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Mercury contamination of rat amylin mimics vasoactivity and cytotoxic effects

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Abstract

Rat amylin differs from human amylin (hIAPP) in that it lacks a fibril-forming capacity. As a consequence, toxic effects have been reported for human but not for rat amylin. This report demonstrates how a mercury contamination of commercial rat amylin imitates peptide-related vasoactive and cytotoxic effects on preparations of isolated blood vessels. The source of mercury contamination was believed related to the peptide synthesis. Thiol groups of cysteine-containing peptides are often protected by acetamidomethyl (Acm) which is cleaved by mercuric acetate.

Keywords: Amylin; Mercury; Vasoactivity; Nitric oxide

1. Introduction

A large number of reports have shown cytotoxic properties for human amylin (hIAPP) and β-amyloid [5,9,10,13]. Both amyloidogenic peptides are characterized by an antiparallel β-sheet conformation of their major protein component and a spontaneus aggregation in the form of fibrils. The 37-amino acid protein amylin is highly conserved between species, but the amino acid residues 18–29, which confer to the amyloidogenic properties, show marked species divergence. In contrast to its structure in humans and cats the amyloidogenic motif is missing in rats and mice and since amylin of these species does not form fibrils, cytotoxic properties are unknown [5,9].

Our initial goal was to study the effect of amylin on the pulmonary circulation of the rat [3]. In an attempt to search for vascular reactivity we applied commercially acquired amylin with rat specific amino acid sequences to isolated pulmonary arterial rings preconstricted by norepinephrine. The effect of amylin was remarkably different from that of vasodilator polypeptides [4,17]. Once applied on a preconstricted isolated blood vessel, vasodilator polypeptides cause an immediate change of vascular tone, but following amylin treatment the vasorelaxation was attenuated without reaching a steady state. In addition, the pulmonary artery ring was no longer responsive to acetylcholine, indicating alterated endothelial function. The loss of endothelial-dependent vasorelaxation in combination with endothelial cell injuries after treatment with rat amylin was unexpected and attracted our attention. Two reports about a mercury complex in commercial samples of rat amylin, published in the early nineties [1,2], led us to consider mercury contamination as a potential cause for the abnormal biological activity seen for amylin.

2. Materials and methods

2.1. Studies of vasoactivity

Experiments were performed on tissues from male Sprague–Dawley rats weighing 300–320 g. 4 mm sections of truncus pulmonalis and aorta thoracalis descendens were incubated with modified Greenberg-Bohr buffer in a

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20 ml double-wall incubation bath, continuously bubbled with 21% O_2, 5% CO_2, 74% N_2, and warmed to 37 °C. Rings were stretched to a passive tension of 1.5 g (pulmonary artery) and 2 g (aorta) for a 1 h equilibration time and a dose–response curve for acetylcholine-relaxation (10^{-7} to 5×10^{-5} M) was performed on norepinephrine (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. Ca-EDTA was added (final concentration 0.026 mmol) to prevent oxygenation of added catecholamines. Propranolol was added to the incubation bath (final concentration 10^{-6} M) to block β-receptors. Rings were contracted by norepinephrine in a dose–response fashion up to 10^{-7} mol/l, which resulted in a submaximal (75%), stable precontraction. Amylin (Suppliers A, B, and C) and $HgCl_2$ were added at varying concentrations (10^{-8} to 10^{-6} M) to test for effects on precontracted vessel rings. After a stable contraction was reached again, a dose–response curve of acetylcholine (10^{-7} to 5×10^{-5} M) or a bolus of 10^{-7} M calcium-ionophore A 23187 was performed as a test for normal endothelial function. In case of a failure of acetylcholine-induced relaxation, a dose–response curve for glyceryltrinitrate (10^{-9} to 10^{-5} M) was performed as a test for smooth muscle function. Experiments were also performed in presence of nitric oxide synthase inhibitor L-NAME (10^{-4} M) to test for possible NO-dependent effects.

2.2. Incubation studies

Rings (4 mm) from aorta thoracalis descendens were incubated for 20 min at 37 °C (continuously bubbled with 21% O_2, 5% CO_2, 74% N_2) in modified Greenberg-Bohr-buffer (control). Experiments were performed with buffer containing either 10^{-7} mol/l or 10^{-6} mol/l $HgCl_2$ and amylin (Suppliers A and B), respectively. After incubation the vessel 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. The experiment

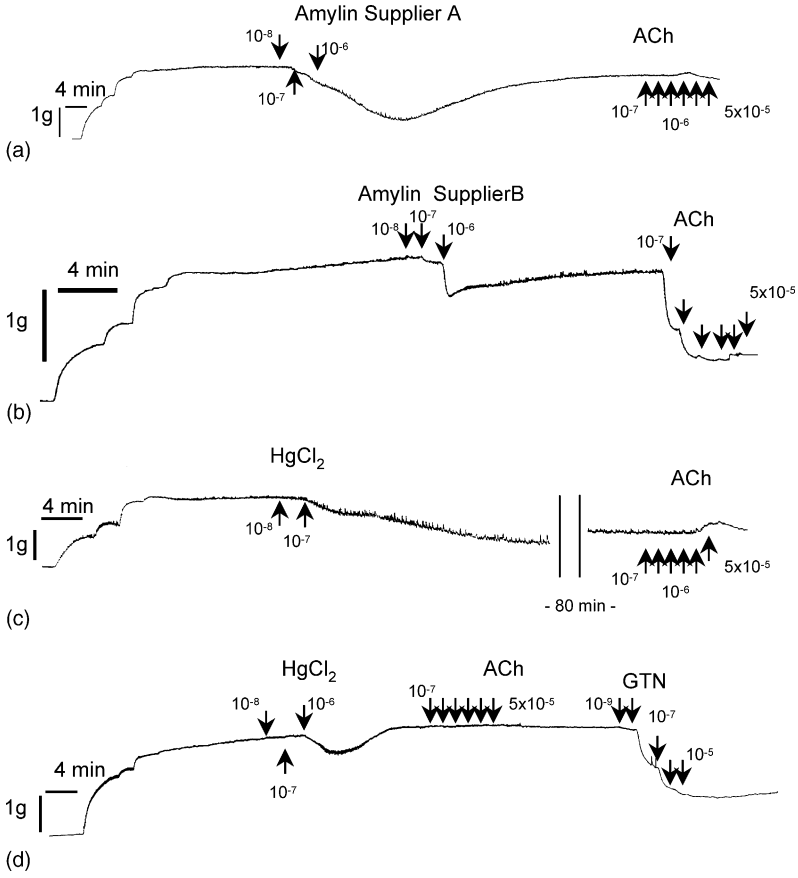

Fig. 1. Original tracing of rat isolated pulmonary artery and aorta rings preconstricted by norepinephrine (10^{-7} M). (a) Amylin (Supplier A) added to pulmonary artery (10^{-8} to 10^{-6} M) noted decline in vascular tone over 20 min then followed by a gradual increase in tone over 25 min to near baseline (10^{-7} M norepinephrine preconstriction). Addition of acetylcholine (ACh) did not result in vascular relaxation, indicating endothelial dysfunction provoked by amylin (Supplier A). (b) Amylin (Supplier B) added to pulmonary artery ring (10^{-8} to 10^{-6} M) immediate decline in vascular tone. Adding acetylcholine resulted in the expected relaxation of the endothelium-intact vessel ring. (c and d) Illustration of vasorelaxation induced by $HgCl_2$ on norepinephrine-preconstricted pulmonary artery and aortic rings. In contrast to pulmonary arterial rings, a 10-fold higher concetration (10^{-6} M) was required to induce vasorelaxation on aortic rings. Loss of acetylcholine-induced vasorelaxation was also manifested after treatment with $HgCl_2$. Since the vessel rings still responded normally to glyceryltrinitrate (10^{-9} to 10^{-5} M), smooth muscle function might be intact.

was repeated three times with blood vessels from different animals.

2.3. Californium-252 plasma desorption mass spectrometry

Amylin samples from Suppliers A and B were received as lyophilized lots, reconstituted in distilled water and used for mass spectrometric analysis without further purification. Nitrocellulose (NC) surfaces were prepared by electrospraying 50 μl of a 2 μg/μl NC (Schleicher & Schuell, Dassel, Germany) solution in acetone onto a Mylar foil. Sample solutions (1–2 μl) were deposited with a microliter syringe and allowed to adsorb for 2 min. The target was then rinsed with 5 μl H_2O, and prepared for measurement by spin-drying technique. Mass spectra were obtained with a modified ^{252}Cf plasma desorption time-of-flight mass spectrometer [7].

2.4. Chemicals

Samples of rat amylin were obtained from three commercial sources, herein designated Suppliers A, B, and C were used without further purification. All other substances were from Sigma Chemicals (Deisenhofen, Germany).

2.5. 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, a 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. Studies of vasoactivity

Commercially acquired amylin (herein designated "Supplier A") with rat specific amino acid sequences applied to isolated pulmonary arterial rings preconstricted by norepinephrine resulted in vasorelaxation (Fig. 1a). To investigate whether the vasorelaxation was related to the source of amylin we used in our experiments, we tested amylin from two additional commercial suppliers (called "Supplier

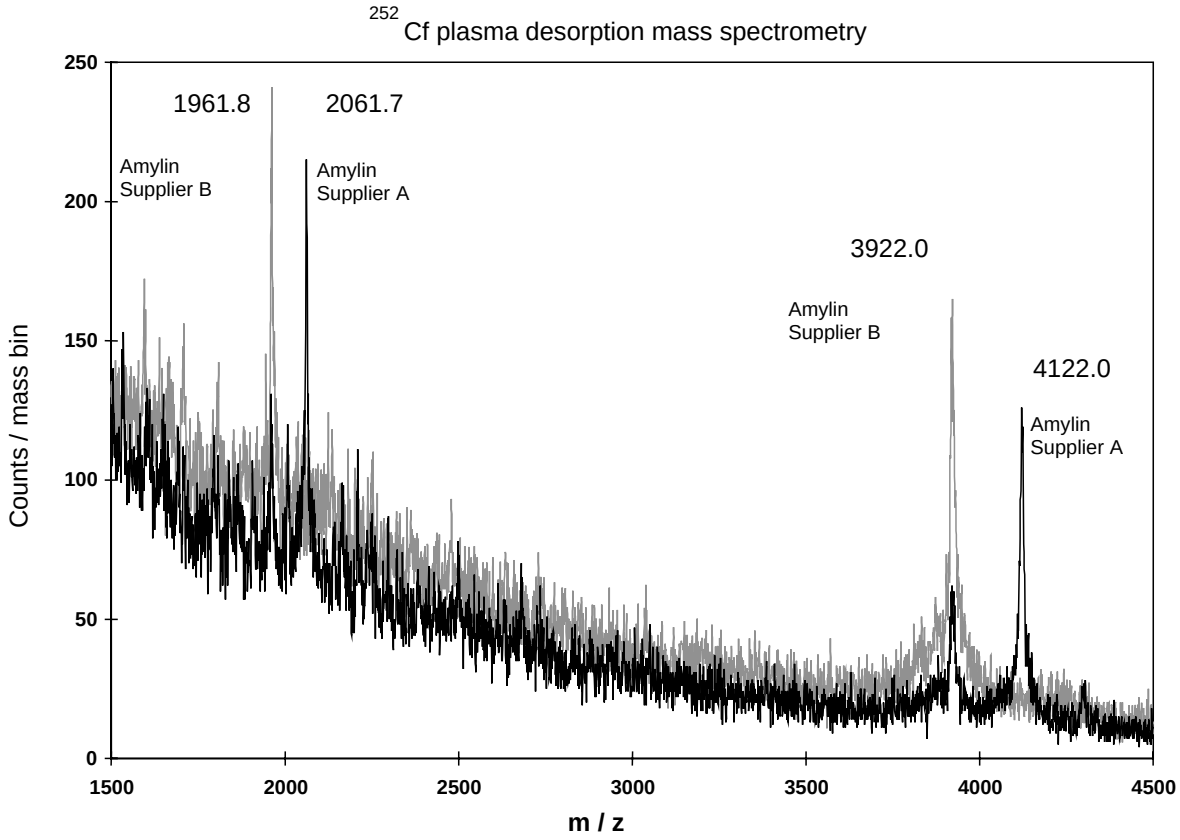

Fig. 2. ^{252}Cf plasma desorption mass spectrogram of two commercial sources of rat amylin (Suppliers A and B). Black tray displays mass spectrum of amylin (Supplier A), whereas gray tray represents amylin from Supplier B. The molecular mass of the sample from Supplier A was 200 Da higher than expected for rat amylin, consistent with the presence of mercury (chemical average mass = 200.6 Da).

B" and "Supplier C"). Fig. 1b shows the effect of amylin (Supplier B) on a norepinephrine-preconstricted pulmonary artery ring. In contrast to amylin (Supplier A), each dose caused a transient, immediate change of vascular tone and the response to acetylcholine was not affected. Similar results were obtained by using amylin from Supplier C (data not shown).

To verify whether mercury alone has the same effects on isolated blood vessels, we applied mercury-II-chloride ($HgCl_2$) on norepinephrine-preconstricted pulmonary artery and aorta rings. Like amylin (Supplier A), $HgCl_2$ provoked a powerful vasorelaxation on either pulmonary and aorta rings. Compared to the pulmonary artery, the vasorelaxation of the aorta rings was less pronounced (Figs. 2 and 3), indicating divergences in the response to mercury within varying types of blood vessels. In addition to the mercury-induced vasorelaxation a loss of acetylcholine-vasorelaxation was noticed after treatment with mercuric chloride (Fig. 1c and d). Pulmonary arteries and aorta rings incubated for 20 min in amylin- (Supplier A) or mercury-containing buffer (pulmonary artery: 10^{-7} M, aorta: 5×10^{-7} M) did not respond to acetylcholine (Fig. 4).

To elucidate the mechanism of amylin and mercury vasorelaxation, aorta and pulmonary arterial rings were preincubated with the nitric oxide-synthase inhibitor L-NAME (10^{-4} M) before adding amylin or $HgCl_2$. L-NAME completely abolished either $HgCl_2$ and amylin (Suppliers A, B, and C) related vasorelaxation on aorta and pulmonary arteries (Fig. 3), indicating an endothelial and nitric oxide-dependent mechanism.

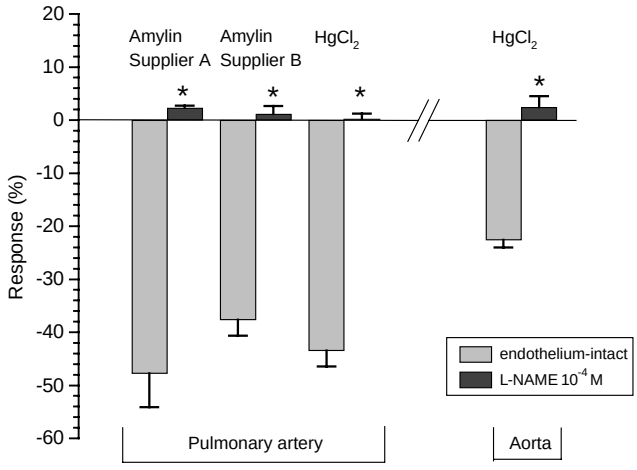

Fig. 3. Effect of amylin (Suppliers A and B) and $HgCl_2$ in absence and presence of the nitric oxide-inhibitor L-NAME. Blood vessels were preconstricted with norepinephrine (10^{-7} M), treated with L-NAME (10^{-4} M) for 15 min before amylin or $HgCl_2$ were applied up to a concentration of 10^{-6} M. Bars represent the mean ± S.E.M. of 10 or more experiments (L-NAME, five experiments). L-NAME abolished amylin (Suppliers A and B) and $HgCl_2$-induced vasorelaxation ($^*P < 0.05$). Compared to pulmonary artery rings $HgCl_2$-induced relaxation was less pronounced on endothelium-intact aorta rings.

3.2. Californium-252 plasma desorption mass spectrometry

We used a plasma desorption mass spectrometry technique [6], to determine the molecular mass in samples of amylin from Suppliers A and B. A 200 Da higher molec-

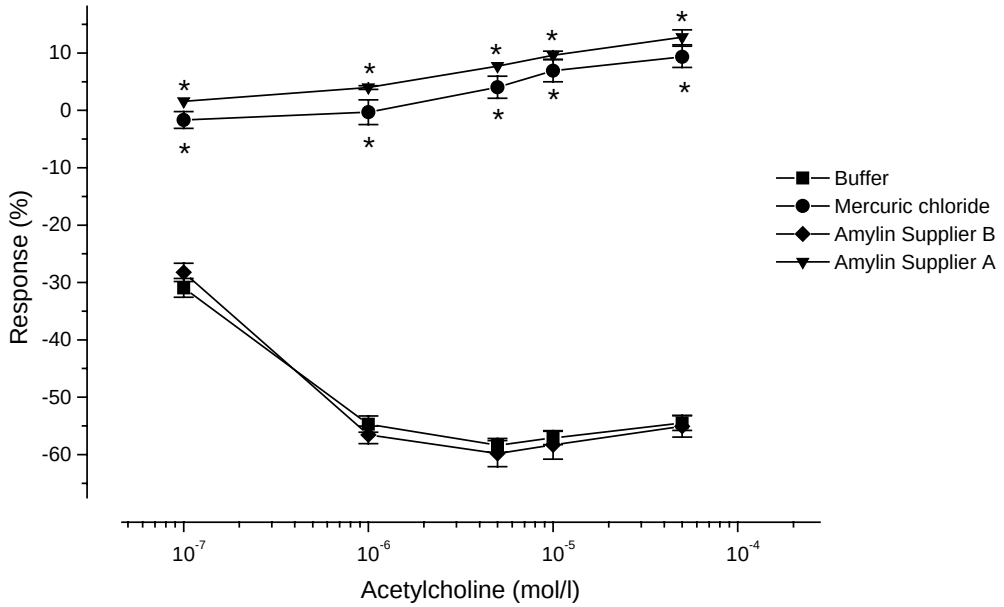

Fig. 4. Response of norepinephrine-preconstricted pulmonary artery rings to acetylcholine (10^{-7} to 5×10^{-5} M). Vessels were preincubated for 20 min either in buffer ($n = 25$), amylin from Supplier A (10^{-7} M, $n = 9$), amylin from Supplier B (10^{-7} M, $n = 7$), or $HgCl_2$ (10^{-7} M, $n = 9$) before acetylcholine dose–response curves were performed. A loss of acetylcholine-induced relaxation was seen for vessels preincubated with $HgCl_2$ and rat amylin (Supplier A), respectively. A normal response was noted for vessels preincubated in Greenberg-Bohr-buffer or rat amylin (Supplier B).

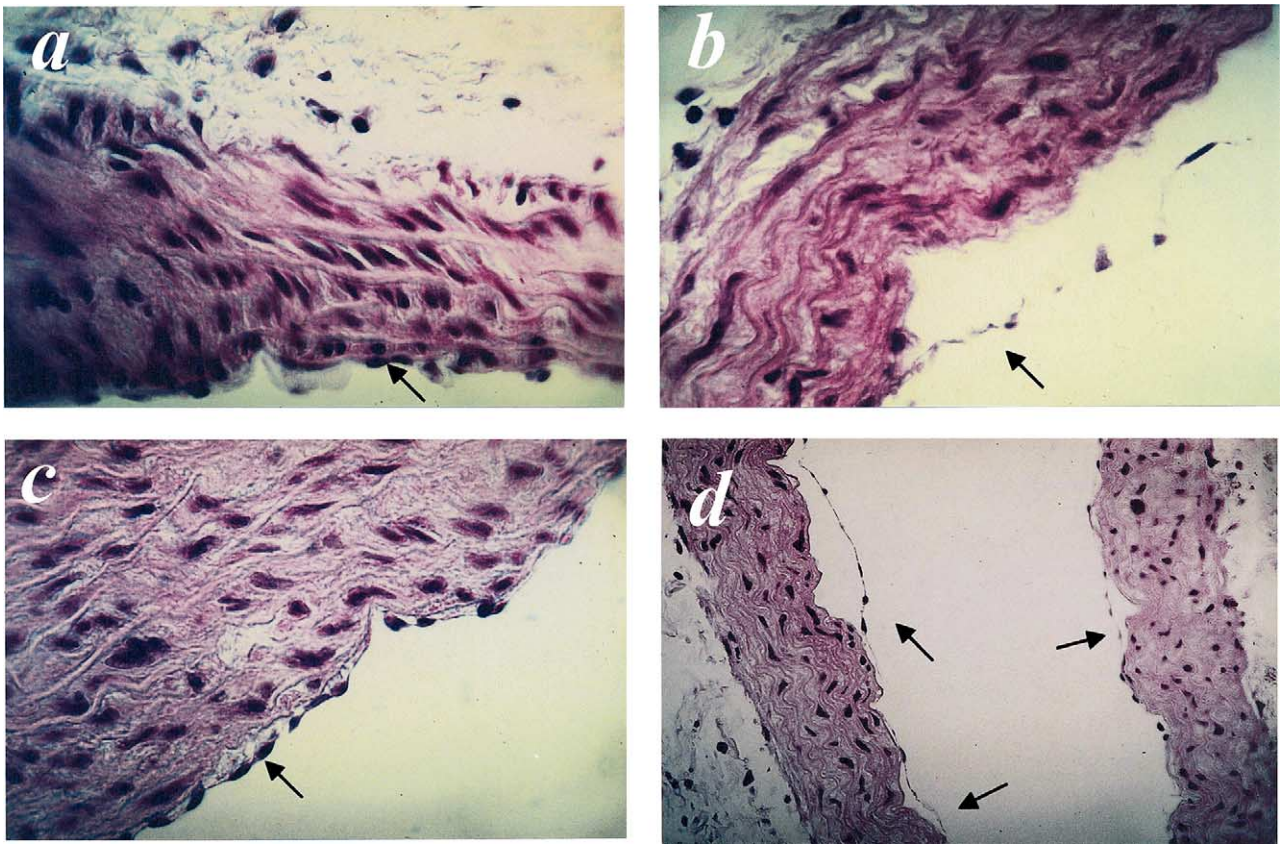

Fig. 5. Effect of amylin and $HgCl_2$ on vascular endothelial cells. Light micrographs showing the lumen of rat aorta rings incubated for 20 min in buffer containing amylin (Suppliers A and B) or $HgCl_2$. The experiment was repeated three times with blood vessels from different animals. (a) Amylin (Supplier B; 10^{-6} M) did not affect endothelial cell structure. The endothelial cell layer (arrow) was intact (magnification 400×). (b) Aorta incubated in amylin (Supplier A; 10^{-6} M) resulted in a destruction of the endothelial cells (magnification 400×). (c) At a concentration of 10^{-7} M amylin (Supplier A) or $HgCl_2$ (shown in this figure) endothelial cells (arrow) were partly lifted from the basal-membrane (magnification 400×). (d) At a concentration of 10^{-6} M $HgCl_2$ nearly all endothelial cells (arrows) were denuded (magnification 100×; note similarities to amylin from Supplier A, shown in (b)).

ular mass (4122) than anticipated for rat amylin was detected in samples from Supplier A, consistent with the presence of mercury (chemical average mass = 200.6 daltons). The measured molecular mass for amylin (Supplier B) was 3922 Da. The mass spectra of the two amylin samples appear in Fig. 2. Further details of the instrumentation and spectral analysis have been described previously [6].

3.3. Histological assessment

Morphological alterations on aortic rings were detected at a mercury concentration of 10^{-7} M. At this concentration the endothelial cells were partly lifted from the basal-membrane (Fig. 5c) and they were denuded from the vessel lumen (Fig. 5d) by increasing the mercury concentration to 10^{-6} M. In contrast to mercury chloride and amylin from Supplier A (Fig. 5b), morphological alterations were not detected by incubating the vessel in either buffer or amylin from Supplier B (Fig. 5a) or Supplier C (data not shown).

4. Discussion

It is impossible to assess the prevalence of contamination in synthetic peptides. Cysteine-containing peptides like calcitonin gene-related peptide (CGRP), calcitonin, amylin (rat and human), adrenomedullin, endothelin, atrial natiuretic peptide (ANP), vasopressin, and somatostatin, all have a risk of mercury contamination since an acetamidomethyl (Acm) group is widely used in protein synthesis to protect the thiol groups from oxidation. Later on the acetamidomethyl group is often removed by mercuric acetate just before disulfide bond formation [8,16]. In case of an insufficient cleavage of mercuric acetate, residues of mercury might remain in the protein samples. Apparently the mercury contamination in one sample of rat amylin we used in our experiments was not an exceptional case—two reports in the early nineties have demonstrated mercury complexes in rat amylin [1,2]. Similarly, a contamination with mercury was also suspected in samples of the vasoactive peptide endothelin [12]. However, the effects of these impurities on biological systems

and the resulting risk of misinterpreting experimental data were not further investigated. In this context it is important to have an appropriate experimental model to become aware of such impurities.

By using isolated blood vessels with intact endothelial cells, we were able to detect the cytotoxic as well as the vasorelaxant effect obtained by the peptide–mercury complex. Our results suggested that the vascular endothelial cells are the main target of mercury action. Whereas endothelial-dependent vasorelaxation was impaired after mercury treatment, glyceryltrinitrate, which directly acts on smooth muscle cells, was able to relax the vessel rings. Since the nitric-oxide-synthase inhibitor L-NAME completely abolished $HgCl_2$-relaxation, mercury is most likely interacting with the L-arginin–NO-pathway in endothelial cells. The observation that amylin without mercury contamination also relaxes blood vessels via the L-arginin–NO-pathway may cause confusion in determining what agent is at work during signal transduction experiments. It may also be confusing that amylin per se is known to be toxic. For instance, human amylin exogenously added to a variety of cultured cells, including primary aortic endothelial cells, causes cell death [9]. In our experiments, the endothelial cell damage obtained by the mercury contamination of rat amylin could easily be misinterpreted as peptide-related. Since rat amylin does not have an amyloidogenic motif or fibril-forming capacity [5,9], peptide-related toxicity could be negated. However, it may be even more difficult to recognize mercury contamination in amyloidogenic peptides like human amylin, calcitonin, and ANP which are all known to be cytotoxic per se [13].

Although the molecular details are not completely understood [14], it became evident that toxicity of amyloidogenic peptides is mediated, at least in part, via oxidative mechanisms [13]. Paradoxically, similar mechanisms are also involved in mercury cytotoxicity. Like amyloidogenic peptides, mercury is known to generate free radicals, alter ionic homeostasis and cause peroxidative injury to proteins and other macromolecules [11,15]. In cases of mercury contamination, it would be almost impossible to separate peptide-related toxic effects from those provoked by mercury. In contrast to human amylin, calcitonin, and ANP, contamination with mercury could practically be ruled out for β-amyloid. This cytotoxic peptide, which is thought to be related to the pathogenesis of Alzheimer's disease [17], does not contain thiol groups and therefore acetamidomethyl protecting groups and mercuric acetate are not used in protein synthesis.

Caution must be observed when using synthetic cysteine-containing polypeptides whenever mercuric acetate is used in deprotection. Misinterpretation of experimental data could be a consequence of unnoticed mercury contamination.

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