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Impact of early developmental arsenic exposure on promotor CpG-island methylation of genes involved in neuronal plasticity

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ABSTRACT

Epigenetic mechanisms are crucial to regulate the expression of different genes required for neuronal plasticity. Neurotoxic substances such as arsenic, which induces cognitive deficits in exposed children before any other manifestation of toxicity, could interfere with the epigenetic modulation of neuronal gene expression required for learning and memory. This study assessed in Wistar rats the effects that developmental arsenic exposure had on DNA methylation patterns in hippocampus and frontal cortex. Animals were exposed to arsenic in drinking water (3 and 36 ppm) from gestation until 4 months of age, and DNA methylation in brain cells was determined by flow cytometry, immunohistochemistry and methylation-specific polymerase chain reaction (PCR) of the promoter regions of reelin (RELN) and protein phosphatase 1 (PP1) at 1, 2, 3 and 4 months of age. Immunoreactivity to 5 methyl-cytosine was significantly higher in the cortex and hippocampus of exposed animals compared to controls at 1 month, and DNA hypomethylation was observed the following months in the cortex at high arsenic exposure. Furthermore, we observed a significant increase in the non-methylated form of PP1 gene promoter at 2 and 3 months of age, either in cortex or hippocampus. In order to determine whether this exposure level is associated with memory deficits, a behavioral test was performed at the same age points, revealing progressive and dose-dependent deficits of fear memory. Our results demonstrate alterations of the methylation pattern of genes involved in neuronal plasticity in an animal model of memory deficit associated with arsenic exposure.

1. Introduction

Numerous recent reports are gradually revealing that environmental and dietary signals reach the deepest levels of cellular organization by modifying epigenetic programs leading to the silencing or over expression of certain genes. This issue is becoming of critical importance for the function of the central nervous system since not only during development but also in fully differentiated neurons, epigenetic mechanisms play a crucial role in neuronal plasticity (Borrelli et al., 2008; Feng et al., 2010).

The continuous creation, reinforcing and elimination of synapses that are characteristic of neuronal plasticity involve changes in gene expression. Levenson et al. (2006) demonstrated *in vitro* that the methylation of the promoter regions of genes involved in neuronal plasticity, such as reelin (RELN) and brain derived neurotrophic factor (BDNF), is a dynamic process, showing also that the inhibition of DNA methylation blocks the induction of long term potentiation (LTP). *In vivo,* employing a mice model of contextual fear conditioning (CFC), Miller and Sweatt (2007) found that the inhibition of DNA methylation blocks memory formation. The process of CFC was associated with decreased methylation of the RELN promoter with a significant increase of protein expression. In contrast, the methylation of protein phosphatase 1 gene (PP1) promoter was associated to a diminution of protein expression. Therefore, the concerted expression of different genes required for synapse reinforcement that underlies memory formation is intimately linked to dynamic changes in DNA methylation. Recently, the inhibition of histone deacetylase 1 (HDAC1) has been reported to ameliorate memory formation in a mice model of Alzheimer disease (Kilgore et al., 2010). Thus, *in vitro*

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and *in vivo* evidence supports the notion that dynamic chromatin remodeling takes place during the process of adult neuronal plasticity. Nowadays, the study of changes of histone acetylation and methylation as well as DNA methylation, among other epigenetic mechanisms, is providing molecular basis for the alterations in gene expression observed in individuals with addiction, depression and neuropsychiatric disorders (Murgatroyd et al., 2009; Gräff and Mansuy, 2009; Tsankova et al., 2007).

Epigenetic mechanisms are potential targets for the neurotoxic action of diverse substances that induce cognitive dysfunction in the human population, mainly when such exposure takes place during prenatal and early postnatal development (Vahter, 2007; Bellinger, 2008). An archetypical example of these substances is arsenic, whose presence in drinking water results in millions of exposed individuals around the world (IARC, 2004; NRC, 2001; WHO, 2001), with a growing number of reports demonstrating cognitive deficits in exposed children (Calderon et al., 2001; Tsai et al., 2003; Wasserman et al., 2004; Vahter, 2007; Rosado et al., 2007). One of the plausible links between arsenic exposure and epigenetic alterations is its biotransformation, which has an effect on DNA methylation by sharing the same methyl donor (S-adenosyl methionine) required for chromatin remodeling (Lin et al., 2002). In this regard, it has been reported that arsenic exposure alters the pattern of DNA methylation in keratinocyte cultures (Reichard et al., 2007). In this context, we hypothesized that developmental arsenic exposure in rats induces learning deficits through the disruption of hippocampal and cortical DNA methylation patterns. For this purpose, we assessed the effect of intrauterine and postnatal arsenic exposure on contextual learning processes and brain DNA methylation. Our data corroborate that arsenic exposure is associated with memory deficit, and suggest that alterations of the methylation pattern of genes involved in neuronal plasticity may contribute to this condition.

2. Experimental procedures

2.1. Reagents and antibodies

Most reagents were molecular biology or analytical grade reagents and were prepared in molecular grade water (18.0 Ω/cm). Solvents, salts and enzymes were obtained from Sigma–Aldrich (St. Louis, MO, USA), Bio-Rad Lab (Hercules, CA, USA) and J.T Baker (Phillisburg, NJ, USA). The rest of the reagents and antibodies were CpG methyltransferase from New England BioLabs (Ipswich, Ma, USA), the wizard DNA clean up system from Promega (Madison, WI, USA), Taq DNA polymerase from Vivantis (Vivantis Technologies Sdn. Bhd. Singapore), anti-5 methyl cytosine antibody (Calbiochem, Darmstadt, Germany), biotinylated rabbit anti-mouse secondary antibody (DAKO, Carpinteria, CA, USA), FITC-coupled secondary antibody (eBioscience, San Diego, CA, USA), RPMI cell culture medium and fetal bovine serum (GIBCO, BRL).

2.2. Animal model and sample collection

The experiments were performed according to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (2003) and the protocol was approved by the ethics committee of Universidad Autonoma de San Luis Potosi. The experiments started by assigning 24 female and 24 male Wistar rats weighting between 200 and 250 g to three experimental groups of eight couples each. One group received arsenic-free drinking water, the second group had access to water containing 3 ppm of sodium arsenite, a concentration that resulted in an approximate ingestion of 0.3–0.4 mg/kg/day of arsenic (an exposure level that has already been reported in human population (Hsieh et al., 2008). At this concentration only morphological alterations in myelinated central tracts have been demonstrated but no other manifestation of toxicity (Rios et al., 2009). Finally a third group received 36 ppm of arsenic (3–4 mg/kg/day), a dosage that has been associated with structural alterations of myelinated tracts in the striatum, smaller and heterochromatic cell nuclei as well as with demyelination (Zarazua et al., 2010). Each mating pair was placed in a cage and maintained under a 12 h light–dark cycle with food (Prolab RMH 2500, PMI Nutrition International, Brentwood, MO) and water *ad libitum*. After 10 days the males were removed and arsenic exposure continued in the pregnant rats throughout gestation and lactation. Offspring were weaned at 4 weeks, separated by sex and subjected to continued arsenic exposure for 1, 2, 3 or 4 months. As the animals aged, the experiments were always performed in groups of rats belonging to different litters for each treatment as follows: (a) Groups of three female rats for immunohistochemical studies, (b) Groups of three females for flow cytometry determinations in brain and blood cells, (c) Groups of five females for the methylation analysis of specific genes, and, (d) Groups of four to five male rats for behavioral test.

2.3. Behavioral test

The training chamber (acrylic, 20 cm × 25 cm × 25 cm) for the contextual fear conditioning (CFC) test was located inside a larger wooden box so as to isolate the chamber from the surrounding environment and to allow only one person to observe the rats. This person was unaware of the treatment to which each animal had been subjected to. Training started by placing the animal inside the chamber and allowing an exploration period of 2 min after which the rat received the conditioning stimulus (CS) consisting of a four second burst of a 4 dB tone followed by 2 second electric shock (US) of 0.75 mA. Three pairs of these CS/US were applied in 30 s intervals after which animals were given 30 additional seconds in the chamber before returning them to their cage. The evaluations were performed in each animal after 1, 6, 24 and 72 h of the conditioning phase. After placing the animal in the cage and allowing a 30 s exploration period, the CS (tone) was applied resulting in a characteristic immobility behavior (Colon et al., 2006). The animal was observed during 5 min in order to obtain the total frozen time and then returned to its cage. The comparison between total freezing times indicated the difference between treatments at each test point, while the analysis of the response over time for each group allowed the evaluation of the extinction of the conditioned response.

2.4. Flow cytometry analysis

For cell isolation from blood and brain samples, the animals were decapitated, blood was collected in heparinized tubes and the brain was extracted from the skull. The rostral third of each hemisphere was manually fragmented with a surgical blade in 1 mL of Hanks balanced solution and incubated for 1 h with 0.1% collagenase at 37 °C. The resulting suspension was then filtered through a 1 mm^2 mesh, centrifuged and the cells obtained by decantation. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation.

The number of 5-methyl cytosine positive cells in brain and PBMC was quantified by using a specific monoclonal antibody and flow cytometry analysis. For this purpose, cells were fixed in 4% paraformaldehyde for 10 min at room temperature and washed with phosphate buffered saline (PBS). After permeabilization with methanol at −20 °C for 20 min, the cells were treated with 2 N HCl at 37 °C for 30 min in order to expose the CpG islands, followed by one step neutralization with sodium borate (0.1 M, pH 8.5) for 1 min. The incubation with the primary antibody (mouse monoclonal anti 5-methylcitosine) was performed at 37 °C during 45 min, followed by the addition of the secondary rabbit anti-mouse IgG secondary antibody coupled to FITC during 20 min. After washing (1% albumin in PBS), cells were fixed with 1% paraformaldehyde, and analyzed in a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA). Results were expressed as the percent of positive cells and negative controls were run in all experiments.

2.5. Immunohistochemistry

At the time of sacrifice, three rats from each experimental group were deeply anesthetized with 50 mg/kg intraperitoneal sodium pentobarbital. Rats were perfused through the heart with a blunted 18-gauge needle that passed through a small incision in the left ventricle to the proximal ascending aorta. Three hundred mL of 0.1 M PBS; pH 7.4 followed by 300 mL of 4% paraformaldehyde and 0.6% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) were delivered by gravity flow through the needle. Brains were removed, the hemispheres separated from the cerebellum and blocked into rostral and caudal sections, through a coronal division approximately 1 mm posterior to bregma (Paxinos and Watson, 2005), and placed in 4% paraformaldehyde and 0.6% glutaraldehyde in 0.1 M phosphate buffer for 24 h at 4 °C and embedded in paraffin. Coronal brain sections (7 μm) were collected in silanized slides from the frontal cortex region between 3.2 and 4 mm anterior to bregma, and the region of the dorsal hippocampus located approximately at −2.1 of bregma. Next, sections were dewaxed, rehydrated and subjected to a sequence of incubation steps in a humidity chamber, starting with sodium citrate at 100 °C (pH 6) during 30 min for epitope recovery (Lorincz and Nusser, 2008), followed by HCl 3.5 N during 15 min at room temperature with the purpose of exposing CpG islands, and finally with 3% hydrogen peroxide in methanol during 15 for blocking endogenous peroxidase. Sections were incubated overnight at 4 °C with 1:200 mouse monoclonal anti-methylcytocine antibody (which is suited for use in paraffin sections, according to the manufacturer instructions) followed by the streptavidin–biotin marked secondary antibody for 15 min at room temperature. Peroxidase activity was visualized by incubating the sections with diaminobenzidine and the sections were counterstained with Harris hematoxiline. All rinses between the incubation steps were performed with TBST (Tris buffer saline–tween, 0.05 M, pH 7.4). The sections were processed in batches that included 2 slides from each treatment (control, low and high arsenic) with 3 sections each. A negative control was included which consisted of tissue sections treated solely without the primary antibody for each batch. The sections were viewed with a Nikon microscope (Nikon, Labophot – 2, Japan) equipped with a digital camera. Three