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Zinc reverses malathion-induced impairment in antioxidant defenses

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ABSTRACT

Malathion toxicity has been related to the inhibition of acetylcholinesterase and induction of oxidative stress, while zinc has been shown to possess neuroprotective effects in experimental and clinical studies. In the present study the effect of zinc chloride (zinc) was addressed in adult male Wistar rats following a long-term treatment (30 days, 300 mg/L in tap water *ad libitum*) against an acute insult caused by a single malathion exposure (250 mg/kg, i.p.). Malathion produced a significant decrease in hippocampal acetylcholinesterase, as well as a decrease in the activity of several hippocampal antioxidant enzymes: glutathione reductase, glutathione S-transferase, catalase and superoxide dismutase. The pretreatment with zinc did not completely prevent acetylcholinesterase activity impairment; however, antioxidant activity was completely restored. Zinc administration significantly increased HSP60, but not HSP70, expression. The HSP60 increase suggests a novel zinc-dependent pathway, which may be related to a counteracting mechanism against malathion effects. Based on these results the following hypothesis can be presented: the published "pro-oxidative" effect of malathion may be related, among others, to compromised antioxidant defenses, while the zinc "antioxidant" action may be related to the preservation of antioxidant defenses. In conclusion, our data points to the inhibition of antioxidant enzymes as an important non-cholinergic effect of malathion, which can be rescued by oral zinc treatment.

1. Introduction

Zinc occurs in hundreds of enzymes and in even more protein domains, participating in a number of cellular processes, including cell proliferation, differentiation, and apoptosis. Zinc participates in the functioning of the immune system, intermediary metabolism, DNA metabolism and repair, reproduction, among others. About 25% of the human population worldwide is at risk of zinc deficiency, and zinc supplementation can be beneficial (Maret and Sandstead, 2006). In acrodermatitis enteropathica, a genetic disorder of zinc metabolism caused by a mutation in the zinc transporter hZip4, zinc supplementation is necessary to limit zinc deficiency (Mao et al., 2007). In the Wilson's disease, zinc supplementation replaces copper and prevents its excess, limiting oxidative damage (Kitzberger et al., 2005). In diabetes, zincuria is a risk factor for zinc deficiency, while zinc supplementation decreases the chances of deficiency and ameliorates several oxidative parameters (Maret and Sandstead, 2006). Zinc supplementation is also postulated as an adjuvant in the therapy of mood disorders (Nowak et al., 2005). In preclinical studies, oral zinc supplementation has been shown to have an antidepressant effect (Brocardo et al., 2007). Zinc also protects against oxidative liver damage induced by chronic alcohol ingestion (Zhou et al., 2005), organophosphate treatment (Goel et al., 2005) or by lithium (Chadha et al., 2008). Given the potential for wide therapeutic use of zinc supplementation we wanted to focus on whether zinc has a neuroprotective function.

In the brain, zinc-rich areas display as much as 300–600 μM of available zinc, particularly in the hippocampus and cerebral cortex (Vallee and Falchuk, 1993; Frederickson et al., 2005). Zinc is stored in synaptic vesicles such as in glutamatergic neurons and released simultaneously with glutamate, acting as a neuromodulator (Frederickson et al., 2005). A significant number of reports describe the protective effects of this endogenous metal against excitotoxic insults (Cole et al., 2000; Cohen-Kfir et al., 2005). However, the mechanism whereby zinc displays its protective action remains to be established. One beneficial aspect on zinc action may be the antagonism of NMDA receptors (Chen et al., 1997; Paoletti et al., 1997). Moreover, zinc can modulate GABAergic neurotransmission by inhibiting GABA transporters in the hippocampus, which could reveal a link between excitatory and inhibitory neurotransmission, especially during epileptic seizures (Cohen-Kfir et al., 2005). In addition, zinc is demonstrated to modulate intracellular

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signaling cascades such as mitogen-activated protein kinases, protein kinase C and Ca^{2+}/calmodulin activated protein kinase II, therefore participating in cell proliferation and differentiation (Beyersmann and Haase, 2001).

Malathion (O,O-dimethyl-S-1,2-bis ethoxy carbonyl ethyl phosphorodithioate) is an organophosphate (OP) pesticide widely employed in agriculture and in domestic pest control. It is considered to be a hazardous compound to human health, pets and wildlife (Flessel et al., 1993). As with other OP agents, malathion is thought to exert its toxic effects by inactivation of serine esterases (Taylor et al., 1995), mainly acetylcholinesterase (AChE; EC 3.1.1.7) as well as butyrylcholinesterase (BuChE; EC 3.1.1.8). The inhibition of AChE leads to the accumulation of acetylcholine in the synaptic terminals of the central and peripheral nervous system with consequent overstimulation of the cholinergic pathways (Kwong, 2002; Bartling et al., 2007). Various studies have reported neurotoxic effects of malathion in both humans (Abdel-Rahman et al., 2004; Rothlein et al., 2006) and animals (Vidair, 2004; Brocardo et al., 2005; da Silva et al., 2006). Malathion is thought to be one of the main agents leading to human OP intoxication in Santa Catarina state in Southern Brazil, according to unpublished data obtained from Toxicological Information Center (Centro de Informações Toxicológicas – CIT) hosted by the Hospital Universitário, Florianópolis, SC, Brazil.

The toxicity induced by OP compounds is also believed to be linked to the pro-oxidative properties of these compounds (Goel et al., 2005; Banerjee et al., 1999; Verma and Srivastava, 2001; Ranjbar et al., 2002). It was described that malathion exposure increases lipid peroxidation in rodent erythrocytes, liver and brain (Hazarika et al., 2003; Akhgari et al., 2003). In a recent report (Brocardo et al., 2007), we showed a neuroprotective action of zinc against malathion which may be related to an up-regulation in neuroprotective effectors (Franco et al., 2008). Among these effectors brain derived neurotrofic factor expression, intracellular signal-regulated protein kinase phosphorylation and GSH synthesis have been postulated (Franco et al., 2008).

Other potential zinc target is the heat shock proteins (HSPs) that work as molecular chaperones, able to protect tissues including brain against cell death (Plumier et al., 1997; Sharp, 1998). It was previously demonstrated that zinc causes increase in heat shock protein 70 kDa (HSP70) expression in a variety of tissues and cell cultures (Lee et al., 2000; Unoshima et al., 2001). The exact role of heat shock proteins in zinc protective/toxic effects is not fully understood. While some authors consider the induction of these molecular chaperones as a beneficial action of zinc (Unoshima et al., 2001; Klosterhalfen et al., 1997; Tons et al., 1997), others, however, believe that such effect is a toxic cellular response (Lee et al., 2000).

In the present study we aimed to investigate whether long-term oral zinc (300 mg/L p.o.) treatment is able to protect the rat brain against toxicity caused by an acute treatment with malathion (250 mg/kg i.p.). Antioxidant activity and the HSPs expression were the endpoints investigated.

2. Materials and methods

2.1. Chemicals and antibodies

Glutathione-disulfide reductase (GR), EC 1.8.1.7, reduced glutathione (GSH), oxidized glutathione (GSSG), *tert*-butylhydroperoxide (*t*-BOOH), 5,5′-dithio-bis(2-nitrobenzoic) acid, cytochrome *c*, xanthine, xanthine oxidase (EC 1.17.3.2), 1-chloro-2,4-dinitrobenzene, acetylthiocholine iodide were purchased from Sigma, São Paulo. NADPH was purchased from Gerbu Biochemicals GmbH, Gaiberg. Zinc chloride was obtained from Merck, Rio de Janeiro and commercial-grade malathion 500 CE (95% purity, CAS 121-75-5) was purchased from BioCarb, Curitiba. The primary antibodies for HSP60 and HSP70 were purchased from StressGen, Ann Arbor, Michigan and secondary antibodies were from Amersham, São Paulo. All other chemicals used in this work were from the highest commercial grade available.

2.2. Animals and treatments

Adult male Wistar rats (3 months old, 250–350 g) were maintained in a room under controlled temperature (23 ± 1 °C). They were subjected to a 12 h light cycle (lights on 7:00 a.m.) with free access to food and water. All procedures used in the present study were approved by the institution ethics committee on the use of animals (CEUA).

Animals were separated to four different groups: (a) the control group was maintained for 30 days and on the 31st day received a saline injection intraperitoneally. (b) In the zinc group, $ZnCl_2$ (300 mg/L) diluted in tap water was offered *ad libitum* during 30 days. Animals received an i.p. saline injection 24 h after zinc treatment was complete. Based on daily liquid consumption, each animal received between 15 and 18 mg/kg body weight of zinc chloride per day. This protocol was based on previous reports (Goel et al., 2005; Domingo et al., 1988). (c) In the malathion group animals received an i.p. injection of malathion (250 mg/kg) 24 h previous to tissue collection. (d) In the zinc/malathion group, animals received zinc for 30 days in the tap water. In order to avoid the acute effect of zinc, 24 h after oral zinc was interrupted animals received an i.p. injection of malathion (250 mg/kg). Animals were sacrificed after 24 h following the respective saline/malathion i.p. injections, i.e., 48 h after oral zinc was interrupted, and tissues were prepared for biochemical analysis.

2.3. Tissue preparation

The hippocampus was rapidly removed to cooled saline and immediately homogenized in 0.02 M HEPES pH 7.0 and centrifuged at 1000 × *g*. An aliquot of the supernatant (S1) was used for measurements of cholinesterase activity and the remaining S1 was centrifuged at 20,000 × *g* for 30 min at 4 °C. The supernatant (S2) was isolated and utilized for measurements of antioxidant enzyme activity. Blood samples were isolated from rat hepatic portal vein, using heparinized syringes, for measurements of plasma acetylcholinesterase activity and AST/ALT activity, as markers of malathion intoxication.

For western blots, tissues were homogenized at 4 °C in a buffer (pH 7.0) containing 50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, 20 mM Na_3VO_4, 100 mM sodium fluoride. The homogenates were centrifuged at 1000 × *g* for 10 min at 4 °C and the supernatants (S1) collected. After protein determination, β-mercaptoethanol was added to samples to a final concentration of 8%. Then samples were frozen at −80 °C for further determination of HSP60 and HSP70 immunocontent. Protein levels were quantified according to Bradford (1976) using bovine serum albumin as standard.

2.4. Enzyme assays

The GR activity was determined according to Carlberg and Mannervik (1985). Glutathione peroxidase (GPx), EC 1.11.1.9, activity was measured indirectly by monitoring the consumption of NADPH at 340 nm according to Wendel (1981) using the *t*-BOOH as a substrate. Glutathione transferase (GST), EC 2.5.1.18, activity was assayed by the procedure of Habig and Jakoby (1981) using 1-chloro-2,4-dinitrobenzene as substrate. Catalase (CAT), EC 1.11.1.6, activity was measured according to Aebi (1984). Superoxide dismutase (SOD), EC 1.15.1.1, activity was based on the decrease in cytochrome *c* reduction (Misra and Fridovich, 1977). Acetylcholinesterase activity was measured according to Ellman et al. (1961). Plasma transaminases, aspartate transaminase (AST, EC 2.6.1.1) and alanine transaminase (ALT, EC 2.6.1.2) activity were determined using commercially available kits (Biotécnica Ltda., Varginha) and expressed as percent (%) of controls.

2.5. Western blot

Samples (10 mg of protein) were separated by SDS-PAGE using 10% gels and transferred to nitrocellulose membrane using 400 mA current (3 h at 4 °C) (Posser et al., 2007). The membranes were blocked with 5% skim milk (1 h), followed by a second blockage (1 h) with 2.5% gelatin, both solutions in TBS (10 mM Tris, 150 mM NaCl, pH 7.5). All steps were followed by three times washing with TBS-T (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5). For HSP detection, rabbit polyclonal antibodies SPA805 (1:5000) and SPA811 (1:5000) (StressGen), anti-human HSP60 and HSP70, were used as primary antibodies. NA934 (1:1000) goat anti-rabbit IgG peroxidase conjugated (Amersham) was used as the secondary antibody for detecting both isoforms. Loading control was checked by probing for β-actin at dilution of 1:25,000 (A3854, Sigma–Aldrich). Immunoblotting was developed using the enhanced chemiluminescence (ECL) system (Amersham Bioscience). HSP60 and HSP70 expression was quantified by densitometric analysis of the immunoreactive bands using the Scion Image® software.

2.6. Statistical analysis

Data are presented as mean ± standard deviation (S.D.). Statistical significance was assessed by two-way ANOVA (malathion and zinc as factors) followed by Tukey's test when appropriate. A value of $P < 0.05$ was considered to be statistically significant.