Abstract

The influence of L-arginine and Nω-nitro-L-arginine (L-NNA) on lead-induced oxidative stress in kidneys of rats with different resistance to hypoxia was studied. The animals were exposed to lead nitrate (3.6 mg/kg, daily) for 30 d and treated with L-arginine (600 mg/kg) or L-NNA (35 mg/kg) 30 min before each injection of lead nitrate. Lead exposure led to a significant increase in the level of thiobarbituric acid reactive substances (TBARS) in rats with high resistance to hypoxia indicating that lead induced renal oxidative stress. Treatment with L-arginine was effective in decreasing TBARS level for this group of animals. L-arginine was ineffective against lead-induced oxidative stress when given to rats with low resistance to hypoxia. In contrast, administration of L-NNA provided significant prooxidative effect on renal lead-induced oxidative stress in rats with low and high resistance to hypoxia. Renal antioxidant defence system remained sensitive to lead-induced oxidative stress and any of the treatments by L-arginine and L-NNA.

Key words: rats, lead-induced oxidative stress, antioxidant defence system, resistance to hypoxia, kidneys, L-arginine, Nω-nitro-L-arginine.

Lead is environmental pollutant with potential public health hazard (5, 6). Its toxicity continues to be a major health problem due to a broad range of physiological, biochemical, and behavioural dysfunctions in animals and human (6). Although the mechanisms by which lead induces oxidative stress are not completely understood, evidence indicates that multiple mechanisms may be involved (5, 27). Recent studies point to the fact that at least some of the effects may occur as a consequence of lead propensity for disrupting the prooxidant and antioxidant balance into mammalian cells (5). Generation of highly reactive species like hydroxyl radical, hydrogen peroxide, superoxide anions, and lipid peroxide in the aftermath of lead exposure may result in depletion of intrinsic antioxidant defences of cells (6).

Multiple data indicate that nitric oxide (NO) plays an essential signalling role in many systems (3, 7, 11, 15). Physiological concentrations of released NO have been reported to cause protective effect in various cell models (4, 9, 24). The role of NO and inhibition of NO synthase in renal failure is debatable. Gupta et al. (9) suggested that NO plays a significant role in the pathophysiology of iron-induced renal failure and administration of NO donors (nitroprusside and L-arginine) can be valuable in the treatment of acute renal failure. L-arginine significantly attenuated the oxidative stress and nephrotoxic effect of cisplatin. L-NAME, an inhibitor of NO synthase, was found to aggravate cisplatin-induced renal oxidative stress (24). On the other hand, Dursun et al. (4) showed that inhibition of NO production by L-NAME in rats exposed to low lead levels increases vascular resistance, decreases renal blood flow and glomerular function, and enhances oxidative stress. Our previous studies showed that pharmacological treatment with L-arginine induced protection during physical loading (18) and lead-induced oxidative stress in the blood of rats with different resistance to hypoxia (26).

The presented study was planned to evaluate the effects of L-arginine on altered oxidative stress parameters before lead-induced oxidative stress and to show that the pharmacological treatment with L-arginine induces protection against lead-induced oxidative stress in the kidneys of rats with different resistance to hypoxia.
Material and Methods

Animals and experimental design. Male white rats (180-220 g) were used in the study. The rats were housed at a constant temperature of 20 ±2°C. The animals had free access to feed and water throughout the experiments. All procedures were done in accordance with guidelines for the care and use of animals in scientific research. Previously, the animals were divided into two groups: rats with low resistance and high resistance to hypoxia. Resistance of rats to hypoxia was evaluated as survival time (min) in the altitude chamber 11,000 m above sea level. Survival time was measured after achieving the altitude. Cessation of breathing served as the criterion for resistance to hypoxia.

Drugs and solutions. The following drugs were used: L-arginine hydrochloride (Sigma Aldrich, USA), Nα-nitro-L-arginine (L-NNA), and lead nitrate. L-arginine hydrochloride and L-NNA were dissolved in isotonic solution. Lead nitrate was suspended in distilled water. All drugs were freshly prepared. All other reagents used were of analytical reagent grade.

Experimental groups. The rats were randomly assigned into four groups: untreated control group (I) consisting six rats with low resistance and six rats with high resistance to hypoxia; Pb group (II) - rats with low resistance (n=6) and high resistance to hypoxia (n=6) receiving daily per os 3.6 mg of lead nitrate/kg b.w. for 30 d; L-arginine and Pb group (III) - rats with low resistance (n=6) and high resistance to hypoxia (n=6) receiving in the same manner 3.6 mg of lead nitrate/kg b.w. and intraperitoneally L-arginine at a dose of 600 mg/kg b.w. 30 min before injection of lead nitrate; L-NNA and Pb group (IV) - rats with low resistance (n=6) and high resistance to hypoxia (n=6) treated like group III, except that they received 35 mg/kg, i.p. of L-NNA, 30 min before injection of lead nitrate.

Tissue isolation. The kidneys were removed from rats after their decapitation. One rat was used for each homogenate preparation. Briefly, the excised kidneys were weighed, washed in ice-cold buffer, and minced. The minced tissues were rinsed with cold isolation buffer to remove blood and homogenised in a glass Potter-Elvehjem homogenising vessel with a motor-driven Teflon pestle on ice. The isolation buffer contained 120 mM KCl, 2 mM K2HPO4, 10 mM HEPES, and 1 mM EDTA; pH of 7.2 was adjusted with KOH.

Biochemical assay. TBARS assay for lipid peroxidation. The malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS) by the method of Kamyszniok (13). Briefly, the reaction mixture consisted of 1.0 ml of 20% trichloroacetic acid solution, 2.1 ml of 10% kidney homogenate (w/v), and 0.8% aqueous solution of 2-thiobarbituric acid. The mixture was heated at 95°C for 10 min. After cooling with ice-cold water, the mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was removed and its absorbance was measured at 540 nm. TBARS were quantified using an extinction coefficient of 1.56·103 M⁻¹ cm⁻¹ and expressed as µmol of TBARS per mg of protein. Protein determinations were performed according to the method described by Bradford (2).

Superoxide dismutase (SOD, E.C. 1.15.1.1). SOD activity was measured spectrophotometrically by the Kostiuk et al. (17) method using quercetin as a substrate after suitable dilution. The reaction mixture in a total volume of 1 ml consisted of 0.1 M sodium phosphate buffer (pH 7.8) and 0.08 mM EDTA in proportion 1:1. Briefly, 0.1 ml of tissue homogenate (dilution 1:1,000) was added to 2.3 ml of distilled water and 1 ml of reaction mixture with EDTA and sodium phosphate buffer. One unit of SOD activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the quercetin (1.4 µM) reduction rates measured at 406 nm in 0 and 20th min. Activity was expressed in units of SOD per mg of protein.

Catalase (CAT, E.C. 1.11.1.6). CAT activity was determined by measuring a decrease in H2O2 in the reaction mixture using a spectrophotometer set at the wave length of 410 nm by the method of Koroliuk et al. (16). The reaction was started by addition of 0.1 ml of tissue homogenate to 2 ml of 0.03% H2O2 and 1 ml of 4% ammonium molybdate solution. One unit of catalase activity was defined as the amount of enzyme required to clear 1 µmol of H2O2 per min per mg of protein.

Glutathione reductase (GR, E.C. 1.6.4.2). GR activity was assayed as described by Glatzle et al. (8) by measuring the oxidation of NADPH2 at 340 nm. The reaction mixture consisted of 67 mM sodium phosphate buffer (pH 6.6), 7.5 mM GSSG, 0.1 ml of kidney homogenate, and 6 mM NADPH2. The specific activity was expressed as nmol NADPH2/min/mg of protein.

Glutathione peroxidase (GPX, E.C. 1.11.1.9). The activity of GPX was measured spectrophotometrically following the method of Moin (19). The assay mixture contained of 0.1 M Tris-HCl with 6 mM EDTA, 12 mM sodium aside (pH 8.9), 4.8 mM GSH, 20 mM t-butylhydroperoxide, and 0.01 M 5,5-dithiobis-2-nitrobenzoic acid. The rate of GSH reducing was followed spectrophotometrically at 412 nm. Glutathione peroxidase activity was expressed as µmol GSH/min/mg of protein.

Statistical analysis. The mean ± SEM values were calculated for each group to determine the significance of inter group difference. Each parameter was analysed separately using one way analysis of variance (ANOVA). To find the difference between the groups Student’s t test was used. P<0.05 was considered to be significant. Correlations between TBARS level and enzyme activities at the set significance level were determined by the regression method. Interactions were established by the Pearson test for linear correlation (32). All statistical calculations were performed on separate data from each individual with STATISTICA version 8.0.
Results

A significant difference in TBARS level in the kidney of rats with high resistance to hypoxia was observed between Pb group (33.45 ±3.16 µmol·mg⁻¹ of protein) at 30 d of intoxication compared to control group (21.47 ±1.15 µmol·mg⁻¹ of protein). For rats with low resistance to hypoxia no significant differences in TBARS level between control and Pb groups were found (Fig. 1).

Administration of L-arginine to lead-exposed rats with high resistance to hypoxia decreased TBARS level by 33.7% (P<0.05). In case of rats with low resistance to hypoxia, L-arginine did not produce any effect on kidney TBARS level. Animals receiving both L-NNA and lead nitrate during 30 d showed higher values of TBARS level. Administration of L-arginine to lead-exposed rats increased TBARS level by 111.6% (P<0.05) and by 61.3% (P<0.05) for rats with low and high resistance to hypoxia, respectively. TBARS level of the Pb group was taken as a reference (Fig. 1).

Statistical significant differences in the SOD activity were observed between Pb and untreated groups during 30-d of intoxication. In Pb group, SOD activity was increased by 78.5% and 95.9% compared with untreated group (P<0.05) and by 61.3% (P<0.05) for rats with low and high resistance to hypoxia, respectively. L-arginine or L-NNA treatment further increased SOD activity significantly (Tables 1 and 2).

In the kidneys, CAT activity increased significantly in Pb group compared to untreated group by 34.4% (P<0.05) and 39.9% (P<0.05) for rats with low and high resistance to hypoxia, respectively. Neither L-arginine, nor L-NNA modified CAT activity significantly. The activity was comparable to that in Pb group. Treatment with L-arginine and L-NNA increased GR activity for rats with low resistance to hypoxia by 72.7% (P<0.05) and by 124.2% (P<0.05), respectively. A significant decrease in kidney GPX activity of rats with high resistance (by 51.9%, P<0.05) was recorded as compared with lead-exposed rats. Treatment with L-NNA before lead intoxication decreased GPX activity of rats with low (by 32.5%, P<0.05) and high resistance (by 57.9%, P<0.05) compared with Pb group.

Several correlations between checked parameters were found (Figs 2, 3). TBARS level of the kidneys from control rats with high resistance correlated inversely with GR activity (r=−0.906, P=0.013) (Fig. 2A). Activity of CAT correlated positively with GPX activity (r=0.829, P=0.041) from lead-exposed rats with high resistance to hypoxia (Fig. 2B).

Thus, CAT activity was connected with GPX activity in the kidneys of lead-exposed rats with low resistance to hypoxia given L-arginine (r=0.870, P=0.024) (Fig. 3A). The relationship between SOD activity and TBARS level was positive in the kidneys of lead-exposed rats with low resistance to hypoxia given L-NNA (r=0.852, P=0.031) (Fig. 3B).

Table 1

<table>
<thead>
<tr>
<th>Antioxidant enzymes activity</th>
<th>Control</th>
<th>Pb</th>
<th>L-arginine and Pb</th>
<th>L-NNA and Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD, U·mg⁻¹ protein</td>
<td>204.70±17.61</td>
<td>365.31±26.34*</td>
<td>520.01±25.51**</td>
<td>577.29±47.52**</td>
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<tr>
<td>CAT, µmol H₂O₂·min⁻¹·mg⁻¹ protein</td>
<td>10.52±0.81</td>
<td>14.14±0.75*</td>
<td>13.49±0.50</td>
<td>14.73±0.52</td>
</tr>
<tr>
<td>GR, nmol NADPH₂·min⁻¹·mg⁻¹ protein</td>
<td>128.29±8.65</td>
<td>114.41±8.92</td>
<td>197.59±16.89**</td>
<td>256.50±19.15**</td>
</tr>
<tr>
<td>GPX, µmol GSH·min⁻¹·mg⁻¹ protein</td>
<td>61.39±2.66</td>
<td>57.18±3.64</td>
<td>50.69±3.21</td>
<td>38.60±4.49**</td>
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</tbody>
</table>

* P<0.05, Pb group was compared vs. untreated group.
** P<0.05, L-arginine and Pb group, L-NNA and Pb group were compared vs. Pb group.

Table 2

<table>
<thead>
<tr>
<th>Antioxidant enzymes activities</th>
<th>Control</th>
<th>Pb</th>
<th>L-arginine and Pb</th>
<th>L-NNA and Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD, U·mg⁻¹ protein</td>
<td>220.24±24.27</td>
<td>431.47±31.64*</td>
<td>940.16±59.55**</td>
<td>888.07±29.52**</td>
</tr>
<tr>
<td>CAT, µmol H₂O₂·min⁻¹·mg⁻¹ protein</td>
<td>10.35±0.98</td>
<td>14.48±0.23*</td>
<td>15.01±0.54</td>
<td>15.04±0.60</td>
</tr>
<tr>
<td>GR, nmol NADPH₂·min⁻¹·mg⁻¹ protein</td>
<td>127.88±11.68</td>
<td>168.09±11.27</td>
<td>178.11±10.59</td>
<td>171.98±7.91</td>
</tr>
<tr>
<td>GPX, µmol GSH·min⁻¹·mg⁻¹ protein</td>
<td>85.31±8.15</td>
<td>41.02±4.07*</td>
<td>52.55±4.03</td>
<td>17.25±2.23**</td>
</tr>
</tbody>
</table>

Symbols as in Table 1.
**Fig. 1.** Effect of L-arginine and L-NNA on TBARS level in the kidney of lead-exposed rats with low and high resistance to hypoxia. Values expressed as mean ± SEM (n=6). * P<0.05, Pb group was compared vs. untreated group (as control). ** P<0.05, L-arginine and Pb group, L-NNA and Pb group were compared vs. Pb group.

**Fig. 2.** Dependences between TBARS level and GR activity (A), between GPX and CAT activity (B) in the kidney of untreated and lead-exposed rats with high resistance to hypoxia, respectively.

**Fig. 3.** Dependences between GPX and CAT activity in the kidneys of L-arginine and lead-exposed rats (A), and between TBARS level and SOD activity in the kidneys of L-NNA and lead-exposed rats (B) with low resistance to hypoxia.
Discussion

The presented study demonstrated differential lipid peroxidation and antioxidant defence system in the kidneys of rats with different resistance to hypoxia. The activity of GPX in the kidneys was higher in rats with high resistance to hypoxia by 39% (P<0.05). This effect could be attributed to its ability to interact with ROS and other radicals to produce less toxic species in the pathogenesis of lead-induced oxidative stress.

Results of the recent studies indicate that ROS generation can constitute the basis of lead-induced oxidative stress (21, 22). ROS inhibit the production of sulphydryl antioxidants and enzyme reactions impairing haeme production, cause inflammation in vascular endothelial cells, damage the nucleic acids, inhibit DNA repair, and initiate lipid peroxidation in cellular membranes (22). The liver, kidneys, and brain have been considered as the target organs for the toxic effects of lead (21). The latest studies suggest that oxidative stress is one of the important mechanisms of toxic effects of lead (5, 22). Results from the present study testify the enhanced lipid peroxide level in lead-exposed rats.

The data presented here show that lead exposure for a period of 30 d resulted in significant (P<0.05) increase in lipid peroxide level in the kidneys of rats with high resistance to hypoxia. Changes in the TBARS level in lead-exposed rats with low resistance to hypoxia did not reach statistical significance (Fig. 1). Our results are in agreement with previous studies and indicate that lead intoxication increases lipid peroxidation level (1, 5, 6, 22).

It has been assumed that the oxidative stress plays a key role in lead-induced toxicity (1, 5, 6, 22, 29). The mechanisms for lead-induced oxidative stress include the effects of lead on membranes, DNA, and antioxidant defence systems of cells (1). Effects of ROS generation have been postulated to be major contributors to lead-exposure related diseases. Recent toxicological studies have reported that lead exposure is capable of generating ROS, and inducing oxidative damage to the brain, heart, kidneys, and reproductive organs with neurological, haematological, gastrointestinal, reproductive, circulatory, and immunological pathology (22). Flora et al. (6) demonstrated that lead causing oxidative burst in the exposed individuals is leading to tissue damage.

Although several mechanisms have been proposed to explain the lead-induced toxicity (22, 27), no mechanisms have been yet determined. Yin and Lin (31) demonstrated lead-catalysed peroxidation of membrane polyunsaturated fatty acids. Nemsadze et al. (20) suggest that the targets for the toxic effects of lead are the haem synthesis pathway, thiol-containing antioxidants, and enzymes (SOD, CAT, GPX, glucose 6-phosphate dehydrogenase, and glutathione). Sivaprasad et al. (25) showed a decline in the thiol capacity of the cell, accompanied by high TBARS levels with lowered activities of SOD, CAT, GPX, and glutathione metabolising enzymes (GR, glucose-6-phosphate dehydrogenase, glutathione-S-transferase) in the kidneys of rats subjected to lead acetate in drinking water for 5 weeks.

Presently, we have noted that significantly elevated SOD and CAT activities in the kidneys of rats with low resistance to hypoxia provided a protection to TBARS levels, suggesting their ability to act as a free radical scavenger and protect cells against toxic effect of lead. In kidneys of rats with high resistance to hypoxia, the main role of prevention against lead-induced lipid peroxidation plays GPX. Inhibition of GPX activity leads to increased TBARS level in the kidneys of lead-exposed rats with high resistance to hypoxia. As anticipated, the relationships between activities of CAT and GPX also agree when the peroxidation process is subsequent to the consumption of antioxidant defence system.

We previously suggested that L-arginine treatment restored blood antioxidant defence systems of lead-exposed animals, especially in rats with low resistance to hypoxia (26). The induction of NO synthesis was proposed for this purpose; its effects on lead-induced oxidative damage were shown to be beneficial (4, 9, 24). L-arginine, NO precursor, seems to be a good antioxidant couple because of its several properties (3, 24). NO has been shown to inhibit the Fenton reaction by binding to ferrous iron and thus preventing the formation of hydroxyl radicals (23) and reduce the ferryl haeme formed from the interaction of haemoglobin with peroxides, and so prevents lipid oxidation. NO may interfere also with the detoxification of hydroperoxides (5, 6).

Therefore, it is plausible to assume that L-arginine inhibits the lipid peroxidation process that is known to be triggered by ROS. Treatment of rats with high resistance to hypoxia by L-arginine reversed the effects of lead on oxidative stress parameters suggesting ROS as a possible contributor to the kidney damage that occurred.

These results are in agreement with other study reporting that NO is a potent antioxidative agent (3). NO is an effective chain-breaking antioxidant in free radical-mediated lipid peroxidation. It reacts rapidly with peroxyl radicals as a sacrificial chain-terminating antioxidant (11). Using low-density lipoprotein as well as model systems, Kelley et al. (14) has been demonstrated that NO can serve as a chain-breaking antioxidant to blunt lipid peroxidation and protect cells against the detrimental effects of ROS. NO terminates can protect the cells against oxidant stress by suppressing iron-induced generation of hydroxyl radicals via the Fenton reaction, interrupting the chain reaction of lipid peroxidation, augmenting the antioxidative potency of reduced glutathione and inhibiting cysteine proteases (3).

Multiple data indicate that NO can both promote and inhibit lipid peroxidation. In the presence of superoxide, NO forms peroxynitrite, a powerful oxidant capable of initiating lipid peroxidation and oxidising lipid soluble antioxidants (10, 28). In light of this, the present study was undertaken to determine the effects of L-arginine against lead-induced oxidative stress in the kidneys of rats with different resistance to hypoxia. The L-arginine, NO precursor, inhibited lipid peroxidation and improved endothelial function in arteries in
hypercholesterolaemic rabbits, as was documented by White et al. (30). The role of L-arginine and NO pathway in glomerular diseases is controversial. Most of the evidence indicates a beneficial change in the renal pathology and function in animals with glomerulonephritides receiving L-arginine. L-arginine-NO pathway has an important role in ameliorating hypertension, renal disease, inflammation, and atherosclerosis (15).

Antioxidant defence system is a potential target for lead toxicity because antioxidant enzymes depend on various essential trace elements for their proper molecular structure and activity (6). SOD catalyses the conversion of superoxide radicals into molecular oxygen and hydrogen peroxide that can in turn be destroyed by CAT or GPX reactions. CAT is an antioxidant enzyme that catalyses the decomposition of H₂O₂ to form water and molecular oxygen. In animals, CAT and GPX detoxify H₂O₂. CAT protects cells from hydrogen peroxide generated within them. Even though, CAT is not essential for some cell types under normal conditions, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response (12). Our results demonstrated that the increase in SOD and CAT activity may result in an increase in lipid peroxidation due to superoxide radicals and H₂O₂ elevation. This situation may reflect the high correlative link between CAT and GPX activity in the kidney from lead-exposed rats (r=0.829, P=0.041).

In our study, the lipid peroxidation level significantly increased in the kidneys of lead-exposed rats treated by L-NNA. The changes in glutathione-mediated antioxidant defence system might either result from reduced glutathione synthesis, GPX activity, or utilisation of glutathione in the detoxification process against induced oxidative stress. The altered glutathione-mediated antioxidant defence system may ultimately affect the thiol disulphide ratio or cellular protein and their functioning. The decrease in GPX activity in lead-exposed animals treated by L-NNA may indicate further consumption of NADPH as a substrate for GR. L-NNA treatment of animals receiving lead for 30 d inhibited GPX activity, which can be explained by the increased need for NADPH.

In summary, our results demonstrated that L-arginine was ineffective against lead-induced oxidative stress when given to rats with low resistance to hypoxia. In contrast, administration of L-NNA provided significant prooxidative effects on renal lead-induced oxidative stress in rats with low and high resistance to hypoxia. Renal antioxidant defence system remained insensitive to lead-induced oxidative stress and any of the treatments by L-arginine and L-NNA.

References


