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Oxidative stress in mice treated with antileishmanial meglumine antimoniate

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ABSTRACT

In order to improve the understanding of the toxicity of pentavalent antimony (Sb^V), we investigated the acute effects of meglumine antimoniate (MA) on the oxidative stress in heart, liver, kidney, spleen and brain tissue of mice. Levels of lipoperoxidation and protein carbonylation were measured to evaluate the oxidative status, whereas superoxide dismutase/catalase activity and glutathione levels were recorded to examine the antioxidative status. We observed that MA caused significant protein carbonylation in the heart, spleen and brain tissue. Increased lipoperoxidation was found in the liver and brain tissue. An imbalance between superoxide dismutase and catalase activities could be observed in heart, liver, spleen and brain tissue. Our results suggest that MA causes oxidative stress in several vital organs of mice. This indicates that the production of highly reactive oxygen and nitrogen species induced by MA might be involved in some of its toxic adverse effects.

1. Introduction

Leishmaniasis is endemic in 98 – mainly developing – countries on five continents and affecting largely the poorest of the poor. At the moment, approximately 350 million people are considered at risk of contracting leishmaniasis. An estimated 0.2–0.4 million cases of visceral leishmaniasis and about 0.7–1.2 million cases of cutaneous leishmaniasis occur every year (Alvar et al., 2012). However, convenient vaccines against leishmaniasis still remain elusive. The drugs currently available for chemotherapy treatment remain unsatisfactory due to a lack of specificity, parasite resistance and in many cases toxicity to humans (de Oliveira et al., 2009; Frézard et al., 2009).

Currently, the most commonly used treatment for leishmaniasis relies on pentavalent antimonial (Sb^V) drugs such as meglumine antimoniate (MA) (Tuon et al., 2008). Even though these antimoniates have been used therapeutically for more than 60 years, the underlying biochemical mechanisms still haven't been uncovered in detail. It is for example known that Sb^V is only active after intracellular reduction to the trivalent form Sb^{III}. Related studies demonstrated that Sb^{III} reacts with reduced forms of trypanothione and inhibits the activity of trypanothione reductase, which induces rapid efflux of trypanothione and glutathione (GSH) (Wyllie et al., 2004). Trypanothione and GSH are important antioxidant systems, which protect parasites and mammalian host cells against the cytotoxic effects of reactive oxygen (ROS) and nitrogen species (RNS) (Romão et al., 1999, 2006; Mukherjee et al., 2009).

The most frequent adverse effects related to systemic administration of MA include urticaria, nausea, lethargy, arthralgia and abdominal pain (Winship, 1987; Guerin et al., 2002). Additional detrimental symptoms such as myalgia, pancreatitis, nephrotoxicity, cardiotoxicity or hepatotoxicity arise during the treatment, due to an accumulation of drug in the cells. These side effects strongly contribute to reduction or withdrawal from the antimony treatment (Croft et al., 2006; Sadeghian et al., 2008; Oliveira et al., 2009). Considering the complex Sb^V resistance mechanisms in *Leishmania*, it is feasible to assume that oxidative stress induced

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by Sb^V during the treatment cycle may be the reason of, or at least contribute to, cellular and organic injury. For example, Rais et al. (2000) have suggested that Sb^V primes macrophages to generate ROS in response to various stimuli and enhance the activity of NADPH oxidase, an enzyme involved in the production of superoxide. Also Sb^V induce *Leishmania*-infected macrophages to produce ROS via PI3K-PKC-Ras/Raf pathways and nitric oxide (NO) via PI3K and p38 MAPK pathways (Mookerjee Basu et al., 2006). It has also been suggested that oxidative stress may contribute to the progression of human cutaneous leishmaniasis (Kocyigit et al., 2003; Vural et al., 2004). Several other reports show in this context that endogenous antioxidant functions are changed during human infections (Kocyigit et al., 1998, 2003, 2005; Erel et al., 1999; Vural et al., 2004; Serarslan et al., 2005). It is well known that exposure to Sb^V compounds can cause cellular and organic injury, particularly in the tissue of the heart, lung, liver and kidney. However, the exact nature of the underlying mechanisms is not well understood (Leonard and Gerber, 1996). In order to gain a better insight into the host toxicity mechanism of Sb^V compounds, we designed this study and investigated the ability of MA to induce oxidative stress in mice after acute treatment.

2. Materials and methods

2.1. Reagents and equipment

Thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (TEP), dinitrophenylhydrazine (DNPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), glutathione reductase, glutathione (GSH), β-Nicotinamide adenine dinucleotide 2′-phosphate reduced (NADPH), albumin from bovine serum and adrenaline were purchased from Sigma Chemical Co. (St. Louis, USA). Hydrogen peroxide, Folin's phenol reagent (phosphomolybdate/phosphotungstate) and trichloroacetic acid were purchased from Vetec Chemical (São Paulo, Brazil). MA was purchased from Aventis Pharma Brazil (Glucantime®, 81 mg Sb^V/ml). An Amersham Biosciences® spectrophotometer or a SpectraMax M2e microplate reader (Molecular Devices) were used for biochemical analyses.

2.2. Animals

CF-1 mice (8 weeks, 30 g) were obtained from the breeding colony of University of Southern Santa Catarina (UNESC). Animals were housed (6 animals/cage) with food and water available *ad libitum*. All animals were kept on alternating light/dark cycles of 12 h (lights on at 7:00 am). All experimental procedures were performed in accordance with the guidelines of the National Institute of Heath, the recommendations of the Brazilian Society for Laboratory Animal Science (SBCAL) and with the approval of the local ethics committee.

2.3. Antimony treatment

Animals (6 mice/group) received daily subcutaneous injections of MA (20, 60 or 120 mg Sb^V/kg/day) over the course of three consecutive days. Prior to the injection, MA was dissolved in a 5% glucose solution. The control group received injections containing only a 5% glucose solution. All animals were killed by decapitation 24 h after the last injection. The heart, brain, liver, kidney and spleen were excised immediately from each animal and stored at −80 °C for posterior analyses. MA dosages used in this study were based on the efficient suppression of *Leishmania* infections in mice and humans (Nakayama et al., 2005; Barroso et al., 2007; Tuon et al., 2008; Lima et al., 2009; Rodrigues et al., 2009).

2.4. General health and behavior of the animals

All mice were weighed and examined for any clinical or behavioral changes on a daily basis. The general appearance of the animals was evaluated by fur checks for cutaneous reactions.

2.5. Tissue analysis

At the end of the acute treatment period, organs were excised and weighed immediately after decapitation. Prior to processing for biochemical analysis, organs were inspected macroscopically for anatomical or pathological abnormalities.

2.6. Analysis of lipoperoxidation

In order to quantify levels of lipoperoxidation, thiobarbituric acid reactive substances (TBARS) generated during acid-heating reactions were measured as described by Draper and Hadley (1990). Samples of tissue homogenate (brain, heart, liver, kidney or spleen) were therefore mixed with trichloroacetic acid (1 mL, 10%) and thiobarbituric acid (1 mL, 0.67%) before being heated in a boiling water bath (15 min). TBARS obtained from the acidic hydrolysis of TEP were used as a standard. TBARS absorbance was measured at 535 nm and results are expressed as malondialdehyde (MDA) equivalents (nmol/mg of protein).

2.7. Analysis of protein carbonylation

The oxidative damage to cell proteins was assessed from the determination of carbonyl groups in tissue homogenate samples, based on the reaction with DNPH (Levine et al., 1990). Proteins contained in examined tissue homogenate samples were briefly precipitated by the addition of trichloroacetic acid (20%), and subsequently quickly dissolved in DNPH, before the absorbance at 370 nm was measured.

2.8. Analysis of catalase (CAT) and superoxide dismutase (SOD) activity

In order to determine CAT activity levels, tissue samples were sonicated in phosphate buffer (50 mM) and the resulting suspension was centrifuged (3000 rpm/10 min). The supernatant was subsequently used for an enzyme assay. The CAT activity level was measured by the decay of the hydrogen peroxide absorbance at 240 nm (Aebi, 1984). The SOD activity level was measured from the inhibition of adrenaline autoxidation (Bannister and Calabrese, 1987).

2.9. Analysis of glutathione levels

Total GSH (reduced and disulphide forms) levels in tissue homogenate samples were measured using the method described by Tietze (1969). Reduced GSH is recycled with glutathione reductase and NADPH, which is linked to the absorption change at 412 nm (associated with the reduction of DTNB). This assay was modified for the use in a microtitre plate reader (SpectraMax M2e Microplate reader, Molecular Devices, Union City, CA, USA) as previously described elsewhere (Romão et al., 2006).

2.10. Quantitative protein analysis

The total amount of proteins for the determination of lipoperoxidation, protein carbonylation and enzyme antioxidant assays was measured using the Lowry protein assay (Lowry et al., 1951). Folin's phenol reagent (phosphotungstate/phosphomolybdate) was therefore added to tissue samples. Folin's phenol reagent binds to