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Lead-induced alteration of apoptotic proteins in different regions of adult rat brain

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ABSTRACT

In our earlier investigations, we have demonstrated the alteration of antioxidant enzymes in adult rat brain exposed to lead. This study was carried out to investigate the effect of lead on inducing apoptosis by choosing poly (ADP-ribose) polymerase (PARP), bcl-2 and caspase-3 expression as marker proteins in the cerebellum, the hippocampus, the brain stem and the frontal cortex. Adult male rats were treated with lead acetate (500 ppm) through drinking water for a period of 8 weeks and parallel controls were maintained on sodium acetate. Both control and exposed rats were sacrificed at intervals of 4 and 8 weeks, brains were isolated and different regions namely the cerebellum, the hippocampus, the frontal cortex and the brain stem were separated and processed to investigate PARP, bcl-2 and caspase-3 expression using western blotting. The results suggest that lead induces region-specific response of expression in apoptotic proteins of rat brain showing more effect in hippocampus and cerebellum and less effect in frontal cortex and brain stem and it is tissue specific. However, results appear to conclude that PARP induced expression in hippocampus and cerebellum was more followed by mitochondrial and cytosolic damage.
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1. Introduction

Apoptosis is a tightly regulated and highly efficient program of cell death which requires interplay of multiple factors. The components of the apoptotic signaling network are genetically encoded and are considered to be usually in place in a nucleated cell ready to be activated. Caspases involved in inflammation and apoptosis, are evolutionarily conserved family of cysteinyl aspartate-specific proteinases (Ishizaki et al., 1995; Weil et al., 1996; Logue and Martin, 2008). The apoptosis can be triggered by various stimuli from outside or inside the cell, such as: (a) by ligation of cell surface receptors, (b) DNA damage as a cause of defects in DNA repair mechanisms, (c) treatment with cytotoxic drugs or irradiation, (d) lack of survival signals, (e) contradictory cell cycle signaling, (f) by developmental death signals (Stellar, 1995). Death signals of such diverse origin eventually activate common cell death machinery leading to the characteristic features of apoptotic cell death. This is often associated with characteristic morphological and biochemical changes with the activation of nucleases that degrade the chromosomal DNA into small oligonucleosomal fragments (Clarke, 1990; Krysko et al., 2008). Because of significant nuclear changes at an early stage of apoptosis, cells are killed by endonucleolytic cleavage of their DNA and by reactive oxygen species (Lau et al., 2008). It has been suggested that apoptosis may be caused by the accumulation of reactive oxygen species in a process that might be controlled by levels of bcl-2 (Hockenbery et al., 1993). According to Giovannetti et al. (2008), the apoptotic mechanisms are used to regulate the development of thymocytes, the shaping of T cell repertoire, its selection and the coordinate events leading to immune responses in the periphery. The activation of caspase cascade is a primary step in apoptosis, which initiates apoptotic signals (Formigili et al., 2000). One of the earliest signs of apoptosis is the externalization of phosphotidyl serine, providing an "eat me" signal for phagocytosing cells (Grimsley and Ravichandran, 2003). Proteins that are cleaved during apoptosis are spectrin, β-catenin, gelsolin, growth arrest-specific-2 and p21 activated kinase-2 (Ziegler and Groscurth, 2004). Interleukin-1-converting enzyme is an intracellular protein cleaving enzyme (a protease) that induces inflammation and this indicates that the death program depends on protein cleavage (Martin, 1998). One of the mechanisms is the granzyme B-mediated cleaving and activation of procaspase cascade during virus infection. At times, stressed cells insert both Fas and Fas-ligand procaspases and kill themselves. In mitochondrial damage, cytochrome *c* is released and it binds with an adaptor molecule (CED-4) required for apoptosis whereas in some cases, cytochrome *c* escapes from mitochondria and activates procaspase-9 increasing

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the speed and efficiency of the death process. However, the accumulation of cancer suppressing protein p53 is one of the pathways for DNA damage and apoptosis (Martin, 1998; Das et al., 2008).

Lead is a potent neurotoxicant and alters *N*-methyl D-aspartate-specific glutamate receptor binding (Rajanna et al., 1997) and ion regulation (Yallapragada et al., 2003) in different regions of the rat brain both *in vitro* and *in vivo*. These disturbances caused by lead can be attributed to an increase in reactive oxygen species in the rat brain as it has a high rate of oxidative metabolism and high content of unsaturated lipids. Recently, we have reported that lead induces alteration of antioxidant enzymes in adult and developing rat brain exposed to lead (Bennet et al., 2007; Kiran Kumar et al., 2008). These imbalances in the cellular compartment result in the accumulation of oxidatively modified molecules (lipids, proteins and nucleotides) which may eventually lead to cell death (Floyd and Hensley, 2002). Moreover, the brain is considered to be a sensitive organ prone to oxidative damage because of its low levels of protective enzymes to eliminate free radicals. Reactive oxygen species formation has been proposed to be the final common pathway for apoptosis by most of the neurotoxicants including lead (Kodavanti, 1999; Bolin et al., 2006). From the available literature, it is evident that the molecular mechanisms of lead-induced apoptosis are not yet completely understood and research information is scanty in this regard, particularly in the brain tissues. In continuation of our earlier investigation on lead-induced free radical generation (Bennet et al., 2007; Kiran Kumar et al., 2008), an attempt has been made to study further the apoptotic proteins poly (ADP-ribose) polymerase (PARP), bcl-2 and caspase-3 in nuclear, mitochondrial and cytosolic fractions respectively from different regions of the rat brain on chronic exposure to lead.

2. Materials and methods

2.1. Materials

Lead acetate (99.8%) and all the chemicals required for the electrophoresis were purchased from Sigma (St. Louis, USA). Polyclonal primary antibodies for caspase-3, PARP, bcl-2 were purchased from Biovision (Mountain View, USA) and secondary antibodies were purchased from Banglore Genei (India).

2.2. Methods

2.2.1. Treatment

The male rats (100–120 g) of Wistar strain were treated with lead acetate (500 ppm) through drinking water for a period of 8 weeks and parallel controls were maintained on sodium acetate throughout the experiment. Both control and exposed rats were sacrificed by cervical dislocation at intervals of 4th and 8th week and the whole brains were isolated immediately. The brains were washed in ice-cold normal saline solution and different regions namely the cerebellum, the hippocampus, the frontal cortex and the brain stem were separated on ice. Due to small amount of tissue, the hippocampus was pooled separately both in exposed and control animals. The tissues were immediately stored at −60 °C until use. The stored tissues were used to determine the apoptotic proteins by monitoring the expression of caspase-3, bcl-2 and poly (ADP-ribose) polymerase (PARP) in the cerebellum, the hippocampus, the brain stem and the frontal cortex of control and exposed animals.

2.2.2. Tissue preparation for blotting

A 10% homogenate of each brain region from control and exposed rats was prepared by using isolation buffer [0.32 M sucrose; 10 mM Tris–HCl, pH 8; 0.1 mM $MgCl_2$; 1 mM EDTA (ethylene diamine tetra acetic acid) and 0.2 mM PMSF (phenazine methionine sulphonyl flouride] (Kim et al., 1997a). Then, the homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C. The crude nuclear pellet obtained was used for detecting PARP protein, and this pellet was suspended in the homogenizing buffer. The resulting supernatant was further centrifuged at 12,300 × *g* for 20 min at 4 °C. The pellet obtained was the crude mitochondrial fraction which was used to determine bcl-2 protein. This pellet was suspended in homogenizing buffer for loading on to the gel. The resulting supernatant was the cytosolic fraction and was used to determine caspase-3. The amount of protein in all these three fractions was estimated by Lowry et al. (1951). A known and equal amount of nuclear, mitochondrial and cytosolic fractions were loaded on to a polyacrylamide gel (12%) (Biotech, India) and later, the gel proteins were electro-transferred by western blotting on to a nitrocellulose membrane to determine the expression of PARP, bcl-2 and caspase-3 (Nihei and Guilarte, 2001).

2.2.3. PARP assay

The gel nuclear proteins that were electro-transferred by western blotting on to a nitrocellulose membrane were probed with primary antibody (1:1000 dilution) (Anti rabbit PARP obtained from Bio-vision, UK) overnight at 4 °C on a rocker after incubating the membrane with blocking solution for 3 h. It was then washed with buffer and probed with secondary antibody (1:500 dilution) (Anti-Antig rabbit obtained from Bangalore Genei, India) overnight at 4 °C on a rocker. After incubation with primary and secondary antibodies, the protein expression of PARP was monitored in the dark with the addition of alkaline phosphatase as a substrate.

2.2.4. bcl-2 assay

The gel mitochondrial protein bands obtained by western blotting were transferred on to a nitrocellulose membrane. Then, this membrane was incubated with blocking solution for 3 h. The membrane was suspended in primary antibody (1:1000 dilution) (Anti rabbit bcl-2 obtained from Bio-vision, UK) solution overnight. Later, the membrane was washed with buffer and probed with secondary antibody (1:500 dilution) (Anti-Antig rabbit obtained from Bangalore Genei, India) overnight at 4 °C on a rocker followed by the addition of alkaline phosphatase as a substrate to examine the expression of bcl-2.

2.2.5. Caspase-3

The cytosolic proteins extracted on to the gel were electro-blotted on nitrocellulose membrane followed by incubating the membrane with blocking solution for 3 h. The transferred proteins were probed with primary antibody (1:1000 dilution) (Anti rabbit caspase-3 obtained from Bio-vision, UK) overnight at 4 °C on a rocker. Later, the membrane was washed with Towbin buffer and then probed with secondary antibody (1:500 dilution) (Anti-Antig rabbit obtained from Bangalore Genei, India) overnight at 4 °C on a rocker. After incubation with primary and secondary antibodies, the protein expression (caspase-3) was monitored in the dark with the addition of alkaline phosphatase as a substrate.

2.2.6. Analysis of the data

The quantification of bands was achieved by using Scion NIH image analysis software (Image J) version 3.5 (USA). The data were analyzed by two-way ANOVA (Snedecor and Cochran, 1967) to study the significant difference between the means of control and exposed in each region of the brain, and the effect of treatment period.

3. Results

Fig. 1 represents the data on the PARP expression in four different regions of control and exposed rat brain. A significant increase in this protein was observed in all the regions of exposed brain with respect to control except the frontal cortex (F: 8.02; $P > 0.05$). This protein expression was found to increase significantly ($P < 0.05$) from 4th week onwards in hippocampus (F: 826.8; $P < 0.05$) and cerebellum (F: 14.0; $P < 0.05$). But this increase was more in the hippocampus compared with the other regions of the brain at both intervals of the exposure (Fig. 1). A similar response was noticed for the cerebellum. In the frontal cortex (F: 1.56; $P > 0.05$) and the brain stem (F: 3.0; $P > 0.05$), the increase in the expression of PARP was not much significant from 4th to 8th week of exposure. Overall, according to two-way ANOVA, the order of the PARP expression is the hippocampus > the cerebellum > the brain stem > the frontal cortex.

The data on bcl-2 protein expression in four regions of the control and the exposed brain are presented in Fig. 2. There was a time-dependent increase in the expression of bcl-2 in the cerebellum and this increase was found to be statistically significant (F: 12.0; $P < 0.05$) compared with their respective controls. However, there was a slight increase in the bcl-2 expression in all the other regions of brain but these values were not statistically significant between treatment periods. At both the treatment periods, the bcl-2 expression of exposed was found to be significantly different from their respective controls regions such as cerebellum (F: 18.77; $P < 0.05$); hippocampus (F: 11.37; $P < 0.05$); frontal cortex (F: 11.28; $P < 0.05$) and brain stem (F: 37.23; $P < 0.05$).

The results on caspase-3 protein expression in all the four regions of the control and the exposed rat brain are presented in

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