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Susceptibility of mitochondrial superoxide dismutase to aluminium induced oxidative damage

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ABSTRACT

Aluminium has been implicated in various neurodegenerative diseases but exact mechanism of action is still not known. Mitochondria being a major site of reactive oxygen species production are considered to be target of oxidative stress and it seems that the oxidative damage to mitochondrial proteins may underlie the pathogenesis of aluminium induced neurodegeneration. Thus, the present study was undertaken to reveal the effects of chronic aluminium exposure (10 mg/kg b.wt, intragastrically for 12 weeks) on the oxidative damage to mitochondrial proteins in male albino Wistar rats. Chronic aluminium exposure resulted in decrease in the activity of mitochondrial superoxide dismutase (MnSOD) and aconitase in different regions of rat brain suggesting increased oxidative stress. This decrease in MnSOD activity in turn might be responsible for the increased protein oxidation as observed in our study. All these processes taken together may cause increased oxidative damage to mitochondrial proteins in general. By taking the advantage of recent immunochemical probe for oxidatively modified proteins, we identified MnSOD to be susceptible to oxidative damage in aluminium treated animals. The quantitative RT-PCR analysis for Lon protease, a protease involved in the removal of oxidatively modified proteins from mitochondria, showed decreased mRNA expression suggesting increased oxidative damage and decreased removal of mitochondrial proteins. The identification of specific proteins as targets of oxidative damage may provide new therapeutic measures to reverse the effects of aluminium induced neurodegeneration.

1. Introduction

Aluminium is a ubiquitous element, comprising approximately 8% of the earth's crust. The almost ubiquitous presence of this element has so heavily contaminated the environment that exposure to it is virtually inescapable. A variety of human and animal studies have implicated learning and memory deficits following aluminium exposure (Kaur et al., 2006; Niu et al., 2007), which can be explained on the basis of this being a cholinotoxic agent. Besides, aluminium also has a strong prooxidant activity inspite of its non-redox status (Exley, 2004). Strong evidence is there that aluminium complexes with superoxide anion forming aluminium superoxide anion, which is a more potent oxidant than superoxide anion on its own and promotes the formation of hydrogen peroxide and hydroxyl radicals that contribute to an oxidizing environment (Exley, 2004). Despite the abundance of reports regarding possible involvement of aluminium in a variety of human maladies, the exact mechanism by which aluminium exerts its toxic effects in the brain is yet to be delineated.

Mitochondria are considered to be the major source of reactive oxygen species (ROS) production and key contributors to neurodegenerative diseases (Leuner et al., 2007). Moreover, mitochondria remain major target for ROS-induced cellular injury (Liang and Godley, 2003) because mitochondria lack protective structural proteins. Toxic consequences resulting in electron transport chain (ETC) dysfunction may ensue mitochondrial damage including oxidation of mitochondrial DNA, proteins and lipids leading to the opening of mitochondrial permeability transition pore, an event associated with neuronal cell death (Halliwell, 1992). The generation of these ROS may induce the oxidative damage to mitochondrial proteins. Increased mitochondrial oxidative damage has also been observed in early pathological events leading to neurodegeneration (Beal, 1996). The mitochondrial proteins represent main target for oxidative modifications and their accumulation may play an important role in neurodegeneration.

The mitochondrial superoxide dismutase (MnSOD) is involved in the cellular defense against oxidative damage to mitochondria by ROS. Studies have indicated increased oxidative damage to be

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associated with altered mitochondrial function in heterozygous MnSOD knockout mice (Williams et al., 1998). Moreover, the over expression of MnSOD in cells has shown protective effects (Keller et al., 1998). Similar to MnSOD, aconitase is another important enzyme present in mitochondrial matrix that plays a key function in cellular energy production. Loss in aconitase activity is commonly used as a biomarker of oxidative damage because of the presence of the iron–sulfur cluster [4Fe–4S] in its active site (Kent et al., 1982). Aconitase has been shown to be sensitive to reactions with superoxide anions (Gardner et al., 1994, 1995; Verniquet et al., 1991) *in vitro*, which may be related to the release of one iron atom from the cluster (Flint et al., 1993). The alteration in aconitase activity has also been observed in increased oxidative stress caused by MnSOD deficiency (Williams et al., 1998) and in some neurodegenerative diseases (Park, 2001; Bradley, 2000; Tabrizi, 1999). If the oxidative stress is prolonged, reversible modulation of aconitase activity progresses to irreversible inactivation resulting in ATP-dependent degradation (Bulteau et al., 2003).

Oxidative stress leads to modifications and accumulation of oxidized proteins, as observed in various cellular dysfunctions (Halliwell, 2006). The post-translational oxidative modifications of proteins may be involved in the structural and functional decline and even to the degradation of proteins from the cell. The addition of carbonyl groups is the most common post-translational oxidative modification of proteins (Levine and Stadtman, 2001) and hence, carbonyl groups are considered as the standard markers of protein oxidation (Berlett and Stadtman, 1997; Stadtman, 1992). The elevation in the total level of protein carbonyls has also been observed in both Alzheimer's disease (AD) and Parkinson's disease (PD) and even in other neurodegenerative diseases (Giasson et al., 2002; Jenner, 2003). Protein carbonylation adds to the irreversible oxidative modification further resulting in either removal of protein by degradation or accumulation of the oxidatively damaged protein.

Cellular proteases are mainly involved in the degradation of oxidized proteins from the cell and in mitochondria this function takes place by a matrix protease known as Lon protease. Down regulation of human Lon protease has been shown to result in disruption of mitochondrial structure, loss of function and cell death (Bota et al., 2005). The decline of Lon protease activity was found to be associated with a decrease in the activity of mitochondrial aconitase, a protein known to be highly sensitive to oxidative inactivation (Bota et al., 2002). This presents the possibility that there is modulation of Lon protease functions in response to oxidative stress.

Previously we reported that aluminium accumulation was higher in hippocampus region of rat brain followed by corpus striatum and cerebral cortex (Kaur et al., 2006; Julka et al., 1995). It has also been shown that aluminium exposure is associated with impairment of mitochondrial functions *in vitro* (Niu et al., 2005) and *in vivo* (Kumar et al., 2008) and increases the ROS production. On the basis of these results, we examined the effect of chronic aluminium induced oxidative stress on the oxidative damage to mitochondrial proteins in different regions of rat brain and their accumulation/degradation within mitochondria.

2. Experimental procedures

2.1. Chemicals

Aluminium was purchased from Fluka Chemicals, Buchs, Switzerland. Acrylamide/bis-acrylamide, ammonium persulfate and N,N,N′,N′-tetramethylenediamine, 2,4-dinitrophenylhydrazine (DNPH), glycine, Tris (hydroxymethyl) aminomethane and agarose were purchased from Sigma Chemicals Co., St. Louis. SDS and glycine were from Sisco Research Laboratory, rabbit anti-DNP antibody was from Sigma Chemicals Co., St. Louis. Rabbit anti MnSOD, mouse anti β-actin, goat anti rabbit HRP labeled IgG and goat anti mouse IgG were from Santa Cruz Biotechnology, CA, USA. Immobilon-P-membrane was from Millipore, Glass redistilled water was used throughout the present investigation.

2.2. Animals and their care

Male albino rats (Wistar strain) in the weight range of 100–120 g were procured from the institute's animal house. The animals were housed in polypropylene cages, and kept in well-ventilated rooms. Animals were provided standard rat pellet diet (Hindustan lever Ltd.; Mumbai, India) and water *ad libitum*.

2.3. Experimental design

The animals were divided into following two groups of 5 animals each.

2.3.1. Control group

In this group, animals were administered an equal volume of sodium lactate (vehicle) as administered to the animals of the aluminium treated group.

2.3.2. Chronic aluminium treated group

Animals received aluminium in the form of aluminium lactate (10 mg/kg b.wt/day) dissolved in distilled water, intragastrically for 12 weeks. The dose used in the present study was same as was used in our previous studies (Kaur et al., 2006; Kumar et al., 2008). During aluminium treatment period, animals were monitored for any change in body weight, dietary intake as well as for behavioral alterations.

After the completion of treatment, animals were fasted overnight and sacrificed by decapitation using sodium pentathol. The brains were removed, rinsed in ice-cold physiological saline (0.9% NaCl) and dissected into following regions as per the guidelines of Glowinski and Iversen (1966): cerebral cortex, corpus striatum and hippocampus. Ethical clearance for killing of animals was duly obtained from institute's Animal Ethical Committee.

2.4. Isolation of mitochondria

Rat brain mitochondria were isolated by the method of Berman and Hastings (1999). Briefly, the brain regions were homogenized in isolation buffer with EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). Homogenate was centrifuged at 1300 × *g* for 5 min at 4 °C. Pellet was resuspended in isolation buffer with EGTA and spun again at 13,000 × *g* for 5 min. The resulting supernatant was transferred to new tubes and topped off with isolation buffer with EGTA and again spun at 13,000 × *g* for 10 min. Pellet containing mitochondrial rich fraction was resuspended in isolation buffer without EGTA. The integrity of mitochondria was checked by assessing respiratory control ratio and marker enzymes (data not shown).

2.5. Superoxide dismutase assay (SOD)

MnSOD activity was measured as described previously (Kumar et al., 2008). MnSOD activity in total solubilized mitochondrial extract was measured by the cytochrome *c* reduction method in the presence of 1 mM potassium cyanide to inhibit both Cu–Zn SOD and extra cellular SOD. The amount of enzyme required to produce 50% inhibition was considered as 1 U of MnSOD activity and results were expressed as U/mg protein.

2.6. Aconitase activity

Mitochondrial suspension was freeze–thawed for three cycles to rupture the mitochondrial membranes and then centrifuged at 16,000 × *g* for 5 min. The aconitase activity was determined in the mitochondrial fractions by the method of Bulteau et al. (2003). Briefly, protein-normalized aliquots of the mitochondrial fraction were incubated at 25 °C in a reaction buffer containing 154 mM Tris, 5 mM sodium citrate, 0.6 mM $MgCl_2$, 0.2 mM $NADP^+$. Absorbance at 340 nm was measured over time as citrate was converted to α-ketoglutarate using the concomitant reduction of $NADP^+$ to NADPH by 1 U/ml isocitrate dehydrogenase. One mU of aconitase activity was defined as the amount catalyzing the formation of 1 nmol of isocitrate per min. Aconitase was reactivated by addition of 0.5 M DTT and 40 mM ferrous ammonium sulphate.

2.7. Quantification of protein carbonyl

Quantitation of protein carbonyl content as an index of protein oxidation in mitochondrial fractions was determined after derivatization with DNPH using protein carbonyl assay kit (Cayman Chemicals, USA). The amount of protein carbonyl was calculated on the basis of molar extinction coefficient of DNPH (0.022 μM^{-1} cm^{-1}). The results were expressed as nmol carbonyl/mg protein.

2.8. Preparation of DNPH derivatized proteins for SDS-PAGE

The brain regions from three different rats were pooled together and mitochondria were isolated. Pellets containing mitochondria were treated with the lysis buffer (1× PBS, 1%NP-40, 0.5% sodium deoxycholate, 0.1%SDS, 250 mM sucrose 20 mM Tris–HCl, pH 7.4, 1 mM DTT, and protease inhibitor) and were incubated on ice for 20 min. The lysate was centrifuged at 10,000 × *g* at 4 °C for 30 min. The resulting