D (15 mg/Kd bw), all injected i.p. and dissolved in the same vehicle (polyethylene–glycol 400, PEG-400). The animals were treated three times a week for 5 weeks. Phase I included the following experimental groups: C-I (treated i.p. with PEG-400); CL25-I, CL50-I and CL100-I (treated i.p. with LA, 25, 50 or 100 mg/kg bw, respectively), T-I (treated i.p. with PM); TL25-I, TL50-I and TL100-I (treated i.p. with PM and simultaneously with 25, 50 or 100 mg LA/kg bw).

In phase II (wash-out period) we carried out a comparative analysis of the biomarkers of oxidative damage, inflammation, and programmed cell death in rats administered with PM during the first 5 weeks followed by suppression of pesticide exposure for another 5 weeks vs. rats never exposed to PM, both groups of animals were treated with different doses of LA. Thus, phase II experiments included the following groups: C-II (treated i.p. with PEG-400 for 10 weeks); CL25-II, CL50-II and CL100-II (treated i.p. with LA 25, 50 or 100 mg/kg bw for 10 weeks); T-II (treated i.p. with PM alone for the first 5 weeks); TL25-II, TL50-II and TL100-II (treated i.p. with PM alone for the first 5 weeks and with 25, 50 or 100 mg LA/kg bw for the entire experimental period).

The dosing schedule (time and doses) of PM and LA were selected in view of previous experimental protocols from our and other laboratories (Sharma et al., 2005a,b; John et al., 2001; Beuret et al., 2005; Daruich et al., 2001; Fitsanakis et al., 2002; Cremer et al., 2006; Arivazhagan and Panneerselvam, 2000; Astiz et al., 2009a,b,c; Bagchi et al., 1995; Nielsen et al., 2006; Nagamatsu et al., 1995; Tang et al., 2007; Skalská et al., 2010). The NOAEL for rats was established at 60 mg LA/kg bw/day for long-term supplementation (Petersen Shay et al., 2009).

Animal maintenance and handling procedures were in accordance with the NIH guide for the care and use of laboratory animals (National Research Council, 1985). All the experiments were approved by the local Laboratory Animal Bioethics Committee, Facultad de Ciencias Médicas, UNLP, Argentina. The model employed was developed to simulate sub-chronic exposure to low doses of pesticide mixtures such as those incorporated from the general environment (Cory-Slechta, 2005). The animals were observed for behavioral performance and possible symptoms of intoxication. A veterinary evaluated the presence of miosis, mouth smacking, salivation or lacrimation, and the rats were also placed in an open field for observation of tremors and gait abnormalities (Moser et al., 2006). There was no significant evidence of differences between control and treated animals. Body weight gain and absolute brain and brain-to-body weight were determined for each experimental group.

2.3. Sample collection

At the end of the treatments (5 weeks for phase I and 10 weeks for phase II), animals were killed by rapid decapitation. Blood was collected using heparin as anticoagulant in ice-cold polypropylene tubes. Plasma was immediately prepared by centrifugation (4000*g*, 10 min) and then stored at −80 °C until analyzed. Brains were excised, washed, weighed and quickly homogenized in ice-cold phosphate buffer 140 mM pH 7.40, with 6 mM of EDTA, in a ratio of 10 mL buffer to 1 g of tissue. In another set of experiments the brains were dissected to obtain the cerebral cortex (CC) (ventromedial areas), hippocampus (HYP), and *substantia nigra* (SN), using the Paxinos and Watson (1998) as guide. In this case samples were stored at −80 °C under nitrogen atmosphere (to avoid peroxidation) until analyzed.

2.4. Analytical methods

2.4.1. Biomarkers of damage

In both phases of the experiment we evaluated the oxidative damage of lipids and proteins, inflammation biomarkers, and the activity of proteases involved in programmed cell death. Thiobarbituric acid reactive substances (TBARS) and protein carbonyls (PCs)

Table 1
Biomarkers of oxidative damage in plasma and in whole brain homogenate prepared from rats treated with the pesticide mixture and/or α-lipoic acid (LA).

Groups	Plasma			Whole brain		
	PCs	TBARS	[NOx]	PCs	TBARS	[NOx]
Phase I						
C-I	3.82 ± 0.14a	2.09 ± 0.11a	0.55 ± 0.03a	4.22 ± 0.15a	1.52 ± 0.06a	18.30 ± 0.75a
CL25-I	3.20 ± 0.08b	1.81 ± 0.12b	0.60 ± 0.10a	4.10 ± 0.12a	1.15 ± 0.06b	17.11 ± 0.62a
CL50-I	3.15 ± 0.13b	1.70 ± 0.15b	0.51 ± 0.12a	3.83 ± 0.11b	1.05 ± 0.10b	17.25 ± 0.16a
CL100-I	2.86 ± 0.20c	1.52 ± 0.05c	0.38 ± 0.05b	3.67 ± 0.15b	0.87 ± 0.06c	15.66 ± 0.12b
T-I	7.17 ± 0.21d	6.68 ± 0.25d	1.32 ± 0.08c	9.22 ± 0.25c	4.88 ± 0.20d	36.87 ± 0.25c
TL25-I	5.42 ± 0.16e	4.40 ± 0.20e	0.90 ± 0.05d	6.66 ± 0.31d	3.50 ± 0.12e	29.54 ± 0.31d
TL50-I	4.11 ± 0.20a	3.11 ± 0.17f	0.59 ± 0.10a	5.14 ± 0.20e	2.31 ± 0.18f	23.22 ± 0.19e
TL100-I	3.98 ± 0.15a	2.32 ± 0.24a	0.46 ± 0.08a	4.37 ± 0.14a	1.67 ± 0.14a	19.65 ± 0.22a
Phase II						
C-II	3.11 ± 0.08b	2.12 ± 0.06a	0.53 ± 0.05a	4.29 ± 0.16a	1.07 ± 0.10b	19.00 ± 0.21a
CL25-II	3.03 ± 0.11b	1.98 ± 0.15a	0.50 ± 0.10a	3.88 ± 0.13b	0.80 ± 0.05c	18.52 ± 0.17a
CL50-II	2.96 ± 0.21b	1.53 ± 0.10c	0.41 ± 0.05b	3.72 ± 0.14b	0.78 ± 0.10c	15.33 ± 0.21b
CL100-II	2.90 ± 0.15b	1.55 ± 0.13c	0.30 ± 0.04b	3.61 ± 0.20b	0.81 ± 0.20c	14.10 ± 0.15b
T-II	5.51 ± 0.23e	3.19 ± 0.14f	1.02 ± 0.05e	5.55 ± 0.31e	2.98 ± 0.14 g	31.11 ± 0.24d
TL25-II	4.30 ± 0.18a	2.15 ± 0.05a	0.61 ± 0.11a	4.41 ± 0.22a	2.22 ± 0.15f	25.02 ± 0.19e
TL50-II	4.18 ± 0.11a	2.01 ± 0.10a	0.53 ± 0.07a	4.30 ± 0.15a	1.65 ± 0.16a	20.12 ± 0.31a
TL100-II	4.22 ± 0.21a	1.81 ± 0.14b	0.55 ± 0.11a	4.18 ± 0.21a	1.58 ± 0.09a	19.89 ± 0.14a

Results were the mean of six animals assayed in duplicate ± SD. Samples were processed as detailed in Section 2. PCs, TBARS and [NOx] were expressed in μmol pyruvate/mg prot., nmol MDA/mg prot.; μmol nitrite/mg prot.; respectively. Results statistically different within the same column are indicated with distinct letters (ANOVA + Tukey test; $p < 0.01$). C, control; CL, treated with LA; T, treated with pesticide mixture; TL, treated simultaneously with pesticide mixture and LA. *Phase I*; 5 weeks of pesticide exposure; *phase II*, 5 weeks pesticide exposure followed by a wash-out period of 5 weeks.

Table 2
Biomarkers of oxidative damage in brain cortex (CC), hipoccampus (HYP) and *substantia nigra* (SN) homogenates prepared from rats treated with the pesticide mixture and/or α-lipoic acid.

Groups	CC			HYP			SN		
	PCs	TBARS	[NOx]	PCs	TBARS	[NOx]	PCs	TBARS	[NOx]
Phase I									
C-I	5.02 ± 0.20a	1.40 ± 0.06a	25.42 ± 1.11a	3.95 ± 0.15a	1.28 ± 0.23a	15.11 ± 0.20a	5.67 ± 0.14a	1.89 ± 0.10a	16.24 ± 0.24a
CL25-I	4.26 ± 0.13a	1.11 ± 0.02b	23.30 ± 0.81a	3.84 ± 0.21a	1.31 ± 0.10a	14.59 ± 0.12a	5.59 ± 0.20a	1.80 ± 0.08a	17.12 ± 0.20a
CL50-I	3.30 ± 0.08b	1.02 ± 0.05b	18.51 ± 0.76b	3.53 ± 0.12b	1.20 ± 0.15a	13.55 ± 0.14a	5.41 ± 0.30a	1.74 ± 0.11a	15.33 ± 0.14a
CL100-I	3.21 ± 0.10b	0.84 ± 0.05c	19.03 ± 0.64a	3.41 ± 0.15b	0.95 ± 0.06b	12.81 ± 0.11a	5.05 ± 0.14b	1.22 ± 0.14b	14.09 ± 0.13a
T-I	11.87 ± 0.31c	4.49 ± 0.25d	42.02 ± 1.03c	7.11 ± 0.20c	2.66 ± 0.15c	20.88 ± 0.14b	15.6 ± 0.18c	7.53 ± 0.21c	23.27 ± 0.15b
TL25-I	9.02 ± 0.40b	2.51 ± 0.14e	33.73 ± 0.51d	4.44 ± 0.13d	2.10 ± 0.08d	15.67 ± 0.11a	8.50 ± 0.11d	4.98 ± 0.16d	20.85 ± 0.10c
TL50-I	7.12 ± 0.22d	1.87 ± 0.20f	29.22 ± 0.44e	4.26 ± 0.22d	1.80 ± 0.10e	16.06 ± 0.21a	6.33 ± 0.20e	3.04 ± 0.12e	17.05 ± 0.15a
TL100-I	5.35 ± 0.31a	1.52 ± 0.16a	23.80 ± 0.57a	4.01 ± 0.08a	1.35 ± 0.15a	14.84 ± 0.11a	5.82 ± 0.14a	2.05 ± 0.23a	16.30 ± 0.17a
Phase II									
C-II	4.88 ± 0.16a	1.33 ± 0.11a	26.62 ± 1.15a	4.05 ± 0.14a	1.31 ± 0.16a	14.95 ± 0.22a	6.01 ± 0.15a	1.80 ± 0.07a	16.07 ± 0.15a
CL25-II	4.01 ± 0.05b	0.90 ± 0.04b	17.51 ± 0.71b	3.72 ± 0.25a	1.28 ± 0.11a	15.08 ± 0.14a	5.87 ± 0.13a	1.76 ± 0.10a	15.55 ± 0.12a
CL50-II	3.10 ± 0.14b	0.78 ± 0.10b	15.83 ± 0.88b	3.01 ± 0.10b	1.20 ± 0.14a	14.53 ± 0.15a	5.70 ± 0.16a	1.71 ± 0.05a	14.26 ± 0.22a
CL100-II	3.05 ± 0.12b	0.65 ± 0.02c	15.20 ± 1.11b	3.15 ± 0.14b	0.95 ± 0.06b	14.44 ± 0.10a	5.12 ± 0.05b	1.66 ± 0.20a	14.04 ± 0.15a
T-II	9.53 ± 0.24b	3.42 ± 0.21 g	35.42 ± 1.13d	5.51 ± 0.16c	2.25 ± 0.15d	21.11 ± 0.10b	8.71 ± 0.26d	4.85 ± 0.33d	20.11 ± 0.15c
TL25-II	6.10 ± 0.57d	1.41 ± 0.13a	29.14 ± 1.22a	5.10 ± 0.12e	1.73 ± 0.12e	15.15 ± 0.10a	6.26 ± 0.15e	2.90 ± 0.21e	16.6 ± 0.22a
TL50-II	5.27 ± 0.31a	1.38 ± 0.09a	27.05 ± 1.17a	4.22 ± 0.13a	1.36 ± 0.12a	13.84 ± 0.25a	5.79 ± 0.25a	2.13 ± 0.11a	15.1 ± 0.15a
TL100-II	5.10 ± 0.18a	1.20 ± 0.05a	25.79 ± 1.20a	4.16 ± 0.11a	1.30 ± 0.15a	14.50 ± 0.20a	5.70 ± 0.16a	1.75 ± 0.15a	14.8 ± 0.24a

Results were the mean of six animals assayed in duplicate ± SD. Samples were processed as detailed in Section 2. PCs, TBARS and [NOx] were expressed in μmol pyruvate/mg prot., nmol MDA/mg prot.; μmol nitrite/mg prot.; respectively. Results statistically different within the same column are indicated with distinct letters (ANOVA + Tukey test; $p < 0.01$). C, control; CL, treated with LA; T, treated with pesticide mixture; TL, treated simultaneously with pesticide mixture and lipoate. *Phase I*; 5 weeks of pesticide exposure; *phase II*, 5 weeks pesticide exposure followed by a wash-out period of 5 weeks.

were used as biomarkers of pro-oxidative damage to lipids and proteins, respectively (Halliwell and Gutteridge, 1999; Dalle-Donne et al., 2006). Lipid peroxidation (LPO) was assayed using the TBARS assay with tetraethoxypropane as standard by the method of Yagi (1976). LPO was expressed as nmol malondihaldehyde (MDA)/mg protein in plasma and brain regions. PCs were determined using the method described by Reznick and Packer (1994) and the results were expressed as μmol pyruvate/mg of protein. The sum of nitrates and nitrites [NOx] was determined as the main end-metabolite of nitric oxide (NO) and peroxinitrite anion ($ONOO^-$) using samples previously deproteinized, reduced with VCl_3 and then subjected to the Griess reaction according to Miranda et al. (2001). Results were expressed as μmoles of nitrites/mg protein.

2.4.2. Antioxidants

Due to the importance of biomembranes as targets for pesticide pollution, we also measured vitamin E (α-tocopherol, Toc) as a non-enzymatic biomarker of the antioxidant defense system within the lipid-soluble cell compartment. Toc was determined after extraction by Buttriss and Diplock's method (1984) using the HPLC technique of Bagnati et al. (1998). The status of the non-enzymatic antioxidant system within the water-soluble cell compartment was assessed by determining total glutathione using the enzymatic recycling Anderson and Meister's method (1984). To calculate the ratio of reduced (GSH) to oxidized (GSSG) glutathione the samples were re-analyzed after derivatization with divinyl-pyridine (3 mM final concentration) using authentic standards of GSH and GSSG (Sigma Chem. Co, Buenos Aires, Argentina).

2.4.3. Programmed cell death

Evaluation of the biological activity of the main protease systems involved in programmed cell death was assessed by determining the activities of caspse-3 (CASP-3) and milli (m)- and micro (μ)-calpains (CALP) in whole homogenates of the three brain regions studied. CASP-3 was measured by a colorimetric assay kit (CASP-3-C), based on the hydrolysis of the synthetic peptide substrate acetyl-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD-pNA) by caspase-3. The release of p-nitroaniline (p-NA) was monitored at 405 nm. Three controls were used for each caspase-3 colorimetric assay: inhibitor-treated homogenate (to measure the non-specific hydrolysis of the substrate), caspase-3 positive control (using commercial caspase-3, 5 mg/mL provided by the kit manufacturer) and a blank of boiled (inactive) homogenate. A calibration curve using a standard solution of p-nitroaniline (p-NA) was run in parallel to calculate the activity of caspase-3 expressed as μmol p-NA released/min mL of sample (Activity = OD × dilution factor/ε (10.5 mM) × time × vol. m-CALP and μ-CALP were determined using an assay involving the hydrolysis of whole ultra-pure casein (Sigma, Chem. Co.) by calpain activity (Botha et al., 2004) and the subsequent detection of trichloroacetic acid (TCA)-soluble peptide fragments at 280 nm. The level of calcium in the medium was regulated (5 or 0.5 mM μM of $CaCl_2$ for m- or μ-CALP, respectively) for the determination of calpain subtypes. Calculations were performed on the basis that a unit of calpain is the amount of enzyme that produces a change of absorbance of 0.01 at 280 nm and the results were expressed as units/min mg of TCP.

2.4.4. Prostaglandin determination

In order to assess $PGF_{2\alpha}$ and PGE_2 content in the brain homogenates, the samples were centrifuged at 15,000*g* for 30 min at 2 °C. Supernatants were filtered though Millipore 0.45 μm membranes and concentrated by liophilization in a Telstar apparatus, Lyobeta Model (Madrid, Spain). The residues were dissolved with HCl 2 N adjusting the pH to 3.5, injected into a 200-mg C_{18} reverse phase Sep-column (Peninsula Lab, Belmont, CA) and then eluted with ethyl acetate. The eluted fractions were evaporated to dryness under a nitrogen stream and reconstituted in buffers for enzyme-immuno assay (EIA) determinations. PGs were determined using commercially available kits (Prostaglandin $F_{2\alpha}$ EIA Kit and Prostaglandin E_2 Express EIA Kit from Cayman, Migliore Laclaustra S.R.L. (Buenos Aires, Argentina) with a minimum detection of 4 and 30 pg/mL, respectively. Intra- and inter-assay coefficients of variation were in the range of 8–12% for both kits.

2.4.5. PLA_2 activity

Phospholipase A_2 (PLA_2) activity was determined with [^{14}C]arachidonoyl-phosphatydylcholine (ARA-PC) (24.0 mCi/mmol,

99% pure) as substrate according to the method by Hirata et al. (1980) with the modifications described in our previous paper (Marra and de Alaniz, 2005). Briefly, tissue homogenates were centrifuged at 15,000*g* for 30 min at 2 °C and 20–40 μg protein was incubated at 37 °C in a metabolic shaker with 0.5 mL of 50 mM TRIS/glycylglycine buffer (pH 8.0), 0.1 mM Mg Cl_2, 5 mM *N*-acetylcysteine, and 1 mM ARA-PC biosynthetically prepared in our laboratory. The lipid was previously scattered, under carefully controlled temperature, in cold potassium buffer plus 0.1 mM sodium deoxycholate (grade II, from Sigma Chem. Co.) by means of three 20-s sonication periods with a Heat System-Ultrasonic Sonicator model W-220F (Plainview, NY) equipped with a 1/8-inch diameter microtip at 50% output. After preincubation at 37 °C for 1 min, the assay was started by the addition of the aliquot assayed. Under these conditions, the reaction was completely linear up to 30 min. It was stopped after 10 min incubation time by the addition of 3 mL isopropyl alcohol/heptane/1 N H_2SO_4 (40:10:1, by vol) followed by 2 mL distilled water plus 2 mL of hexane (Carlo Erba, Milan, Italy) containing 5 μg/tube unlabelled arachidonic acid, thorough mixing and incubation for 10 min at room temperature. Unlabeled acid was added as a carrier to facilitate quantitative extraction. Labeled fatty acids, released by the action of phospholipase A_2, were recovered from the upper phase (at least 95% yield) and counted by liquid scintillation (ACS II Scintillation Cocktail, Amersham Pharmacia Biotech., Buckinghamshire, England) using a Wallac Rackbeta Liquid Scintillation Counter (Turku, Finland) with 90% efficiency for ^{14}C. Blanks consisted of equivalent aliquot samples boiled at 100 °C for 5 min. Formation of unlabeled lyso-PC was routinely checked by recovering the phospholipid components followed by thin-layer chromatography and radioscanning (radio-TLC) in a TLC-Proportional Radioactivity Scanner System (Berthold LB-2832, Widbad, Germany) equipped with a Hewlett–Packard 3396-A Data Station.

Protein content was determined by Bradford's method (1976).

2.5. Statistical analysis

All values represent the mean of six rats analyzed in duplicate and are expressed as the mean ± standard deviation (SD). Data were analyzed using one way ANOVA plus Tukey's test with the aid of Systat (version 15.0 for Windows) from SPSS Science (Chicago, IL). The results were also plotted and analyzed using Sigma Scientific Graphing Software (version 11.0) from Sigma Chem. Co. (St. Louis, MO). Distinct letters are used for indicating the statistical significance of differences ($p \leqslant 0.01$). This means that two compared data with different letters are statistically significant between them at a $p < 0.01$ level.

3. Results

The groups treated with 100 mg/kg bw of LA showed a significant decrease in body weight gain, final body weight and food efficiency ratios (data not shown). There was a drop of approximately 15% in the weight gain curve that became statistically significant from day 14 and persisted up to the end of the experimental period. Thus, on the day of sacrifice there was a difference of almost 29% for phase I and 38% for phase II between the final weight of rats treated with LA 100 mg/kg bw and the other groups (data not shown). The administration of 25 or 50 mg LA /kg bw gave rise to lower decreases in body weight gain which were not statistically significant compared to controls. Absolute and relative brain weights (brain weight/rat weight) were indistinguishable between experimental groups in both phase I and phase II experimental protocols.

In phase I we evaluated the protective effect of LA on the oxidative damage to lipids and proteins induced by the sub-chronic administration of the PM (Table 1). The administration of pesticides provoked significant oxidative damage to protein and lipids, reflected at the systemic level by an increase in PCs and TBARS, respectively, and substantial nitrative stress as indicated by the elevation in the peripheral level of [NOx] (Table 1). The co-administration of PM and LA produced a dose-dependent reduction of these three biomarkers, achieving basal values with the higher dose of LA. In addition, control rats treated with LA showed a reduction in basal protein damage and lipid peroxidation as reflected by the results obtained for both phases of the experimental protocol. The results of phase II experiments indicate that the suppression of PM administration is not enough to restore the values of any damage biomarker to those of the control group: administration of LA was necessary to completely normalize the levels of the three biomarkers tested. The alterations observed in plasma were clearly reflected in the whole brain (Table 1). The dose-dependent protective effect of LA was also required to normalize levels in brain during phase II.

The damage induced by PM exposure in brain cortex, hippocampus and *substantia nigra* and the protective effect displayed by LA administration is summarized in Table 2. A similar pattern was observed for all three regions studied; however, the biomarkers of protein (PCs) and lipid (TBARS) oxidative damage appear to be more sensitive than changes in nitrate + nitrite concentration ([NOx]). Moreover, the accumulation of PCs and TBARS under PM exposure was particularly high in SN, exhibiting a three and fourfold increase over control values, respectively. The lowest dose of LA used (25 mg/bw) already showed a protective effect against PM administration, but higher doses were able to diminish the formation of PCs and TBARS to basal levels. It was evident during both experimental phases that all brain regions were sensitive to the antioxidant protection of LA. As in the case of the whole brain, in phase II the restorative effect was complete and depends on the continuous administration of LA during the wash-out period (Table 2).

The observed increase in biomarkers of OS-induced brain damage positively correlates with the levels of endogenous water- and lipid-soluble antioxidants (Table 3). The basal levels of α-tocopherol (Toc) decreased in the order cortex > hippocampus > *substantia nigra* in pesticide-treated animals. Also, exposure to PM strongly affected the GSSG/GSH ratio by more than tripling the basal values in all brain regions. An increase in the total (GSH + GSSG) concentration of glutathione was observed, especially in SN. Toc content was partially restored by suppression of the PM exposure and completely restored under LA treatment with 50 or 100 mg/kg doses of LA at phase I, or with any of the three doses assayed at phase II (Table 3). A similar restorative effect was observed for both the total amount of glutathione and the GSSG/GSH ratio.

We next examined the activity of phospholipase A_2 as the source of the arachidonate acting as substrate for endogenous prostaglandin E_2 and $F_{2\alpha}$ production (Fig. 2). Enzyme activity in whole brain was not significantly modified by PM exposure; however, we observed a dose-dependent reduction in the groups treated with LA in both phase I and phase II experiments which was more evident during the wash-out period. In connection with this finding, prostaglandin biosynthesis was strongly altered by both PM exposure and LA treatment (Fig. 3). The basal level of PGE_2 was higher than that of $PGF_{2\alpha}$ and both were reduced by the sole administration of LA at 100 mg/kg. PM exposure increased both prostaglandins and these effects were significantly reduced by simultaneous administration of LA in a clear dose-dependent fashion. As observed for the biomarkers of damage and antioxidant levels, the amount of prostaglandins did not normalize spontaneously after PM suppression. At least during our tested wash-out period, the restorative effect was only achieved by LA administration.

Since oxidative stress and inflammation are both strongly linked to the activation of cell death process, we explored the

Table 3
Antioxidant levels in brain cortex (CC), hipoccampus (HYP) and substantia nigra (SN) homogenates prepared from rats treated with the pesticide mixture and/or α-lipoic acid.

Groups	CC			HYP			SN		
	GSH + GSSG	GSSG/GSH	Toc	GSH + GSSG	GSSG/GSH	Toc	GSH + GSSG	GSSG/GSH	Toc
Phase I									
C-I	886.5 ± 45.3a	0.11 ± 0.02a	0.31 ± 0.02a	708.2 ± 40.5a	0.08 ± 0.01a	0.25 ± 0.02a	620.2 ± 48.8a	0.08 ± 0.01a	0.22 ± 0.01a
CL25-I	896.8 ± 51.0a	0.12 ± 0.02a	0.32 ± 0.02a	717.8.0 ± 51.1a	0.10 ± 0.02a	0.26 ± 0.02a	630.8 ± 60.0a	0.08 ± 0.02a	0.23 ± 0.02a
CL50-I	902.7 ± 44.2a	0.08 ± 0.02a	0.32 ± 0.02a	722.6 ± 48.5a	0.06 ± 0.01a	0.26 ± 0.01a	632.2 ± 48.1a	0.06 ± 0.01a	0.22 ± 0.02a
CL100-I	890.2 ± 61.3a	0.06 ± 0.01b	0.35 ± 0.03b	715.5 ± 51.7a	0.05 ± 0.01b	0.28 ± 0.02b	623.8 ± 50.5a	0.04 ± 0.01b	0.25 ± 0.02b
T-I	1489.9 ± 74.0b	0.35 ± 0.03c	0.22 ± 0.01c	1202.2 ± 88.3b	0.28 ± 0.02c	0.18 ± 0.01c	1345.8 ± 112.0b	0.25 ± 0.02c	0.15 ± 0.01c
TL25-I	1159.3 ± 53.3c	0.22 ± 0.02d	0.26 ± 0.01d	933.0 ± 44.4c	0.18 ± 0.02d	0.21 ± 0.01d	812.3 ± 43.1c	0.15 ± 0.01d	0.18 ± 0.01d
TL50-I	1005.1 ± 42.2d	0.18 ± 0.01e	0.30 ± 0.03a	804.1 ± 50.1d	0.14 ± 0.01e	0.24 ± 0.01a	704.4 ± 41.5d	0.13 ± 0.01e	0.21 ± 0.01a
TL100-I	955.3 ± 70.7a	0.14 ± 0.01a	0.31 ± 0.02a	766.0 ± 42.5a	0.11 ± 0.01a	0.25 ± 0.02a	658.8 ± 40.9a	0.10 ± 0.01a	0.22 ± 0.02a
Phase II									
C-II	902.4 ± 45.5a	0.12 ± 0.01a	0.33 ± 0.03a	722.6 ± 55.0a	0.10 ± 0.01a	0.26 ± 0.02a	631.0 ± 40.3a	0.08 ± 0.01a	0.23 ± 0.01a
CL25-II	886.5 ± 54.1a	0.08 ± 0.02a	0.34 ± 0.03a	715.2 ± 45.6a	0.06 ± 0.02a	0.27 ± 0.03a	621.1 ± 44.5a	0.06 ± 0.02a	0.24 ± 0.02a
CL50-II	870.3 ± 78.2a	0.07 ± 0.01b	0.34 ± 0.03a	699.3 ± 58.2a	0.06 ± 0.01b	0.27 ± 0.02a	611.2 ± 55.6a	0.05 ± 0.01b	0.24 ± 0.02a
CL100-II	896.1 ± 67.0a	0.06 ± 0.01b	0.33 ± 0.02a	717.2 ± 55.5a	0.05 ± 0.01b	0.26 ± 0.02a	627.4 ± 45.8a	0.04 ± 0.01b	0.23 ± 0.03a
T-II	1222.8 ± 61.1c	0.26 ± 0.02d	0.28 ± 0.01e	978.5 ± 66.6c	0.20 ± 0.02d	0.22 ± 0.01e	856.5 ± 51.7c	0.18 ± 0.01d	0.19 ± 0.01e
TL25-II	1104.2 ± 50.5c	0.19 ± 0.01e	0.31 ± 0.02a	883.3 ± 40.9d	0.15 ± 0.01e	0.25 ± 0.02a	773.8 ± 49.5c	0.13 ± 0.01e	0.22 ± 0.02a
TL50-II	906.3 ± 45.8a	0.13 ± 0.01a	0.30 ± 0.04a	725.3 ± 51.2a	0.10 ± 0.01a	0.24 ± 0.02a	643.2 ± 40.2a	0.10 ± 0.01a	0.21 ± 0.02a
TL100-II	911.0 ± 65.4a	0.12 ± 0.01a	0.31 ± 0.02a	729.5 ± 45.1a	0.09 ± 0.01a	0.25 ± 0.01a	638.4 ± 45.9a	0.07 ± 0.02a	0.22 ± 0.01a

Results were the mean of six animals assayed in duplicate ± SD. Samples were processed as detailed in Section 2. PCs, TBARS and [NOx] were expressed in nmoles GSH or α-tocopherol/mg prot. Results statistically different within the same column are indicated with distinct letters (ANOVA + Tukey test; $p < 0.01$). C, control; CL, treated with LA; T, treated with pesticide mixture; TL, treated simultaneously with pesticide mixture and lipoate. *Phase I*; 5 weeks of pesticide exposure; *phase II*, 5 weeks pesticide exposure followed by a wash-out period of 5 weeks.

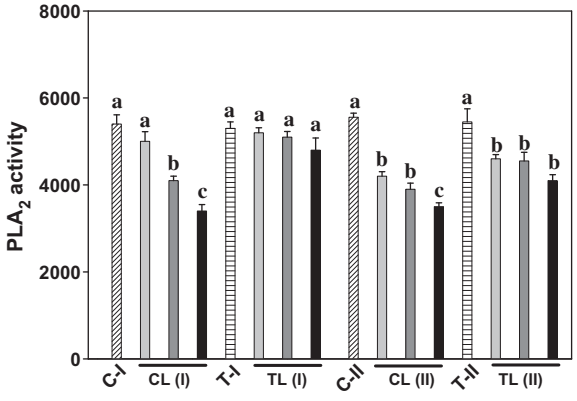

Fig. 2. Phospholipase A_2 activity in whole brain homogenates prepared from rats treated with pesticide mixture and/or lipoic acid (LA). Results were obtained according to the procedure described in Section 2.4.5 and are expressed as the mean ± SD of six animals assayed in duplicate. Control groups corresponding to phases I and II of the experimental protocol are indicated with sloping lines. Treated groups of the two phases are indicated with horizontal lines. Doses of LA are denoted with gray, dark gray or black bars (for 25, 50 or 100 mg LA/kg bw, respectively). Distinct letters are used to indicate the statistical significance of differences (ANOVA + Tukey test; $p < 0.01$). Thus, two compared data with different letters are statistically significant (at the level $p < 0.01$) between them.

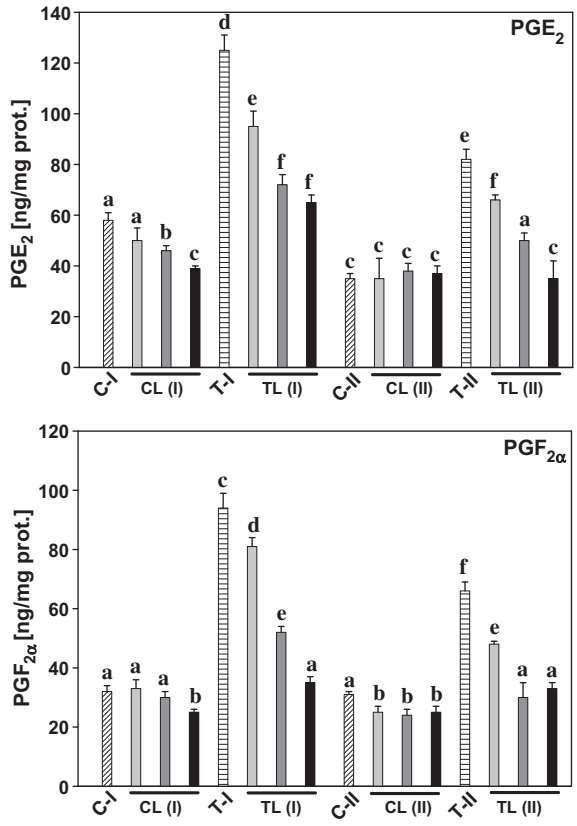

Fig. 3. Prostaglandin E2 (upper panel) and F2α (lower panel) determined in whole brain homogenates prepared from rats treated with pesticide mixture and/or lipoic acid (LA). Results were obtained using the procedure described in Section 2.4.4 and are expressed as the mean ± SD of six rats assayed in duplicate. Control groups corresponding to phases I and II of the experimental protocol are indicated with sloping lines. Treated groups of the two phases are indicated with horizontal lines. LA doses are denoted with gray, dark gray or black bars (for 25, 50 or 100 mg LA/kg bw, respectively). Distinct letters are used to indicate the statistical significance of differences (ANOVA + Tukey test; $p < 0.01$). Thus, two compared data with different letters are statistically significant (at the level $p < 0.01$) between them.

activities of the two main proteases involved. Caspase-3 (CASP) activity was tested in all experimental groups. We observed a non-statistical significant increase ($p = 0.061$) in the rats receiving the PM (Fig. 4). However, LA was able to reduce basal CASP activity in control rats in a clear dose-dependent manner and also in PM-exposed animals. The results of phase II experiments showed that LA administration had similar inhibitory effect on CASP activity (Fig. 4). Calpain isoform activities were modified by both PM and LA administration and by a combination of the two (Fig. 5). Treatment with 50 or 100 mg/kg bw of LA reduced the basal activity of m-CALP and the three doses assayed reduced the activity of μ-CALP. PM exposure activated both isoforms with a proportionally higher increase in μ-CALP activity than m-CALP activity.

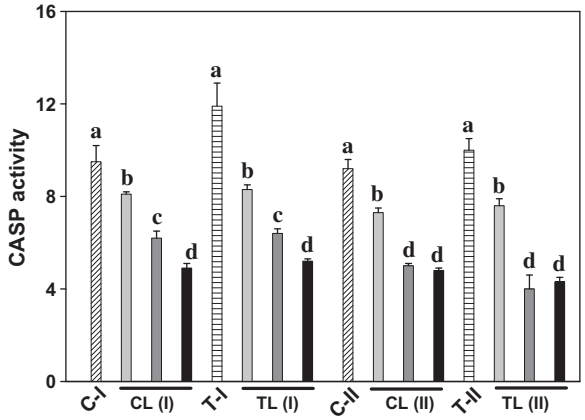

Fig. 4. Caspase-3 activity in whole brain homogenates prepared from rats treated with pesticide mixture and/or lipoic (LA) acid. Results were obtained according to the procedure described in Section 2.4.3 and are expressed as the mean ± SD of six rats assayed in duplicate. Control groups corresponding to phases I and II of the experimental protocol are indicated with sloping lines. Treated groups of the two phases are indicated with horizontal lines. LA doses are denoted with gray, dark gray, or black bars (for 25, 50 or 100 mg LA/kg bw, respectively). Distinct letters are used to indicate the statistical significance of differences (ANOVA + Tukey test; $p < 0.01$). Thus, two compared data with different letters are statistically significant (at the level $p < 0.01$) between them.

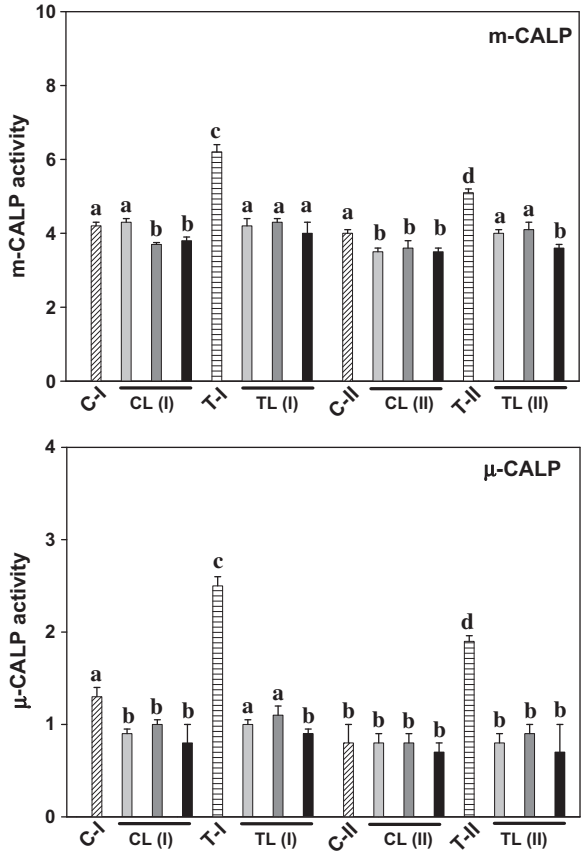

Fig. 5. Activity of milli-(upper panel) and micro-(lower panel) calpain isoforms determined in whole brain homogenates prepared from rats treated with pesticide mixture and/or lipoic acid (LA). Results were obtained according to the procedure described in Section 2.4.3 and are expressed as the mean ± SD of six rats assayed in duplicate. Control groups corresponding to phases I and II of the experimental protocol are indicated with sloping lines. Treated groups of the two phases are indicated with horizontal lines. LA doses are denoted with gray, dark gray or black bars (for 25, 50 or 100 mg LA/kg bw, respectively). Distinct letters are used to indicate the statistical significance of differences (ANOVA + Tukey test; $p < 0.01$). Thus, two compared data with different letters are statistically significant (at the level $p < 0.01$) between them.

Simultaneous administration of LA – at any dose – completely normalized the activities of both calpains to control values. We also observed a spontaneous reduction in these preotease activities during the wash-out period; however, complete normalization after suppression of PM exposure required LA administration of (Fig. 4).

4. Discussion

Our results show that PM exposure provoked a marked OS condition, clearly evident at the systemic level and reflected throughout the whole brain and in the specific brain regions studied. This finding agreed with previous reports from our laboratory (Astiz et al., 2009a,b,c). LA treatment caused a general improvement in this condition; however, the administration of LA also produced a significant loss of weight gain in those groups receiving 100 mg/kg bw. This could be due to the anti-obesity effect of lipoic acid previously documented by Cremer et al. (2006) and Kim et al. (2004) and/or other unknown effects, all of which should be taken into consideration when evaluating the protective properties of the drug. A study on the toxic effect of LA administration in rats demonstrated that the drug is biosafe for up to 2 years at doses up to 180 mg/kg-day (Cremer et al., 2006). For chronic treatment (up to 5 years) in humans, LA is usually indicated in three daily doses of 200 mg each, for patients of approximately 70 kg, which is equivalent to 9 mg LA/kg bw (Jacob et al., 1999; Ruessmann, 2009; Baillie et al., 2009; López-D'alessandro and Escovich, 2011); however, in some clinical circumstances this indication is increased to 1800 mg/day (26 mg LA/kg bw) (Ziegler et al., 2006), 3600 mg/day (51 mg LA/bw) (Ziegler et al., 1995, 1999) or even higher (daily doses of 600–800 mg LA administered intravenously) (Ziegler, 2004; Petersen Shay et al., 2009; Evans and Goldfine, 2000). Pharmacological and clinical studies have demonstrated that LA is safe for humans in doses of 1800–2400 mg/day (26–35 mg LA/kg bw) for at least three months (Petersen Shay et al., 2009; Yadav et al., 2010; Gu et al., 2010), even in patients with severe renal failure (Teichert et al., 2005). Furthermore, it has been demonstrated that the rapid gastrointestinal uptake of LA and its appearance in plasma is followed by an equally rapid clearance (Petersen Shay et al., 2009). After i.p. administration of 100 mg LA/kg bw, the antioxidant is primarily accumulated in liver, heart and skeletal muscle after which it efficiently crosses the blood–brain barrier to accumulate in several brain regions, particularly striatum, hypothalamus and cerebral cortex (Arivazhagan et al., 2002; Chng et al., 2009).

Our findings on the palliative effect of LA on protein oxidation and lipid peroxidation in brain are in agreement with previous reports (Arivazhagan et al., 2000, 2002; Shila et al., 2005; Gadelha Militão et al., 2010; Pirlich et al., 2002; Ferreira et al., 2009). In addition to the pesticide-induced oxidative damage to proteins (PCs) and lipids (TBARS) in the central nervous system, we also observed a higher concentration of [NOx] as the end metabolic product directly related to peroxinitrite over-production. The observed increases in the levels of [NOx] could be the consequence of iNOS activation, the expression of which is especially high in activated microglia. This phenomenon adds a new dimension to the damage associated with pesticides since it indicates the establishment of brain nitrosative stress derived from the reaction between NO and superoxide, giving rise to the powerful pro-oxidant peroxinitrite (Halliwell and Gutteridge, 1999) involved in the pathogenesis of many neurodegeneratrive disorders (Duncan and Heales, 2005). Interestingly, a study by Fen Liang and Akaike (2000) demonstrated that LA could be a potent inhibitor of iNOS, even more effective than N^G-monomethyl-L-arginine, without any proven

citotoxicity. Our results on the effect of LA on [NOx] levels are also coincident with those of Freitas (2009).

Though the antioxidant properties of LA are widely recognized, opinions differ as their implications A limited number of studies on the pro-oxidant action of both LA and its metabolite, dihydrolipoic acid (DHLA), has been performed in recent years (Moini et al., 2002; Cakatay, 2006; Petersen Shay et al., 2009). Sparking controversy about the beneficial effect of these antioxidants still remains unsolved. In our experimental model the antioxidant protection exerted by LA was evident at both the systemic (peripheral plasma) and the local (brain homogenates) levels. This finding is in agreement with the great bulk of evidence proposing lipoate as the most powerful and versatile antioxidant and the reason for which LA has been labeled "the antioxidant of antioxidants") (Bilska and Włodek, 2005). Scientific literature abounds with examples of the multiple protective actions displayed by LA including its activity as a metal chelator agent, ROS/RNS scavenger, and its role in the preservation of other antioxidant molecules from the water- and soluble-cell compartment (Bilska and Włodek, 2005; Holmquist et al., 2007). Moreover, the dose-dependent protective effect of LA – as evidenced by the reduction in the levels of the damage biomarkers – was even observed in control animals not exposed to the pro-oxidative condition produced by PM administration. This fact *per se* is of interest from the clinical point of view, suggesting that LA could be considered as a supplement to be used in cases of suspected sub-clinical free radical formation, for example exposure to environmental pollutants causing oxidative stress (Arnal et al., 2010, 2011; Astiz et al., 2011; Cristalli et al., 2011).

Interestingly, we demonstrated a complete restorative effect in both damage biomarkers levels and in the concentration of the antioxidant molecules in the three brain regions studied. It is well-known that α-tocopherol (Toc) can be indirectly recycled by LA through the reduction of dihydroascorbate to ascorbate (Kagan et al., 1992; Guo and Packer, 2000). The restoration of Toc concentration by LA may acquire additional importance in the central nervous system, where PUFAs play a critical and relevant role in several physiological aspects of brain metabolism (Arivazhagan et al., 2000). Moreover, results obtained in rats from phases I and II of our protocol, which had never been exposed to PM, also demonstrated the role of LA as an efficient buffer of antioxidants of the hydrophobic and hydrophilic cell compartments. Glutathione levels were also normalized under LA administration, in agreement with a previous finding by Suh et al. (2004). The observed increase in the total glutathione content in rats exposed to PM (mainly at expense of the oxidized form) is a phenomenon previously described in living animals and culture cells subjected to pro-oxidative conditions generated by pesticide or other pollutants such as copper (Barlow et al., 2005; Arnal et al., 2011; Astiz et al., 2009a,b,c). Probably, the higher levels of total glutathione are a consequence of an increase in (or induction of) the biosynthesis of GSH owing to the higher expression of the key enzyme γ-glutamyl-cysteinyl synthetase. This response has been previously reported particularly in the central nervous system under oxidative stress conditions (Dringen and Hirrlinger, 2003). Also, Arivazhagan et al. (2000) demonstrated that the intraperitoneal administration of LA to aged rats led to a time-dependent reduction in hydroxyl radicals together with an elevation of GSH/GSSG ratio and glutathione-reductase activity. Thus, LA prevents the reoxidation of GSH and protects the glutathione-related enzymes from peroxidative or nitrosative damage (Arivazhagan et al., 2000). In addition, the ratio GSH/GSSG has been recommended as an important biomarker to determine the oxidative status in human neurodegenerative disorders (Owen and Butterfield, 2010). In view of the well-documented deficiency of the antioxidant defense system in brain tissue (Chong et al., 2005), the above-mentioned facts are obviously important when considering lipoate as a candidate to protect the nervous system, in particular the dopaminergic neuron population from OS caused by pollutants and counteract inflammation and redox-dependent cell death signals (Singh and Dikshit, 2007). The SN is the brain region involved in the pathogenesis of PD and a clear association has been drawn between PD incidence and exposure to environmental pollutants such as the pesticides studied in this work (Le Couteur et al., 1999; Paolini et al., 2004; Di Monte, 2003).

Our results also indicate that LA may substantially improve the restoration of the redox condition after the suppression of pesticide exposure. Since human life without pesticide exposure is practically inconceivable, supplementation with antioxidants, especially those of an amphiphilic nature such as lipoate, remains a good strategy not only as a therapeutic tool but also as a preventive resource (González-Pérez and González-Castaneda, 2006). A previous paper has demonstrated the beneficial effect of LA administration in testicles from rats receiving a mixture of the pesticides chloropyrifos and fenitrothion (Gawish, 2010).

Increased oxidative stress in response to PM exposure is likely involved in pro-inflammatory conditions and activation of the programmed cell death pathway. Pesticides did not modify phospholipase A_2 activity, suggesting that the availability of arachidonate was not substantially altered. However, administration of PM produced a strong hyperproduction and/or lower catabolic rate of PGE_2 and $PGF_{2\alpha}$ in brain tissue. LA treatment gave rise not only to a dose-dependent inhibitory effect on PLA_2 activity, but also to a significant reduction in the levels of both prostaglandins, displaying a clear anti-inflammatory effect. This finding is in agreement with the previously reported effect of LA as an inhibitor of inflammation in many experimental systems (Cho et al., 2004; Kolgazi et al., 2007; Choi et al., 2010; Foo et al., 2011; Odabasoglu et al., 2011; Khabbazi et al., 2012; Deiuliis et al., 2011) including those involving the nervous system (Jomova et al., 2010; Chaudhary et al., 2011), and specifically Alzheimer's disease (Maczurek et al., 2008) and brain stroke (Panigrahi et al., 1996; Gonzalez-Perez et al., 2002; Garcia-Estrada et al., 2003; Wang et al., 1998, 2011a,b; Connell et al., 2011; Richard et al., 2011). COX-2 protein expression has a region-specific activity in brain tissues (Yokota et al., 2004; Kawaguchi et al., 2005); however, the proportion of PGE_2 to $PGF_{2\alpha}$ coincides with the levels found in our study. The anti-inflammatory effect of LA likely resides in the fact that COX-2 and phospholipase A_2 – the main source of the substrate for prostaglandin biosynthesis – are both inhibited by lipoic acid (Ha et al., 2006; Jameel et al., 2006; Ho et al., 2007). It is well-known that prostaglandins derived from arachidonic acid play a prominent role in neurodegenerative disorders such as Alzheimer and Parkinson's diseases (Takemiya et al., 2007; Casadesus et al., 2007; Strauss, 2008). Thus, LA administration could have important implications in clinical situations hallmarked by pesticide (or pollutant)-induced inflammation of the central nervous system.

Finally, we address the matter of LA effect on the modification of the protease activity involved in programmed cell death. As stated before, inflammation and overproduction of ROS/RNS are both directly linked to the activation of programmed cell death. PM treatment clearly stimulates both calpain isoforms but not caspase-3 activity. This result coincides with our previous finding in a similar experimental model (Astiz et al., 2009b). The question of which protease system is responsible for programmed cell death remains a matter of controversy. It is widely known that calpains are activated in response to pro-apoptotic stimuli such as increased ROS and/or RNS (Chandra et al., 2000). Furthermore, caspase-3 is known to be the main effector protein common to the intrinsic and extrinsic pathways of apoptosis and that ROS overproduction is effectively involved in both mechanisms. Moreover, some studies demonstrate that both calpains are activated in apoptotic and necrotic pathways (McGinnis et al., 1998; Van Raam et al.,

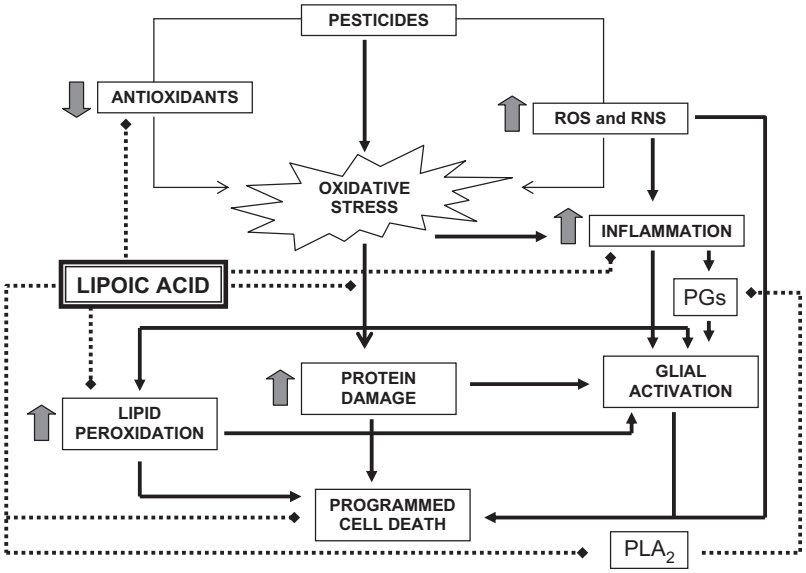

Fig. 6. Graphical representation summarizing the protective effects of LA on pesticide-induced damage in rat brain. PM-induced oxidative stress causes antioxidant depletion and production of ROS/RNS. This condition led to inflammation and probably glial over-reactivity. Accumulation of nitrogen and oxygen reactive species and the subsequent lipid and protein damage-directly or indirectly activates programmed cell death. Lipoate administration restores antioxidant levels, quenches free radicals, decreases phospholipase A_2 activity and prostaglandins E_2 and $F_{2\alpha}$ levels, and inhibits the calpain activation involved in programmed cell death.

2008; Guha et al., 2010). Our present findings suggest one of two possibilities: either exposure to PM preferentially activates calpains instead of caspase-3 or calpain activation prevents caspase-3 activation. The striking similarity between the substrates for caspases and calpains raises the possibility that both protease families could contribute to the loss of cells under ROS and/or RNS overproduction (Raynaud and Marcilhac, 2006). Moreover, caspases have been reported to up-regulate calpain activities through modification of calpastatin (an endogenous calpain inhibitor) by proteolytic cleavage (Wang et al., 1998, 2011a,b). On the contrary, several studies suggest that calpains are able to cleave and inactivate endogenous caspases such as caspase-3, -7, -8, and -9 (Chua et al., 2000). Although there is multiple cross-talk between caspases and calpains, the exact signaling pathway linking the two protease families remains to be elucidated (Bizat et al., 2003a,b; Chong et al., 2005; Chen et al., 2006; Samantaray et al., 2007). The predominant activation of calpain proteases rather than caspases was observed by other authors in models of chronic injury (Wang et al., 1998, 2011a,b; Chua et al., 2000). In addition, induced OS (of any origin) may also contribute to the programmed cell death in other ways. Evidence from other laboratories demonstrates that activation of caspase-3 is strongly dependent on the maintenance of a thiol/redox status (Orrenius, 2004) in which the glutathione level is crucial. Thus, depletion of GSH (or increased concentration of GSSG), as observed in rats exposed to PM, may explain the failure of caspase-3 activation in our experimental system.

5. Conclusion

Fig. 6 summarizes the main effects of LA administration in brain of pesticide-treated rats. The present results show that LA has excellent antioxidant properties in both lipophilic and hydrophilic brain cell environments due to its ability to prevent oxidative damage to lipids and proteins and the concomitant pro-inflammatory condition induced by pesticide pollution. LA also seems to block the activation of programmed cell death via the calpain system. Our experimental protocol constitutes a promising model to investigate questions concerning the dosage and administration routes, biomarkers of damage with probable clinical utility, and the impact of LA administration on the components of the antioxidant defense system and programmed cell death machinery in different tissues.

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