Low mercury concentrations cause oxidative stress and endothelial dysfunction in conductance and resistance arteries


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Low mercury concentrations cause oxidative stress and endothelial dysfunction in conductance and resistance arteries. Am J Physiol Heart Circ Physiol 295: H1033–H1043, 2008. First published July 3, 2008; doi:10.1152/ajpheart.00430.2008.—Increased cardiovascular risk after mercury exposure has been described, but the underlying mechanisms are not well explored. We analyzed the effects of chronic low mercury concentrations on endothelial modulation of vascular responses in aorta and mesenteric resistance arteries (MRA). Wistar rats were treated with mercury chloride (1st dose 4.6 μg/kg, subsequent dose 0.07 μg·kg⁻¹·day⁻¹ im, 30 days) or vehicle. Blood levels at the end of treatment were 7.97 ± 0.59 μM. Mercury treatment: 1) did not affect systolic blood pressure; 2) increased phenylephrine-induced vasoconstriction; 3) reduced acetylcholine-induced vasodilation; and 4) reduced in aorta and abolished in MRA the increased phenylephrine responses induced by either endothelium removal or the nitric oxide synthase (NOS) inhibitor N⁰-nitro-L-arginine methyl ester (L-NAME, 100 μM). Superoxide dismutase (SOD, 150 U/ml) and the NADPH oxidase inhibitor apocynin (0.3 mM) decreased the phenylephrine-induced contraction in aorta more in mercury-treated rats than controls. In MRA, SOD did not affect phenylephrine responses; however, when coinubated with L-NAME, the L-NAME effect on phenylephrine response was restored in mercury-treated rats. Both apocynin and SOD restored the impaired acetylcholine-induced vasodilatation in vessels from treated rats. Endothelial NOS expression did not change in aorta but was increased in MRA from mercury-treated rats. Vascular O₂⁻ production, plasmatic malondialdehyde levels, and total antioxidant status increased with the mercury treatment. In conclusion, chronic exposure to low concentrations of mercury promotes endothelial dysfunction as a result of the decreased NO bioavailability induced by increases in oxidative stress. These findings offer further evidence that mercury, even at low concentrations, is an environmental risk factor for cardiovascular disease.

 mercury; oxidative stress; nitric oxide; arteries

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The current study aims to explore the chronic effect of low-dose mercury exposure on vascular responses and the possible role of oxidative stress in the effect. We have accordingly developed an experimental model of chronic exposure to low-dose mercury chloride in rats that produces blood mercury levels similar to those found in humans exposed to mercury (5, 16, 28). We have then analyzed the effects of this treatment on: 1) blood pressure; 2) the endothelial modulation of vasoconstrictor and vasodilator responses in conductance and resistance arteries; and 3) ROS production and its participation in these vascular responses. Our findings provide first ever evidence that chronic exposure to low mercury dose causes an endothelial dysfunction in resistance and conductance arteries that is probably due to increases in oxidative stress.

MATERIALS AND METHODS

Animals. Male Wistar rats (3 mo old) were obtained from the Animal Quarters of the Universidad Autónoma de Madrid. Rats were housed during treatment at a constant room temperature, humidity, and light cycle (12:12-h light-dark) with free access to tap water and fed with standard rat chow ad libitum. Rats were divided into two groups: control (vehicle-saline solution im) or treated with mercury chloride for 30 days (1st dose 4.6 μg/kg, subsequent dose 0.07 μg·kg⁻¹·day⁻¹ im to cover daily loss). Systolic blood pressure was measured weekly by a tail cuff plethysmography method. Some rats were housed individually in plastic shoebox cages to determine whether mercury treatment affected either daily water consumption or urinary excretion. Experiments were conducted in accordance with the American Physiological Society guidelines for animal use, the Guiding Principles in the Use of Animals in Toxicology, and with the current Spanish and European laws (RD 1201/2005 Ministerio de Agricultura, Pesca y Alimentación and 609/86). The experimental protocol was approved by the Universidad Autónoma de Madrid Ethics Committee.

Rats were anesthetized with CO₂ and killed by bleeding 30 days after initiating treatment. The thoracic aorta and the third-order mesenteric resistance arteries (MRA) were dissected and placed in Krebs-Henseleit solution (KHS, in mM: 115 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄·7H₂O, 2.5 CaCl₂, 1.2 KH₂PO₄, 11.1 glucose, and 1.18 calcium chloride). Systolic blood pressure was measured weekly by a tail cuff plethysmography method. Some rats were housed individually in plastic shoebox cages to determine whether mercury treatment affected either daily water consumption or urinary excretion. Experiments were conducted in accordance with the American Physiological Society guidelines for animal use, the Guiding Principles in the Use of Animals in Toxicology, and with the current Spanish and European laws (RD 1201/2005 Ministerio de Agricultura, Pesca y Alimentación and 609/86). The experimental protocol was approved by the Universidad Autónoma de Madrid Ethics Committee.

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0.01 Na2EDTA) at 4°C. For analysis of protein expression, some arteries were rapidly frozen in liquid nitrogen and kept at −80°C until the day of analysis.

Blood samples were collected in tubes containing 15% K3EDTA (BD Vacutainer Systems; Preanalytical Solutions, Plymouth, UK) and placed in ice. Blood samples were centrifuged at 1,500 g for 15 min at 4°C. The resulting plasma was kept at −80°C until used to determine malondialdehyde (MDA) concentration and total antioxidant status (TAS).

**Mercury contents.** The mercury levels in acid-digested samples of whole blood at 30 days of treatment were measured in duplicate by atomic fluorescence spectrometry (PSA Analytical, Model 1025 Millenium System) in the Centro de Espectrometría Atómica (Universidad Complutense de Madrid). The accuracy of the mercury analysis was checked with an external reference material (theoretical value, 3.8 ng/ml; experimental value, 3.85 ng/ml).

**Vascular reactivity measurements.** Aortic segments (2 mm in length) were mounted between two parallel wires (75 μm in diameter) in organ baths at 37°C containing KHS gassed with 95% O2-5% CO2 (pH 7.4). Arterial segments were stretched to an optimal resting tension of 1.5 g. Isometric tension was recorded using a force displacement transducer (Grass FT03C) connected to an acquisition system (MacLab/41 ADInstruments, Castle Hill, Australia). MRA segments (2 mm in length) were mounted in a small-vessel dual-chamber myograph for measurement of isometric tension according to Mulvany and Halpern (31). Segments were stretched to their optimal lumen diameter for active tension development. This was determined based on the internal circumference-to-wall tension ratio of the segments by setting their internal circumference, L0, to 90% of what the vessels would have if they were exposed to a passive tension equivalent to that produced by a transmural pressure of 100 mmHg.

After a 45-min equilibration period, MRA and aorta were exposed to 120 and 75 mM KCl, respectively, to check their functional integrity. Concentration-response curves to ACh or diethylamine (DEA)-NONOate were then performed in arteries previously contracted with phenylephrine at a concentration that produced 50% of the contraction to KCl in each case. After 60 min washout, concentration-response curves to phenylephrine were determined. Single concentrations were performed in each segment. Effects of N5-nitro-L-arginine methyl ester (L-NAME), superoxide dismutase (SOD), L-NAME plus SOD, apocynin, and catalase (all from Sigma Chemical, St. Louis, MO) were investigated in parallel by their addition to the organ bath 30 min before either ACh or phenylephrine.

In some experiments, the role of the endothelium on the contractile responses was studied by removing this vascular component. Effectiveness of endothelial removal was confirmed by the inability of 10 μM ACh to induce relaxation.

**Western blot analysis.** Proteins from homogenized arteries (5 μg for Cu/Zn- and Mn-SOD and 20 μg for extracellular (EC)-SOD and endothelial nos (eNOS)) were separated by 7.5% (eNOS) or 12% (Cu/Zn-, Mn-, and EC-SOD) SDS-PAGE. Proteins were transferred to polyvinyl difluoride membranes that were incubated with either mouse monoclonal antibodies for eNOS (1:1,000; Transduction Laboratories, Lexington, UK) or rabbit polyclonal antibodies for Cu/Zn-SOD (0.1 μg/ml; StressGen, Victoria, Canada), Mn-SOD (0.05 μg/ml; StressGen), or EC-SOD (10 μg/ml; StressGen). After being washed, membranes were incubated with anti-mouse (1:5,000; StressGen) or anti-rabbit (1:2,000; Bio-Rad Laboratories, Hercules, CA) immunoglobulin antibody conjugated to horseradish peroxidase. After being washed thoroughly, immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus; Amersham International, Little Chalfont, UK) and subjected to autoradiography (Hyperfilm ECL International). Signals on the immunoblot were quantified with a National Institutes of Health Image V1.56 computer program. The same membrane was used to determine α-actin expression using a mouse monoclonal antibody (1:300,000; Sigma Chemical). Homogenates from human endothelial cells and rat brain were used as a positive control for the eNOS and SOD isoforms, respectively.

**RT-PCR real time assay.** NOX-1 mRNA was determined in MRA and aortic segments from control and mercury-treated rats. Total RNA was obtained using TRizol (Invitrogen Life Technologies, Philadelphia, PA). A total of 0.5 μg of DNase I-treated RNA was reverse-transcribed into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a 10-μl reaction. PCR was performed in duplicate for each sample using 1 μl of cDNA as a template for NOX-1, 1× TaqMan Universal PCR Master Mix (Applied Biosystems), and 10× of Taqman Gene Expression Assays (Rn00586652_m1; Applied Biosystems) in a 20-μl reaction. Quantitative RT-PCR was carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the following conditions: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. As a normalizing internal control, we amplified β2-microglobulin (Rn00560865_m1). To calculate the relative index of gene expression, we employed the 2-DDC method (25) using the untreated samples as calibrator. Results are expressed as the relative expression of mRNA in mercury-treated compared with untreated rats.

**In situ detection of vascular O2− production.** The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate O2− production in situ, as previously described (21). Hydroethidine freely permeates cells and is oxidized in the presence of O2− to ethidium bromide, which is trapped by intercalation with DNA. Ethidium bromide is excited at 546 nm and has an emission spectrum of 610 nm. Frozen tissue segments were cut into 4-μm-thick sections and placed on a glass slide. Serial sections were equilibrated under identical conditions for 30 min at 37°C in Krebs-HEPES buffer (in mM: 130 NaCl, 5.6 KCl, 2 CaCl2, 0.24 MgCl2, 8.3 HEPES, and 11 glucose, pH = 7.4). Fresh buffer containing DHE (2 μM) was applied topically on each tissue section, covered with a cover slip, incubated for 30 min in a light-protected humidified chamber at 37°C, and then viewed with a fluorescent laser-scanning confocal microscope (Leica TCS SP2 equipped with a krypton/argon laser, ×40 objective) using the same imaging settings in control and mercury-treated rats. Fluorescence was detected with a 568-nm long-pass filter. For quantification, four rings per animal were sampled for each experimental region and averaged. The mean fluorescence densities in the target region were calculated.

**TAS measurement.** TAS was measured using the Calbiochem TAS assay kit (Calbiochem-Novabiochem, Bad Soden, Germany) according to the manufacturer’s instructions.

**Measurement of MDA production.** Plasmatic MDA levels were measured by a modified thiobarbituric acid (TBA) assay (38). Plasma was mixed with 20% trichloroacetic acid in 0.6 M HCl (1:1 vol/vol, HCl).

**Table 1. Effect of endothelium removal and L-NAME on vasoconstrictor responses to phenylephrine (Rmax and pD2) in aorta and MRA from untreated and HgCl2-treated rats**

<table>
<thead>
<tr>
<th></th>
<th>Aorta</th>
<th></th>
<th>MRA</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>pD2</td>
<td>Rmax</td>
<td>pD2</td>
<td>Rmax</td>
</tr>
<tr>
<td>Untreated E+</td>
<td>6.68 ± 0.06</td>
<td>86.3 ± 2.5</td>
<td>5.61 ± 0.08</td>
<td>115.8 ± 3.5</td>
</tr>
<tr>
<td>E−</td>
<td>7.50 ± 0.08†</td>
<td>146.8 ± 12.6†</td>
<td>5.91 ± 0.07†</td>
<td>137.6 ± 5.8†</td>
</tr>
<tr>
<td>L-NAME</td>
<td>7.38 ± 0.07†</td>
<td>130.0 ± 1.7†</td>
<td>6.22 ± 0.13†</td>
<td>121.8 ± 3.3</td>
</tr>
<tr>
<td>HgCl2, E+</td>
<td>6.68 ± 0.04</td>
<td>112.8 ± 3.1</td>
<td>6.01 ± 0.10</td>
<td>124.3 ± 2.4</td>
</tr>
<tr>
<td>E−</td>
<td>7.38 ± 0.08†</td>
<td>121.9 ± 4.4</td>
<td>6.22 ± 0.09</td>
<td>123.6 ± 3.8</td>
</tr>
<tr>
<td>L-NAME</td>
<td>7.32 ± 0.07†</td>
<td>130.1 ± 3.1†</td>
<td>6.05 ± 0.07</td>
<td>127.6 ± 2.5</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE of the no. of animals shown in Figs. 1–3. MRA, mesenteric resistance arteries; Rmax, maximal effect; pD2, log one-half Rmax; E+: endothelium intact; E−, endothelium removal; l-NAME, N5-nitro-l-arginine methyl ester. P < 0.05 vs. untreated rats (*) and vs. E+ (†).
and tubes were kept in ice for 20 min to precipitate plasma components and so avoid possible interferences. Samples were centrifuged at 1,500 g for 15 min before adding TBA (120 mM in 260 mM Tris, pH 7) to the supernatant in a proportion of 1:5 (vol/vol); next, the mixture was boiled at 97°C for 30 min. Spectrophotometric measurements at 535 nm were made at 20°C.

**Data analysis and statistics.** Vasoconstrictor responses induced by phenylephrine were normalized to the contraction induced by the higher concentration of KCl used (75 or 120 mM KCl in aorta and MRA, respectively) and expressed as a percentage of this contraction. Vasodilator responses are expressed as a percentage of the previous contraction. For each concentration-response curve, the maximum effect (R_{max}) and the concentration of agonist that produced one-half of R_{max} (EC50) were calculated using nonlinear regression analysis (GraphPad Prism Software, San Diego, CA). The sensitivity of the agonists was expressed as pD2 (-log EC50). To compare the effects of L-NAME or endothelium denudation on contractile responses to phenylephrine, some results were expressed as “differences” in the area under concentration-response curves (dAUC) to phenylephrine in control and experimental situations. AUC were calculated from the individual curve plots (GraphPad Prism Software), and differences were expressed as percentages of the AUC of the corresponding control situation. These values give information of whether the magnitude of effect of either endothelial denudation or L-NAME is different in untreated or mercury-treated rats.

![Graph](image)

**Table 2. Effect of HgCl2 treatment on vasodilator responses (R_{max} and pD2) to ACh and DEA-NONOate in aorta and MRA from untreated and HgCl2-treated rats**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aorta</th>
<th>MRA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pD2</td>
<td>R_{max}</td>
</tr>
<tr>
<td>ACh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>7.42±0.11</td>
<td>80.1±3.6</td>
</tr>
<tr>
<td>HgCl2</td>
<td>6.92±0.14*</td>
<td>64.5±1.1*</td>
</tr>
<tr>
<td>DEA-NONOate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>6.64±0.16</td>
<td>101.7±1.6</td>
</tr>
<tr>
<td>HgCl2</td>
<td>6.65±0.18</td>
<td>103.0±1.7</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE of the no. of animals shown in Fig. 1. DEA, diethylamine. *P < 0.05 vs. untreated rats.

Fig. 2. Effect of endothelium removal (E−) and Nω-nitro-L-arginine methyl ester (L-NAME, 100 μM) on the concentration-response curve to phenylephrine in aortic rings from untreated and HgCl2-treated rats. *P < 0.05 by ANOVA. No. of animals used is indicated in parentheses. Insert shows differences in area under the concentration-response curve (dAUC) in endothelium-denuded and intact segments and in the presence and absence of L-NAME. *P < 0.05 by Student’s t-test.
For protein expression, data are expressed as the ratio between signals on the immunoblot corresponding to the studied protein and α-actin.

Results are expressed as means ± SE of the number of rats indicated; differences were analyzed using Student’s t-test or two-way ANOVA followed by a Bonferroni test. *P < 0.05 was considered significant.

RESULTS

In rats exposed to 30 days mercury chloride treatment, the whole blood mercury level was 7.97 ± 0.59 ng/ml (n = 5). Systolic blood pressure remained unchanged by treatment (control: 133 ± 11.4 mmHg, n = 9; mercury-treated: 126 ± 8.4 mmHg, n = 9, P > 0.05). No differences in body weight between groups were observed either before (control: 353 ± 7.7 g, n = 9; mercury-treated 343 ± 9 g, n = 9; P > 0.05) or after (control: 419 ± 8 g; mercury-treated 395 ± 11.7 g; P > 0.05) treatment. No differences in volumes of daily food intake, drinking water, or urine were observed during the treatment (results not shown).

**Effect of mercury treatment on vasoconstrictor and vasodilator responses.** Mercury treatment increased the contractile responses induced by phenylephrine (1 nM-30 μM) in rat aorta.
Thus mercury increased \( R_{\text{max}} \) but not sensitivity to phenylephrine (Table 1). The response to phenylephrine (0.01–30 \( \mu M \)) in MRA was also increased by treatment (Fig. 1). However, mercury treatment only increased sensitivity but not \( R_{\text{max}} \) in these arteries (Table 1). Mercury treatment did not affect the response to KCl in either aorta (control: 27.9 \( \pm \) 3.4; mercury: 27.2 \( \pm \) 4.6 mN, \( P > 0.05, n = 28–36 \)) or MRA (control: 9.9 \( \pm \) 0.9; mercury: 11.1 \( \pm \) 0.1 mN, \( P > 0.05, n = 28 \)).

ACh (1 nM-30 \( \mu M \)) induced concentration-dependent relaxations in aorta that were reduced by mercury treatment (Fig. 1). Similarly, a decrease in relaxation induced by ACh (0.1 nM-3 \( \mu M \)) was observed in MRA after mercury treatment. In aorta and MRA, mercury treatment diminished both \( R_{\text{max}} \) and sensitivity (Table 2). However, the vasodilator response induced by the NO donor DEA-NONOate (0.01–100 \( \mu M \)) was unaffected by mercury treatment in either aorta or MRA (Fig. 1 and Table 2).

Endothelium removal and incubation with the NOS inhibitor l-NAME (100 \( \mu M \)) both left-shifted the concentration-response curve to phenylephrine in aortic segments from either group, and these effects were smaller in preparations from mercury-treated than control rats, as shown by dAUC values (Fig. 2). In MRA from control rats, both endothelium removal and the incubation with l-NAME left-shifted the concentration-response curves to phenylephrine. However, in MRA from mercury-treated rats, neither endothelium removal nor l-NAME modified the contractile response to phenylephrine (Fig. 3).

These results indicate that mercury treatment induces endothelial dysfunction in both conductance and resistance arteries,
thereby reducing the vasodilator response to ACh and endothelial NO modulation of vasoconstrictor responses. Because eNOS alterations can produce endothelial dysfunction, the expression of this protein was measured. The mercury treatment did not modify eNOS protein expression in aorta although it did increase it in MRA (Fig. 4).

Effect of mercury treatment on oxidative stress parameters. Basal $O_2^-$ production was greater in aorta and MRA from rats treated with mercury chloride than from controls (Fig. 5). The blood oxidant state, as determined by plasmatic MDA levels, and the blood antioxidant state, as determined by plasmatic TAS, were greater in mercury-treated than control rats (Fig. 5). Western blot analysis revealed similar levels of Cu/Zn- and Mn-SOD protein expression in aorta from both groups, but the EC-SOD expression was greater in mercury-treated rats (Fig. 6). However, the MRA protein expression of the three SOD isoforms was similar in both groups.

To determine if the increased $O_2^-$ production observed in arteries from mercury-treated rats contributes to the observed endothelial dysfunction, the effects on vasoactive responses of the superoxide anion scavenger SOD and the NADPH oxidase inhibitor apocynin were analyzed. SOD (150 U/ml) did not modify vasoconstrictor responses to phenylephrine in aortic segments from control rats although it reduced these responses in segments from mercury-treated rats (Fig. 7A). Apocynin (0.3 mM) reduced phenylephrine responses in aortic segments from both groups, and this effect was greater in mercury-treated rats (Fig. 7A). Thus phenylephrine responses were similar in control and mercury-treated rats in the presence of either SOD or apocynin (Fig. 7A). SOD or apocynin did not modify ACh-induced responses in aortic segments from control rats (data not shown); however, the two drugs restored the impaired vasodilator response in segments from mercury-treated rats (Fig. 7B).

In MRA, SOD did not modify phenylephrine responses in either control or mercury-treated rats (data not shown). We next analyzed the effect of SOD on the observed effects of l-NAME on vasoconstrictor responses. In control rats, coincubation of SOD plus l-NAME did not modify the effect of l-NAME alone. However, in mercury-treated rats, SOD restored the l-NAME effect, increasing phenylephrine responses (Fig. 8A). As in aorta, neither SOD nor apocynin (0.3 mM) modified ACh-induced relaxation in MRA from control rats (data not shown); however, both drugs improved the vasodilator response in segments from mercury-treated rats (Fig. 8B). Moreover, catalase (1,000 U/ml) restored the ACh response in mercury-treated vessels (Fig. 8B).

mRNA levels of the NOX-1 subunit of NADPH oxidase were unaffected by mercury treatment in both aorta (control: $1.06 \pm 0.14$; mercury treated: $0.94 \pm 0.16$; $P \geq 0.05$) and MRA, although a tendency to increase was observed in MRA from mercury-treated animals (control: $1.00 \pm 0.17$; mercury treated: $1.33 \pm 0.25$; $P = 0.09$ by Mann-Whitney test).

![Western blot analysis of Cu/Zn-, Mn-, and extracellular (EC)-superoxide dismutase (SOD) protein expression in aorta and MRA from control and HgCl2-treated rats.](http://ajpheart.physiology.org/)

Fig. 6. Densitometric analysis of the Western blot for Cu/Zn-, Mn-, and extracellular (EC)-superoxide dismutase (SOD) protein expression in aorta and MRA from control and HgCl2-treated rats. No. of animals used is indicated in parentheses. *$P < 0.05$ by Student’s $t$-test. Representative blots are also shown.
DISCUSSION

Major findings from the present study demonstrate, for the first time, that a 30-day treatment with a low mercury dose induces endothelial dysfunction in resistance and conductance arteries, probably due to decreased NO bioavailability provoked by increased O$_2$/H$_2$O$_2$ production. This is evidenced by the following effects of treatment. Vasoconstrictor responses to phenylephrine were increased, and NO endothelial modulation of these responses was decreased. The endothelium-dependent vasodilator response induced by ACh was also decreased. Meanwhile, vascular O$_2$$_2$ production, plasmatic MDA levels, and plasmatic TAS were all increased. Both the O$_2$_2 scavenger SOD and the NADPH oxidase inhibitor apocynin restored the NO endothelial modulation of phenylephrine responses and the impaired ACh-induced vasodilatation in vessels from the mercury-treated rats.

The United States Environmental Protection Agency’s recommended reference blood concentration of mercury of 5.8 ng/ml, and below this level exposure is considered to be without adverse effects (33, 36, 41). Some studies have reported that the blood mercury concentration in the control population is ~1 ng/ml, whereas in workers exposed to mercury or in residents of Guizhou (China), an area that is known to suffer mercury contamination, the levels are between 7 to 10 ng/ml (5, 16). In a recent biomonitoring study in New York City adults, the blood mercury concentration was 2.73 ng/ml, whereas in regular fish consumers it reached 5.65 ng/ml (28). In the present study, we used low dose of mercury chloride and attained a blood mercury content of around 8 ng/ml, close to the human exposed levels reached.

Mercury is an identified risk factor for cardiovascular diseases in humans (19). An association between mercury exposure and hypertension in humans (3, 13, 24, 42) and increased blood pressure in rats chronically treated with a high dose of mercury (0.5 mg·kg body wt$^{-1}$·day$^{-1}$ or 200 µg/ml of mercury chloride in drinking water for 180 days) have been reported (4, 46). However, we did not observe changes in systolic blood pressure in our rats after mercury treatment. This is probably due to either the lower blood mercury concentration that was reached in the present work or the shorter treatment period. Additionally, in a previous study using acute mercury administration in anesthetized rats, we described reduced arterial pressure despite mercury-induced vasoconstriction (7). This issue was explained by the reduction of cardiac output observed (39). Despite the lack of change in blood pressure after mercury treatment, phenylephrine-induced contractile responses were increased in conductance and resistance arteries in our study. In agreement, acute exposition to mercury increased sensitivity to phenylephrine in perfused rat tail artery (47). Other authors have found that acute exposure to mercury induces vasoconstriction (7, 9), although vasorelaxation or reduction of the norepinephrine contractile responses has also been reported (15). The specific action of mercury increasing the vasoconstrictor effect of phenylephrine without affecting the response to potassium chloride in both vessels might suggest alterations in the endothelial modulation of these responses after mercury treatment. However, we cannot rule out the possibility that there are more receptors for phenylephrine or that the intracellular signaling pathways are altered in the mercury-treated rats.
Endothelial cells are important regulators of vascular tone through the production of a variety of mediators such as NO, ROS, prostanoids, and endothelin. Moreover, endothelial dysfunction has been widely associated with different cardiovascular diseases. In vitro exposure to mercury chloride or methyl mercury induces cytotoxicity in endothelial cells (15, 23, 27, 48). In addition, acute exposure to a high dose of mercury chloride (1 or 5 mM) impaired ACh-induced relaxation of rat aorta and tail vascular bed (7, 15). In agreement, in the present study, endothelium-dependent relaxation to ACh was reduced in both aorta and MRA from mercury-treated rats. These results demonstrate that a 30-day treatment with a low mercury dose induced endothelial dysfunction in rat resistance and conductance arteries. Because it has been described that ACh-induced relaxation is greatly mediated by NO in both vascular beds (12), we might suggest that the effect of mercury on ACh relaxation was affecting NO production or availability although we cannot discard that mercury treatment also alters the participation of other endothelium-derived factors in the ACh response. In accordance with our hypothesis, we found a leftward shift in the phenylephrine concentration-response curve in rat aorta after endothelial removal or after incubation with the NOS inhibitor l-NAME that was stronger in control than in mercury-treated animals. Similar results were found in perfused tail artery after acute exposition to nanomolar concentrations of mercury chloride (47). In MRA, the effect of mercury treatment was even greater because neither endothelial removal nor l-NAME modified the contractile response to phenylephrine after mercury treatment.

Methyl mercury inhibits NO production and NOS activity in cultured human umbilical vascular endothelial cells (23). The present study shows no change in aorta and even increased eNOS protein expression in MRA. The participation of resistance vessels in the control of peripheral resistance and hence in blood pressure regulation is well known. We might speculate that resistance vessels develop compensatory mechanisms (eNOS upregulation) against the endothelial dysfunction induced by mercury treatment to maintain a normal vascular function. In any case, these results would exclude the possibility that changes in eNOS protein are responsible for the endothelial dysfunction observed after mercury treatment. However, we cannot discard a possible reduction in the activity of this isoform or that uncoupled eNOS due to BH4 deficiency might result in a switch from NO to O2 production (11). Alterations in the NO relaxation mechanism might also explain the inhibitory effect of mercury on endothelial function. However, mercury treatment did not modify the relaxation induced by the NO donor DEA-NONOate in either vessel. Likewise, other authors have shown that acute exposure of rat aortic segments to mercury chloride (1 μM) did not change relaxation induced by glyceryl trinitrate (15).
Several studies have analyzed the relationship between increased ROS production and impairment of endothelium-dependent relaxation in cardiovascular diseases (10, 43). The reaction of O$_2^-$ with NO effectively reduces the bioavailability of endothelium-derived NO. Mercury exposure in animals or humans induces generation of ROS with the subsequent oxidative damage to several organs and systems and alters antioxidant defense systems (5, 19, 20, 22, 26, 29, 35). Herein, we provide evidence that the endothelial dysfunction after mercury treatment would be due to an increased ROS production in aorta and MRA: 1) incubation with exogenous SOD diminished the contractile response to phenylephrine only in aorta from mercury-treated rats and restored the effect of 1-NAME on phenylephrine response in resistance vessels; 2) apocynin diminished vasoconstrictor responses to phenylephrine more strongly in aorta from mercury-treated than from control rats; and 3) both SOD and apocynin restored the impaired acetylcholine-induced relaxation observed in vessels from mercury-treated rats. In addition, in our experimental model of mercury exposure, we found increased plasmatic MDA levels and vascular superoxide anion production in aorta and MRA. Recently, several authors have reported different actions of apocynin independently of its ability to inhibit NADPH oxidase (17, 37). In any case, apocynin has been reported to reduce ROS availability through its antioxidant properties (17). We do not know the exact mechanism of apocynin in our experimental model. The important finding is that both apocynin and SOD increase endothelial function in mercury-treated rats that participate in vascular responses. Unexpectedly, mRNA levels of NOX-1 subunit of NADPH oxidase were unchanged after mercury treatment, although a tendency to increase was observed in MRA from mercury-treated animals. It is possible that protein expression of NADPH oxidase or, even more probably, NADPH oxidase activity is affected by mercury treatment. However, the link between mercury and NADPH oxidase requires further experiments. Apocynin fully recovered the ACh response in both aorta and MRA from mercury-treated animals, whereas SOD had somehow less effect. It might be possible that other ROS different from O$_2^-$ also participate in the impaired ACh response. In fact, it is possible that O$_2^-$ is converted to hydrogen peroxide by endogenous SOD. The fact that catalase also improved the ACh response in mercury-treated vessels suggests that hydrogen peroxide also participates in the endothelial dysfunction described.

The plasmatic TAS was also increased after mercury treatment and the Cu/Zn- and Mn-SOD protein expressions were similar in vessels from both groups. Even more, increased EC-SOD was observed in aorta but not in MRA from mercury-treated rats. As mentioned, exogenous SOD decreased the phenylephrine response in aortic rings from mercury-treated rats. However, in MRA, SOD did not significantly affect the phenylephrine contractile response in either experimental group. These results might suggest that O$_2^-$ modulates phenylephrine responses in aorta more than in MRA. It is therefore possible that, in this specific vascular bed, compensatory mechanisms (SOD upregulation) are developed against the increase in O$_2^-$ after mercury treatment. Other authors have also reported augmented antioxidant defenses after mercury exposure (2, 5, 14). Wolf and Baynes (48) have described that, whereas high concentrations of mercury (>$3–5$ $\mu$M) induced GSH depletion and inhibition of the activity of the thiol enzymes glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase in bovine pulmonary artery endothelial cell monolayer, low concentrations (1–2 $\mu$M) increased GSH and thiol enzyme activities. It is therefore possible that antioxidant mechanisms might become activated in mercury-exposed rats to protect cells against the increased oxidative stress.

In conclusion, we demonstrate that treatment with a low mercury dose alters vascular reactivity and induces endothelial dysfunction. This might be attributable, at least partly, to reductions in NO bioavailability due to the increased ROS production. Environmental contamination has exposed humans to various metal agents, including mercury. This exposure seems to be more common than otherwise expected with as yet unclear health consequences. The present data show that chronic low dose of mercury have an important and deleterious effect on vascular function. This impact could be compared with that produced by traditional cardiovascular risk factors such as hypertension, diabetes, or hypercholesterolemia. Therefore, mercury could be considered an important risk factor for cardiovascular disease that could participate in development of cardiovascular events. Whether this effect can increase consequences of traditional risk factors, or whether it can play a primary role in those patients with low cardiovascular risk, needs to be determined.

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REFERENCES


