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Toxicology

Melatonin attenuates the effects of sub-acute administration of lead on kidneys in rats without altering the lead-induced reduction in nitric oxide

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ABSTRACT

Exposure to lead induces oxidative stress and renal damage. Although most forms of oxidative stress are characterized by simultaneous elevation of nitrogen and oxidative species, lead-induced oxidative stress is unusual in that it is associated with a reduction in nitric oxide (NO) levels in the kidney. The role of NO in kidney injury is controversial; some studies suggest that it is associated with renal injury, whereas others show that it exerts protective effects. Concentration-dependent effects have also been proposed, linking low levels with vasodilatation and high levels with toxicity. The aim of this study was to evaluate the effects of melatonin co-exposure on the lead-induced reduction in renal NO levels. We found that sub-acute intraperitoneal administration of 10 mg/kg/day of lead for 15 days induced toxic levels of lead in the blood and caused renal toxicity (pathological and functional). Under our experimental conditions, lead induced an increase in lipid peroxidation and a decrease in NO. Melatonin co-treatment decreased lead-induced oxidative stress (peroxidation level) and toxic effects on kidneys without altering the lead-induced reduction in renal NO. These results suggest that, in our experimental model, the reduction in renal NO levels by lead exposure is not the only responsible factor for lead-induced kidney damage.

Introduction

Pollution by lead, a heavy metal widely used in industry, is a current global problem [1–3]. Lead induces toxic effects in the body [4], particularly the kidneys, which are among the most affected organs after long-term occupational exposure to lead [5]. The risk of renal cell carcinoma and kidney failure is elevated in people with the highest cumulative exposure to lead [6]. Some studies have shown that kidney cancer mortality increases in workers in the printing industry occupationally exposed to inorganic lead [7].

One mechanism by which lead induces toxicity is oxidative stress, which is an imbalance between concentrations of reactive oxygen and nitrogen species on the one hand and antioxidant species on the other [8]. In cells, reactive oxygen species (ROS) are interrelated with reactive nitrogen species [9]. Oxidative stress may arise from interactions with infectious agents, xenobiotics or diseases, such as diabetes, simultaneously inducing oxidative and nitrosative stress.

Nitric oxide (NO) is a free radical gas with many physiological functions: it is an endothelium-derived relaxing factor in the cardiovascular system, a neurotransmitter, and a cytotoxic agent in the immune defense network [10]. NO is produced by oxidation of L-arginine to L-citruline through nitric oxide synthase (NOS). There are three NOS forms: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). eNOS and nNOS are constitutively expressed and are activated by a rise in intracellular calcium. In contrast, iNOS expression is not regulated by calcium, but is instead regulated transcriptionally by cytokines, such as interferon-γ, interkeukin-1β and tumor necrosis factor-α, as well as by oxidative stress.

The hormone melatonin, which is secreted by many organs but mainly by the pineal gland, has many functions in the body. Anti-inflammatory actions and pro-inflammatory actions of melatonin suggest a role in the immune response [11]. Importantly, melatonin reduces oxidative stress, an action that it accomplishes through two mechanisms. First, it acts as a scavenger of both oxygen- and nitrogen-based reactive molecules [12]. Second, it acts

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through melatonin receptors to increase the expression of antioxidant enzymes, for example upregulating superoxide dismutase and glutathione reductase in corneal fibroblasts [13] and inducing the synthesis of glutathione (GSH) in hamster ovary cells [14]. Moreover, melatonin *in vivo* reduces the oxidative stress of lead [15] and protects the kidney and liver against its toxic effects [16].

Melatonin reduces NO production by inhibiting iNOS *in vitro* [17]. In rats, short-term treatment with melatonin (10 mg/kg/day) has been shown to decrease NO production induced by carbon tetrachloride (0.8 g/kg/bw) in the rat liver [18]. Recently, synthetic melatonin analogs have been synthetized as inhibitors of iNOS and nNOS forms [19].

Lead induces an unusual oxidative stress in the kidney characterized by a reduction in NO levels. The effects of inhibiting iNOS in the kidneys implicate this isoform in the mechanism underlying this lead-induced reduction in NO [20]. In the kidneys, NO is synthesized in glomeruli, vessels and tubular segments, including the macula densa and inner medullary segments. The reduction in kidney NO level following exposure to lead is associated with an increase in vascular resistance, deterioration of renal function, and hypertension.

The aim of this work was to study the sub-acute effect of co-administration of melatonin and lead acetate on oxidative stress and NO levels in the kidneys. These effects were correlated with renal functions and pathological kidney alterations. Moreover, body weight, kidney weight, blood effects, and DNA damage in lymphocytes were analyzed.

Materials and methods

Chemicals

Histopaque-1077 and all other compounds were purchased from the Sigma Chemical Company.

Animals and experimental design

Male, 5-week-old Sprague-Dawley rats were used in these experiments. Rats were housed in steel cages with an illumination period of 12 h/day, and provided a commercial diet and purified water *ad libitum*. After a 1-week acclimation period, animals were divided into four groups (n = five rats/group): (1) control, (2) 10 mg melatonin/kg body weight/day, (3) 10 mg Pb/kg body weight/day, and (4) 10 mg Pb/kg body weight/day plus 10 mg melatonin/kg body weight/day at different times of day. All groups were very similar in age and body weight. Melatonin and lead acetate were administered by intraperitoneal (ip) injection. The number of animals used was the minimum necessary to obtain relevant results. All animals were handled in accordance with the guidelines of our institute and followed international norms of animal care and maintenance.

Body weight

The body weight of each animal was measured weekly.

Melatonin administration

Melatonin (10 mg/kg body weight) was administered daily (ip) to animals during the designated night period.

Lead administration

Rats were administered *lead acetate* (ip) at doses of 10 mg/kg body weight, 5 days per week for 3 weeks.

Lead quantification

For lead determination, 100 μL of internal standard (^{115}In and ^{209}Bi) was added to kidney samples (400 μL), and a wet digestion was carried out with 500 μL of concentrated nitric acid for 2 h at 120 °C. In the second step, 200 μL of H_2O_2 (30%) was added and the mixture was again incubated at 120 °C for 1 h. Finally, after cooling to room temperature, the volume was brought to 5 mL with deionized water and the sample was centrifuged. The sample was then analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) using a Model 7500ce ICP-MS system (Agilent Technologies, Tokyo, Japan) with a Meinhard nebulizer and Peltier-cooled spray chamber (2 °C). Each analysis was carried out in triplicate, and blanks were run in parallel. The instrument operating conditions were as follows: forward power, 1500 W; plasma gas flow rate, 15 L/min; carrier gas flow rate, 0.89 L/min; make-up gas flow rate, 0.15 L/min; sampling depth, 10 mm; dwell time, 300 ms per isotope; platinum sampling and skimmer cones were used. The isotopes ^{206}Pb, ^{207}Pb, ^{208}Pb were monitored and standardized to ^{115}In and ^{209}Bi. Calibration was performed using Agilent commercial standards at lead concentrations of 0, 0.2, 0.4, 1.0, 2.0, 5.0 and 10 μg/L with the internal standards Bi and In (5.0 μg/L each). The lead detection limit was 0.02 μg/L.

Histopathological examination

Kidneys were fixed in a 10% formalin solution for 2 weeks. Tissues were sectioned at a thickness of 4 μM and embedded in paraffin. Kidney sections were stained with hematoxylin and eosin (H&E) and examined for histological changes.

NO measurement

NO levels were measured as described by the manufacturers of a colorimetric assay kit (NB98; Oxford Biomedical Research) based on an enzymatic reduction by nitrate reductase that converts nitrate into nitrite. The amount of nitrite was quantified by adding Griess Reagent and monitoring the completed reaction at 540 nm.

POx measurement

The level of peroxides in the kidney was measured with a Peroxi Detect kit (PD1 KT Sigma), which is based on the conversion of Fe^{2+} into Fe^{3+} ions by peroxides under acidic conditions. The resulting Fe^{3+} ions form a colored adduct with xylenol-orange, which is measured at 560 nm. The endogenous iron value was subtracted from the sample value obtained using the color reagent from the sample value obtained using the reagent blank. This assay was carried out in accordance with the manufacturer's protocol.

Comet assay

The comet assay used was based primarily on a previously described protocol [21]. In brief, four folded comet slides were made for each treatment. Lymphocytes (2×10^4) with ~90% viability (estimated by trypan blue exclusion) were mixed with 100 μL of 0.7% agarose, and the mixture was transferred onto a frosted slide (ES 370; Erie Scientific) pre-coated with normal-melting agarose (1%). A coverslip was added and the slide was cooled on ice to allow the agarose to harden. After the embedding procedure, slides were treated for 1 h with cold lysis buffer (10 mM Tris pH 10, 2.5 M NaCl, 100 mM EDTA, 200 mM NaOH, 1% [v/v] Triton X-100, 10% [v/v] DMSO). After lysis, slides were washed and immersed in cold alkaline unwinding electrophoresis solution (0.3 M NaOH and 1 mM Na_2 EDTA in deionized water, pH 13.5)