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Nitric oxide-dependent vasorelaxation and endothelial cell damage caused by mercury chloride

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Abstract

Mercury and its derivatives are known to constrict vascular smooth muscle cells. However, little is known about the role of endothelial cells in mercury-induced vasoreactivity. Using isolated, norepinephrine preconstricted rat aorta and pulmonary artery rings with intact endothelium, we demonstrate that mercury chloride ($HgCl_2$) induces an endothelial-dependent vasorelaxation which was totally blocked by the nitric oxide inhibitor L-NAME. Besides this vasorelaxant effect, treatment with $HgCl_2$ resulted in functional and morphological alterations of the endothelial cells. On aortic rings, endothelial cells were partly lifted from the basal membrane when incubated for 20 min in $HgCl_2$ (10^{-7} M)-containing buffer. At a concentration of 10^{-6} M, the endothelial cells were completely denuded and acetylcholine vasorelaxation was abolished. Endothelial cell structure and function was preserved by incubating the vessels in $HgCl_2$-containing rat blood instead of buffer. We conclude that $HgCl_2$ induces an endothelial-dependent vasorelaxation and alters structure and function of vascular endothelial cells.

Keywords: Mercury chloride; Nitric oxide; Vasorelaxation; Endothelial cells

1. Introduction

The pathophysiological role of heavy metals on smooth muscle function has been well described. Mercury and its derivatives are known to contract vascular smooth muscle cells and to induce arterial hypertension (Schroeder and Vinton, 1962; Perry and Yunice, 1965;Perry et al., 1967a,b; Perry and Erlanger, 1971, 1974; Massaroni et al., 1995). The hypertensive effects were either evoked by acute and chronic administration and through different routes of application. In accordance with the in vivo hypertensive response, mercury provoked constriction of isolated blood vessels (Perry et al., 1967a,b; Tomera and Harakal, 1986; Evans and Weingarten, 1990). Most studies which have investigated the effects of mercury on the vasculature have focused on smooth muscle cells. How-

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ever, little is known about their actions on endothelial cells. The vascular endothelium is an important regulatory organ in maintaining cardiovascular homeostasis. Endothelial cells, as the inner lining of blood vessels, are strategically located between circulating blood cells and the vascular smooth muscle. The endothelium controls the tone of the underlying vascular smooth muscles through the production of a variety of vascular mediators, such as nitric oxide, prostacyclin (PGI_2) and endothelin (Lüscher and Barton, 1997). A swelling of endothelial cells has been demonstrated on isolated perfused kidneys straight after mercuric chloride administration (Russell, 1975). Histological findings in $HgCl_2$-induced renal failure demonstrated a fibrinoid damage in the media segments of preglomerular renal vessels which additionally were dilated (Zimmermann et al., 1977). In mercury treated guinea pigs an endothelial cell swelling of mitochondrial disintegration and protrusion of endothelial cell cytoplasm herniating into the lumen of the vessel associated with altered vascular permeability was observed in labyrinthine blood vessels (Anniko and Sarkady, 1977). In the present study, we investigated the effect of mercury chloride on vasoreactivity by using isolated blood vessel rings with an intact endothelial cell layer. Since treatment with mercury chloride resulted in both functional and structural damage to vascular endothelial cells, we further investigated whether the composition of the incubating buffer used in the organ bath experiments affected the endothelial cell injury.

2. Materials and methods

Male Sprague–Dawley rats weighing 300–320 g (Charles River Wiga, Sulzfeld, Germany) were used for in vitro experiments. All animal husbandry and animal care procedures conformed to the 'Guide for Care and Use of Laboratory Animals', Department of Health and Human Services (National Institutes of Health). Animals were anesthetized with pentobarbital sodium (30 mg/kg ip), and their lungs were removed carefully. Under microscopic control, 4 mm sections (rings) of the truncus pulmonalis, and aorta thoracalis descendens were dissected out and prepared free of surrounding fatty and connective tissues. The vascular rings were incubated with modified Greenberg–Bohr buffer ((mmol): 119 NaCl, 4.7 KCl, 1.17 $MgSO_4 \cdot 7H_2O$, 19.05 $NaHCO_3$, 1.18 KH_2PO_4, 5.5 glucose, 50 sucrose, and 1.6 $CaCl_2$) in a 20 ml double-wall incubation bath (modified Schuller bath, HSE, March-Hugstetten, Germany), adjusted to pH 7.4, continuously bubbled with 21% O_2, 5% CO_2 and 74% N_2 and warmed to 38 °C. Rings were mounted on steel connectors, which were inserted through the vessel lumen without injuring the endothelium, and attached to a strain gauge isometric pressure transducer unit (K 30-Biegestab, HSE). The isometric pressure of isolated rings was measured over a bridge amplifier (type 302, HSE) and a four-channel record system (Kompensograph C1013, Siemens). The vessel rings were stretched to a passive tension of 1.5 g (A. pulmonalis) and 2 g (Aorta). After an equilibration of 1 h, a concentration–response-curve for acetylcholine-relaxation (10^{-7} to 5×10^{-5} M) was performed on norepinephrine (5×10^{-9} to 10^{-7} M) precontracted vessel rings to test for vessel viability. Thereafter, the incubation bath was flushed several times, and the isometric tension was returned to baseline values. The rings were contracted by norepinephrine in a concentration–response fashion up to 10^{-7} mol/l, which resulted in a submaximal (75%), stable precontraction. The rings were then exposed to buffer-dissolved $HgCl_2$ at different concentrations (10^{-9} up to 10^{-6} M). Experiments were also performed in the presence of the nitric oxide synthase inhibitor L-NAME (10^{-4} M) to test for possible NO-dependent actions. After reaching a stable plateau, acetylcholine (10^{-7} to 5×10^{-5} M), glyceryltrinitate or isoproterenol (10^{-8} to 10^{-5} M) were applied to the rings to test for preserved endothelial and smooth muscle function.

2.1. Incubation studies

Rings (4 mm) from aorta thoracalis descendens were incubated for 20 min at 38 °C (continuously bubbled with 21% O_2, 5% CO_2 and 74% N_2) in modified Greenberg–Bohr-buffer (control) and $HgCl_2$-containing buffer (10^{-7} and 10^{-6} M). Experiments were also performed by using $HgCl_2$-containing (10^{-6} M) rat blood (4 ml) as incubating media. Rat blood cells were separated by centrifugation (5000 U/min, 5 min), washed and resuspended in modified Greenberg–Bohr-buffer (4 ml) before the $HgCl_2$ (10^{-6} M) was added. In addition, aortic rings

were incubated in fresh or heat-inactivated (3 min; 90 °C) rat plasma containing mercuric ions. After incubation for 20 min, rings were fixed in 4% buffered formaldehyde solution, dehydrated, and embedded in paraffin using standard procedures. Sections were stained with hematoxylin–eosin and subsequently examined by light microscopy.

2.2. Chemicals

All substances were obtained from Sigma Chemicals (Deisenhofen, Germany).

2.3. Statistical analysis

All values are presented as mean ± S.E.M. Statistical evaluation of each value was performed using an one-way analysis with Bonferroni for multiple comparisons. Results of curves were compared by using a two-way analysis of variance for repeated measures. If the *P*-value was significant, an one-way analysis of variance with Bonferroni for multiple comparisons was employed to allow comparisons of individual means. Values were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Vasoreactivity

$HgCl_2$ resulted in a powerful relaxation of norepinephrine preconstricted rat aortic and pulmonary artery rings (Fig. 1a and b). Pulmonary artery rings

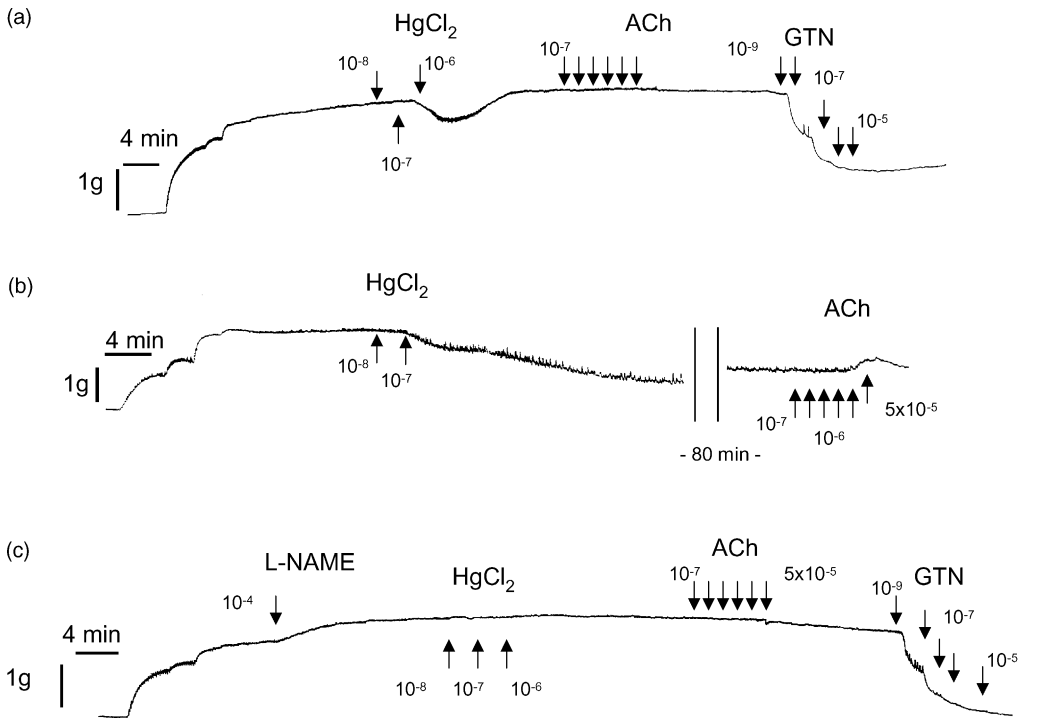

Fig. 1. Original tracing of experiments that demonstrate the relaxant effect of $HgCl_2$ on: (a) rat aortic and (b) pulmonary artery rings. The vessel rings were preconstricted by norepinephrine (5×10^{-9} to 10^{-7} M), which resulted in a submaximal (75%), stable precontraction before a concentration response-curve for $HgCl_2$ was performed. As a test for normal endothelial and smooth muscle function, the vessel rings were further treated with acetylcholine (10^{-7} to 5×10^{-5} M) or glyceryltrinitrate (10^{-9} to 10^{-5} M); (c) pulmonary artery rings preincubated with L-NAME (10^{-4} M) before treatment with $HgCl_2$.

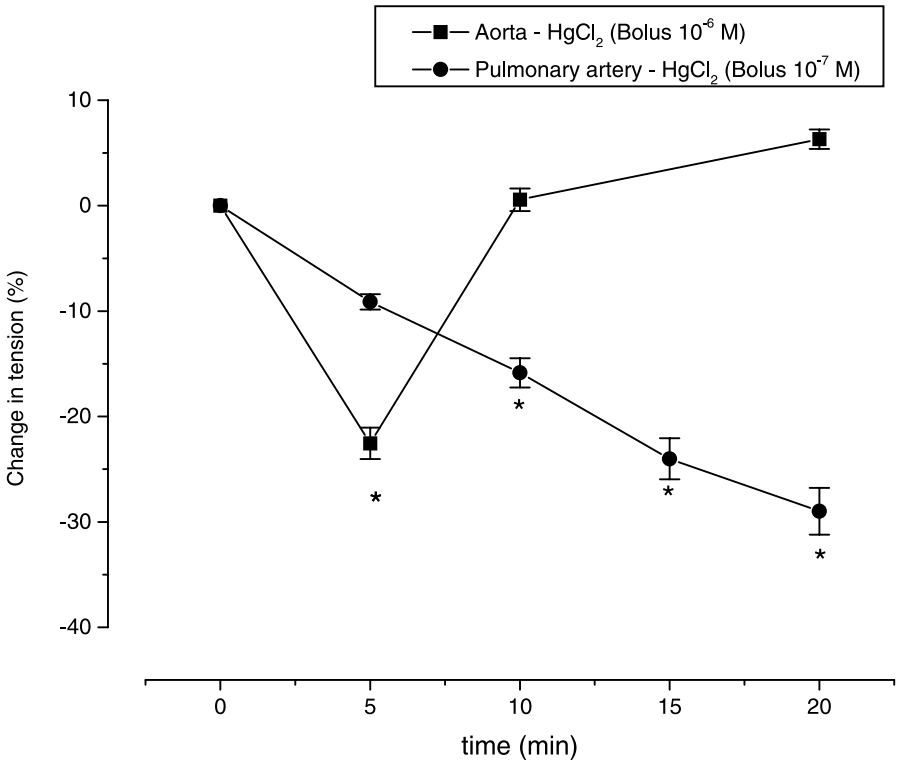

Fig. 2. Time-course of $HgCl_2$-induced relaxation on isolated, norepinephrine (10^{-7} M) preconstricted rat aortic ((■) $n = 31$) and pulmonary artery rings ((●) $n = 19$). The application of 10^{-7} M $HgCl_2$ resulted in a long-lasting relaxation of pulmonary artery rings. Rat aortic rings were treated with $HgCl_2$ at a concentration of 10^{-6} M to provoke a significant but transient vasorelaxation. Values represent the mean ± S.E.M. (∗) $P < 0.05$ compared to control.

were more sensitive to mercury chloride when compared to aorta rings. A concentration of 10^{-7} M resulted in a 28% reduction in the vascular tone (Fig. 2), whereas no response was observed on aortic rings (Fig. 3). By increasing the mercury concentration to 5×10^{-7} M, a 7% (23% is 10^{-6} M) reduction in vascular tone was noted in aortic rings (Fig. 3). The vasorelaxation of pulmonary artery rings persisted longer than that of the aortic rings. Eighty minutes after mercury application the pulmonary arteries remained relaxed (Fig. 1b), whereas aortic rings returned to baseline conditions 10 min following treatment with mercury chloride (Figs. 1a and 2).

To elucidate the mechanism of mercury vasorelaxation, we incubated aorta and pulmonary arteries with the nitric oxide-synthase inhibitor L-NAME (10^{-4} M) before adding $HgCl_2$. The mercury-induced relaxation of rat pulmonary artery (Fig. 1c) and aortic rings (Fig. 3) was completely abolished by using either the nitric oxide-synthase inhibitor L-NAME (10^{-4} M), or after mechanical removal of the endothelial cells (data not shown).

3.1.1. Endothelial cell and smooth muscle function

To investigate whether mercury treatment provoked an alteration of endothelial cell and smooth muscle function of the vessel ring, we tested both endothelial-dependent and -independent vasoreactivity. Twenty minutes after application of $HgCl_2$ (5×10^{-7} M) on aortic rings, an irreversible loss of acetylcholine-induced vasorelaxation was apparent (Fig. 4). However, glyceryltrinitrate (GTN) or isoproterenol (10^{-8} to 10^{-5} M) induced a vasorelaxation which did not differ from the contol (Fig. 5), demonstrating that the vascular smooth muscle cells responded normally to vasorelaxant substances. On

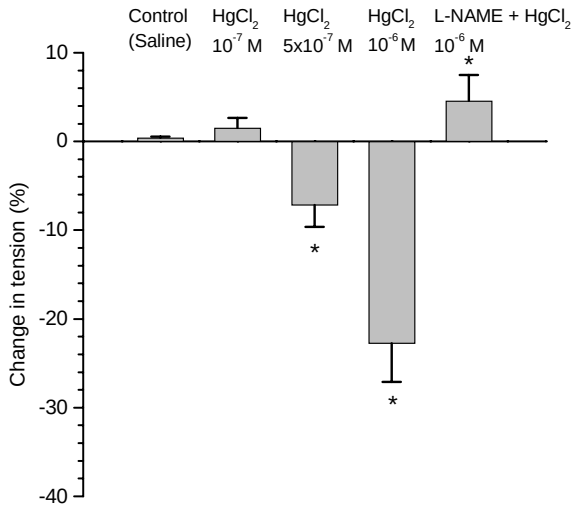

Fig. 3. Effect of different concentrations of $HgCl_2$ (10^{-7}, 5×10^{-7} and 10^{-6} M) on norepinephrine (10^{-7} M) preconstricted rat aortic rings. The maximal relaxation induced by $HgCl_2$ is noted. L-NAME (10^{-4} M) abolished $HgCl_2$-induced relaxation. Values represent the mean ± S.E.M. of at least 11 experiments each group ($HgCl_2$ 5×10^{-7}, $n = 6$). (∗) $P < 0.05$ compared to control.

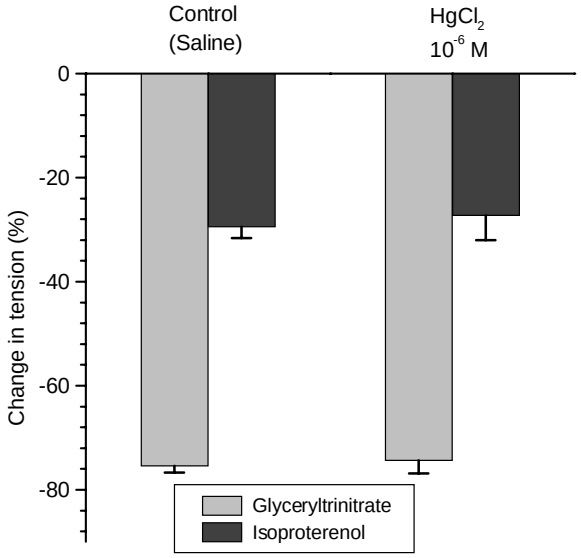

Fig. 5. To test for smooth muscle cell function, concentration–response-curves were performed for: glyceryltrinitrate (10^{-7} to 10^{-5} M; gray bar) and isoproterenol (10^{-7} to 10^{-5} M; black bar) on rat aortic rings pretreated for 20 min with $HgCl_2$ (10^{-6} M). Bars represent the maximal response to glyceryltrinitrate and isoproterenol of at least six experiments each group. Values represent the mean ± S.E.M.

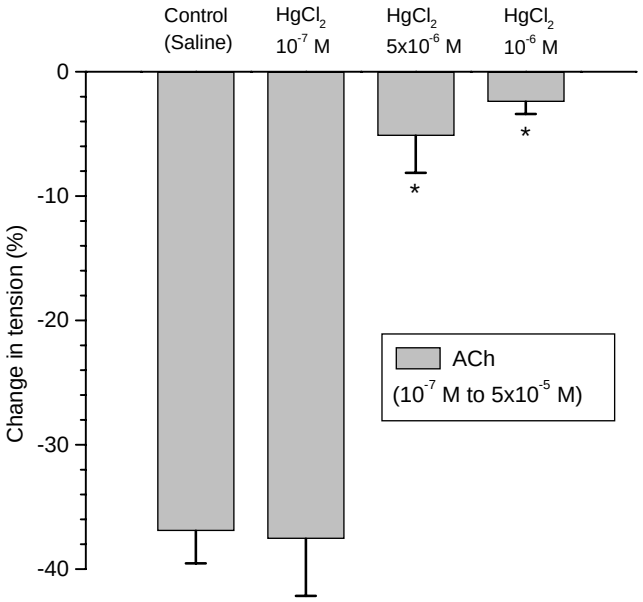

Fig. 4. Effect of $HgCl_2$ on endothelial-dependent vasorelaxation. Aortic rings were treated for 20 min with $HgCl_2$ (10^{-7}, 5×10^{-7}, and 10^{-6} M, respectively) before testing endothelial-dependent relaxation provoked by acetylcholine (10^{-7} to 5×10^{-5} M). Bars represent the maximal response to acetylcholine of at least eight experiments each group ($HgCl_2$ 5×10^{-7}, $n = 5$). Values represent the mean ± S.E.M. (∗) $P < 0.05$ compared to saline-control.

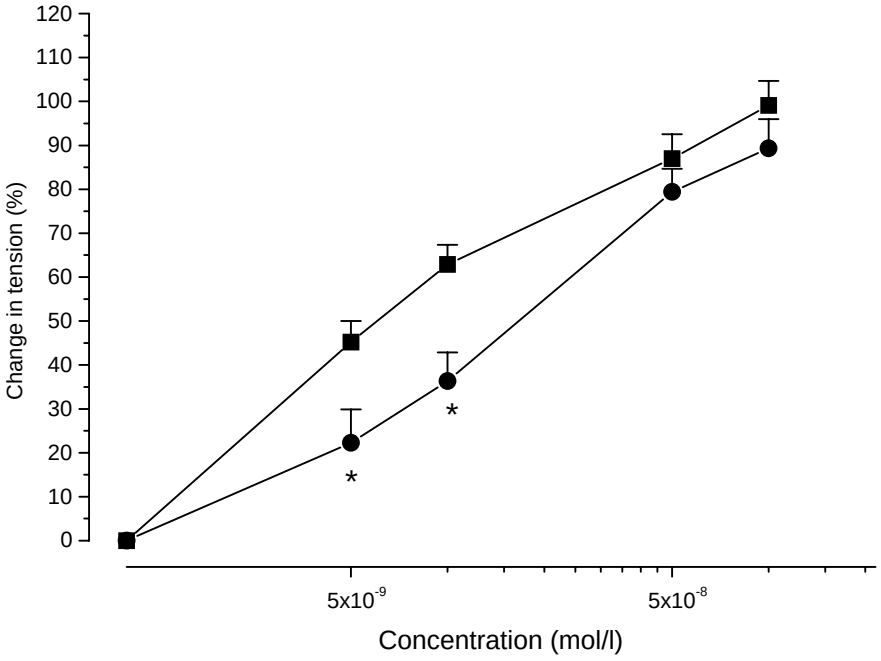

Fig. 6. Effect of $HgCl_2$ on norepinephrine vasoconstriction. Rat aortic rings were incubated for 20 min in $HgCl_2$-containing (10^{-6} M) Greenberg–Bohr-Buffer ((●) $n = 12$) before perfoming a concentration–response-curve for norepinephrine (5×10^{-9} to 10^{-7} M). Control: Greenberg–Bohr-buffer without $HgCl_2$ ((■) $n = 12$). Values represent the mean ± S.E.M.

the other hand, a right shift of the norepinephrine concentration–response-curve was detected after application of $HgCl_2$ (10^{-6} M), indicating that the smooth muscle cells response to vasoconstrictive substances was altered (Fig. 6). We conclude that the endothelial cell function as well as the smooth muscle cell function is affected by mercury treatment. In support of our functional data, we detected damage to the endothelial cell layer by light microscopy. Endothelial cells of the aorta were partly lifted from the basal membrane while incubating the vessel rings for 20 min in $HgCl_2$ (10^{-7} M)-containing buffer (Fig. 7A). Interestingly, at this point in time the vessel rings did not show a reduction of the endothelial-dependent relaxation (Fig. 4). By increasing the mercury concentration up to 10^{-6} M the endothelial cells were completely detached from the vessel rings with only fragments of the cells remaining attached (Fig. 7B and C) resulting in complete abolishment of the endothelial cell dependent relaxation (Fig. 4). To test whether the composition of the incubating buffer used in the organ bath experiments could affect mercury-toxicity, we incubated rat aortic rings in rat blood containing mercury (10^{-6} M) before performing the concentration–response curves for acetylcholine. No endothelial damage was seen under these conditions, and the endothelial-dependent relaxation provoked by acetylcholine was unaffected (Fig. 8).

In order to uncover which component of the rat blood prevented toxicity, we tested rat serum and rat blood cells, dissolved in Greenberg–Bohr-buffer. Both the incubating media contained $HgCl_2$ at a concentration of 10^{-6} M, but only rat serum and not the blood cells protected the endothelial cells from mercury toxicity (Fig. 8). However, heat-inactivated rat serum failed to provide full protection, since acetylcholine-induced vasorelaxation was significantly decreased (Fig. 8).

4. Discussion

In the present study, we demonstrate an endothelial-dependent vasorelaxation of isolated norepinephrine-

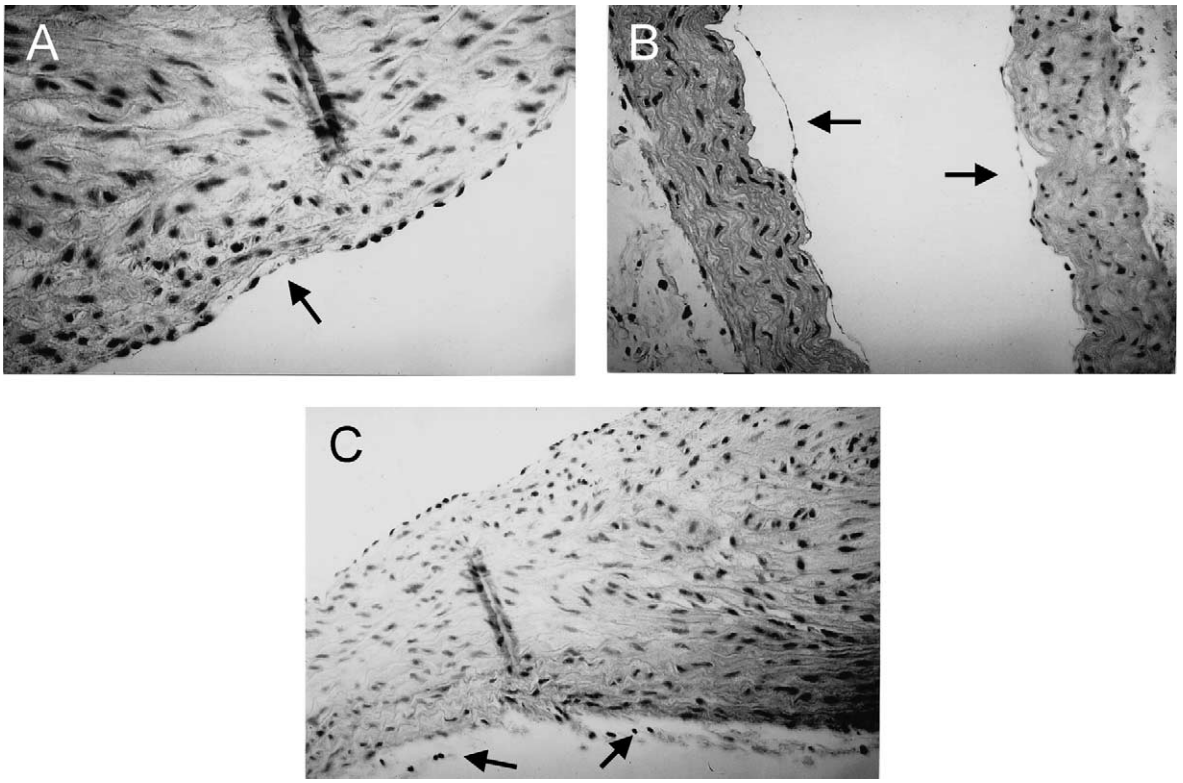

Fig. 7. Light micrographs showing the lumen of rat aortic rings incubated for 20 min in Greenberg–Bohr-Buffer containing either $HgCl_2$: (A) 10^{-7} M or (B and C) 10^{-6} M. At a $HgCl_2$-concentration of 10^{-7} M, some endothelial cells (arrows) were partly lifted from the basal membrane. At a concentration of 10^{-6} M $HgCl_2$ the endothelial cells were mostly denuded from the basal-membrane; (A and C) magnification 400×; (B) magnification 100×.

preconstricted rat aortic and pulmonary artery rings caused by mercury chloride. Further, we demonstrate that mercury chloride provokes an irreversible, functional and structural damage to the endothelial cells. This toxic effect was observed while incubating the vessels in mercury-containing Greenberg–Bohr-buffer. It did not occur by using mercury-containing rat blood or serum as incubation media instead of the protein- and cell-free Greenberg–Bohr-buffer.

Studies have demonstrated that the average whole-blood total and inorganic mercury concentrations in humans were about 2.55 and 0.54 μg/l, respectively (Kingman et al., 1998). The US Environmental Protection Agency's recommended reference blood concentration of mercury for below which exposure are considered to be without adverse effects is 5.8 μg/l (National Academy of Sciences, 2000). In our in-vitro experiments, we used a mercury concentration of 10^{-9} to 10^{-6} M (0.26–20.6 μg/l). We believe that the chosen concentration reflects mercury concentrations found in people with and without mercury intoxication.

To our knowledge, this is the first time that mercury has been shown to mediate an endothelial cell and nitric oxide dependent vasorelaxation. In contrast to this finding, the manifestation of arterial hypertension with tachycardia and excess of catecholamine excretion has been reported in people with acute mercury intoxication (Torres et al., 2000; Wossmann et al., 1999; Henningsson et al., 1993). An increase in blood pressure was also noted in a variety of animal experiments (Perry and Yunice, 1965; Perry et al., 1967a,b; Perry and Erlanger, 1971, 1974; Massaroni et al., 1995). However, a recent study reported a decrease in left ventricular systolic pressure in anes-

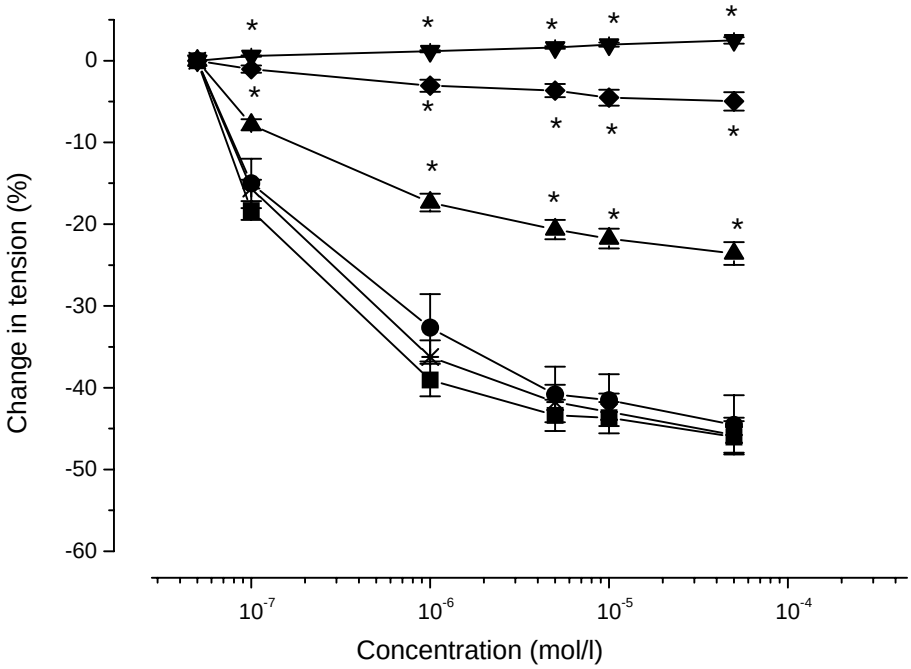

Fig. 8. Effect of the incubation media (containing $HgCl_2$ 10^{-6} M) on the concentration–response-curve for acetylcholine (10^{-7} to 5×10^{-5} M). Aortic rings were incubated for 20 min in diverse media and preconstricted with norepinephrine (10^{-7} M): (■, control) buffer without $HgCl_2$ ($n = 15$); (▼) buffer containing $HgCl_2$ (10^{-6} M, $n = 8$); (◆) buffer containing $HgCl_2$ (10^{-6} M) + resuspended blood cells, $n = 12$); (×) rat blood containing $HgCl_2$ (10^{-6} M, $n = 13$); (●) rat serum containing $HgCl_2$ (10^{-6} M, $n = 7$); (▲) heat-inactivated rat serum containing $HgCl_2$ (10^{-6} M, $n = 4$); values represent the mean ± S.E.M. (∗) $P < 0.05$ compared to control.

thetized rats after intra-venous application of $HgCl_2$ (Rossoni et al., 1999). These hemodynamic effects might be caused by reduction of cardiac contractility and cholinergic mechanism but direct vasorelaxant effects could also be, at least in part, responsible for the effects. Whether the endothelial-dependent vasorelaxation of mercury ions is related to the reported hypotension in rats is not known. However, caution must be observed by comparing in vitro with in vivo results.

By using preparations of isolated blood vessels with intact endothelium we investigated the effect of mercury chloride on vascular smooth muscle cells interacting with endothelial cells. In earlier studies, modest knowledge about endothelial cell biology and function may account for the failure to detect the mercury-induced vasorelaxation. Until Furchgotts discovery of the 'endothelium-derived relaxing factor' a putative role for endothelial cells modulated vasoreactivity had not been postulated (Furchgott and Zawadzki, 1980). Therefore, experiments focused on the response of smooth muscle cells, but did not address the possibility of endothelial-dependent vasoreactivity. For example, experiments performed with vascular spirals (Perry et al., 1967a,b; Solomon and Hollenberg, 1975) in place of vascular rings, do not detect endothelial-dependent responses, presumably because the endothelial cell layer is destroyed during preparation of the spirals. It is also comprehensible that only vasoconstrictive effects were detected on dogfish shark '*Squalus acanthias*' aorta rings treated with mercury chloride (Evans and Weingarten, 1990) since the endothelial cells were explicitly removed for a better detection of smooth muscle-mediated effects. Mercury chloride applied on blood vessels, which were not further preconstricted with vasoconstrictors, e.g. norepinephrine, exclusively resulted in vasoconstriction (Solomon and Hollenberg, 1975;

Tomera and Harakal, 1986). Under these conditions, also acetylcholine, a substance known to relax blood vessels in an endothelial cell-dependent fashion, constricts blood vessels (Furchgott and Bhadrakon, 1953).

In addition to the vasorelaxant effect, mercury chloride causes functional and structural damages to the vessels. The loss of the endothelial-dependent vasoreactivity and the destruction of the endothelial cell layer suggests that the endothelial cells were the main target for mercury toxicity. In accordance with our data a loss of acetylcholine-induced vasorelaxation has been recently observed for the isolated rat tail vascular bed treated with $HgCl_2$ (5 μM) for 20 min (da Cunha et al., 2000). In our experiments, using rat aortic rings a loss of acetylcholine-induced vasorelaxation was already manifested at a $HgCl_2$-concentration of 5×10^{-7} M and morphological changes were even detectable at a concentration of 10^{-7} M. Light-micrographs demonstrate that endothelial cells are partly lifted from the basal-membrane at this concentration. Despite these morphological alterations the endothelial nitric oxide-releasing mechanism is still intact. The loss of endothelial-dependent vasorelaxation appeared when the endothelial cells were structurally more damaged, e.g. denuded from the vessel lumen. Although the response to glyceryltrinitrate and isoproterenol was not attenuated, the smooth muscle cells were also affected by $HgCl_2$. After treatment with $HgCl_2$ (10^{-6} M) a right-shift of the norephinephrine concentration–response-curve was observed, indicating alterations in the mechanism of smooth muscle vasoconstriction.

Functional integrity of the endothelium is crucial for the maintenance of blood flow and anti-thrombotic capacity, because the endothelium releases humoral factors that control relaxation and contraction, thrombogenesis and fibrinolysis, and platelet activation and inhibition. Thus, the endothelium contributes to blood pressure control, blood flow and vessel patency (Lüscher and Barton, 1997). Although local endothelial damage has been reported in humans when mercury was injected intra-venously by a drug addict vivo (De Ruggieri et al., 1989) it is presently not known whether mercury intoxication leads to the manifestation of a dysfunctional endothelium.

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