Cadmium Is a Novel and Independent Risk Factor for Early Atherosclerosis Mechanisms and In Vivo Relevance

Barbara Messner, Michael Knoflach, Andreas Seubert, Andreas Ritsch, Kristian Pfaller, Blair Henderson, Ying H. Shen, Iris Zeller, Johann Willeit, Günther Laufer, Georg Wick, Stefan Kiechl, David Bernhard

Objectives—Although cadmium (Cd) is an important and common environmental pollutant and has been linked to cardiovascular diseases, little is known about its effects in initial stages of atherosclerosis.

Methods and Results—In the 195 young healthy women of the Atherosclerosis Risk Factors in Female Youngsters (ARFY) study, cadmium (Cd) level was independently associated with early atherosclerotic vessel wall thickening (intima-media thickness exceeding the 90th percentile of the distribution; multivariable OR 1.6[1.1–2.3], P=0.016). In line, Cd-fed ApoE knockout mice yielded a significantly increased aortic plaque surface compared to controls (9.5 versus 26.0 mm², P<0.004). In vitro results indicate that physiological doses of Cd increase vascular endothelial permeability up to 6-fold by (1) inhibition of endothelial cell proliferation, and (2) induction of a caspase-independent but Bcl-xL-inhibitable form of cell death more than 72 hours after Cd addition. Both phenomena are preceded by Cd-induced DNA strand breaks and a cellular DNA damage response. Zinc showed a potent protective effect against deleterious effects of Cd both in the in vitro and human studies.

Conclusion—Our research suggests Cd has promoting effects on early human and murine atherosclerosis, which were partly offset by high Zn concentrations. (Arterioscler Thromb Vasc Biol. 2009;29:1392-1398.)

Key Words: cadmium, zinc • endothelial • dysfunction • injury • permeability • necrosis • ApoE • atherosclerosis • vascular • pathophysiology • risk factor • intima media thickness • apoptosis • cell death

Since the use of Cd in manifold industrial applications, sources for and the amount of Cd uptake by humans has increased dramatically. Cd is, for example, released into the air through the burning of fossil fuels (coal, oil) and the incineration of municipal waste (Environmental Protection Agency, 2000). The most relevant sources for Cd uptake by humans are, however, cigarette smoking (one cigarette contains ≈1 to 2 µg; daily uptake of Cd ≈1 to 3 µg per pack smoked) and food for nonsmokers (daily intake ≈30 µg; daily uptake ≈1 to 3 µg), as well as exhaust gases (Agency for Toxic Substances and Disease Registry, 1999). After inhalation or ingestion of Cd, it is transferred into the bloodstream (whole blood and serum Cd concentrations range between ≈0.2 and ≈20 nmol/L¹-²), where Cd is transported either as a free ion or protein-bound, eg, attached to albumin or metallothioneins. Cd is taken up by cells of Cd target organs (liver, kidneys, and testis) via solute carriers, calcium and manganese channels, and iron transporters.³⁻⁵ In 2001, Abu-Hayyeh et al⁶ demonstrated that the aortic vessel wall is another under-recognized target organ for Cd accumulation (aortic wall concentrations of Cd are up to 20 µmol/L). Epidemiologically, high Cd level was found to be associated with hypertension, stroke, and cardiac arrest,⁷⁻⁹ but confirmatory data are sparse and the mechanistic basis for these interactions remains unclear. Houtman et al observed a higher than expected frequency of atherosclerosis in a Cd-contaminated area in the Netherlands.¹⁰ Coronary arteries in Cd-exposed rabbits showed enhanced atherosclerosis,¹¹ but the precise role of Cd in the initiation of disease remained unresolved. The first report on an interaction of Cd with endothelial permeability stems from Alsborg and Schwartzke who observed a purple discolouration of testis after subcutaneous injection of Cd.¹² In 1983 Sacerdote et al suggested a potential reason for this phenomenon by demonstrating that a subcutaneous injection of Cd in rats causes disruption of endothelial adherence junctions of capillaries.¹³ More recent findings on the effects of Cd on ECs were summarized by Prozialeck et al.¹⁴ Especially, Cd effects on cell structure and

Received December 11, 2008; revision accepted June 10, 2009.

From the Cardiac Surgery Research Laboratory, Department of Cardiac Surgery (B.M., I.Z., G.L., D.B.), the Departments of Neurology (M.K., J.W., S.K.) and Medicine (A.R.), and the Division of Histology and Embryology (K.P.), Innsbruck Medical University, Austria; the Department of Chemistry (A.S.), Philipps-University of Marburg, Germany; the Laboratory of Autoimmunity (B.H., G.W.), Biocenter, Innsbruck, Austria; the Department of Surgery (Y.H.S.), Baylor College of Medicine, Houston, Tex; and Cardiothoracic Surgery, Department of Surgery (G.L.), Medical University of Vienna, Austria.

Correspondence to PD Dr David Bernhard, Cardiac Surgery Research Laboratory, Department of Surgery, Medical University of Vienna, Ebene 8G09/07; Währinger Gürtel 18-20, A-1090, Vienna, Austria. E-mail David.Bernhard@i-med.ac.at

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.109.190082

1392
induction of cell death have been described. Structural changes like breaking down of cell–cell contacts and reorganization of intermediate fibers were ascribed to an interaction of the metal with VE-cadherins. A variety of mechanisms underlying Cd-induced cell death have been suggested including JNK-, p38/MAPK-, p53, or bc12 family member-dependent pathways, but the data available are not consistent and suggest a high level of cell-type specificity. Interestingly, several in vitro studies reported on the protective interaction of elements like manganese or Zn with Cd-mediated processes, but again CVD-relevant interactions in vivo remain largely unclear.

**Methods**

For details and further methods see the supplemental materials.

**Association Between Intima Media Thickness and Serum Metal Concentrations in Healthy Young Females: the ARFY Study**

Classical vascular risk factors, lifestyle behaviors, and family history were assessed in 195 female participants aged 18 to 22 years as detailed elsewhere. Characteristics of study participants are summarized in supplemental Table I (available online at http://atvb.ahajournals.org). Mean maximum IMT of the right and left common (CCA) and internal carotid artery (ICA) was quantified by high resolution B-mode ultrasound (supplemental Figure I). High IMT was predefined as exceeding the 90th percentile of the site-specific IMT distribution (CCA, ICA, or both) (supplemental Figure II). In addition, serum metal concentrations of 11 different metals including Cd were measured by induced-coupled plasma mass spectrometric analyses (ICP-MS). The association between Cd and high IMT was analyzed by means of multivariate logistic regression analysis adjusted for classical risk factors. Differential effects of Cd on high IMT according to Zn levels were tested by inclusion of an appropriate interaction term.

**Cell Culture**

The isolation and culture of human umbilical vein ECs (HUVECs) has been described elsewhere.

**Quantification of Cell Death**

The detection and quantification of cell death with the Annexin/PI method and light scatter analyses were performed as previously described.

**Lactate Dehydrogenase Release Assay**

The amount of lactate dehydrogenase (LDH) released from cells was quantified using the LDH cytotoxicity kit II (Biovision) according to the manufacturer’s instructions.

**Monolayer Permeability Assay**

Analyses of endothelial permeability were performed on a Transwell-based assay system that was developed by our group. After the incubation times with different concentrations of Cd, endothelial permeability was determined by the amount of horseradish peroxidase permeation through the cell layer.

**Detection of DNA Strand Breaks**

The detection and quantification DNA strand breaks was performed with the in situ cell death detection kit, POD (Roche) according to the manufacturer’s instructions.

**Western Blotting**

Western Blotting was performed as previously described.

**Caspase 3 Activity Assay**

Caspase-3 activity was performed as described elsewhere.

**Analysis of the Number of Viable Cells**

Quantification of the number of viable cells was done by the XTT assay (Biomol GmbH).

**Treatment of Animals and Assessment of Atherosclerotic Plaque Area**

Female ApoE KO mice were divided randomly into 4 groups. Group 1 received normal water; group 2 100 mg/L of CdCl2 in drinking water; group 3 400 mg/L ZnCl2; and group 4 100 mg/L CdCl2 plus 400 mg/L ZnCl2. In addition, all mice were fed a Western type diet. After 12 weeks of treatment blood samples were taken and the aorta was excised and subjected to staining and analysis of atherosclerotic plaques.

**Fixation and Scanning Electron Microscopy of Mouse Aortas**

After anesthesia, rinsing of the vasculature, and fixation of animals, aortas were carefully removed, tissues dehydrated and desiccated, mounted, sputtered, and examined with a Zeiss DSM 982 Gemini scanning electron microscope. The images were taken from the central nonplaque containing parts of the aortas.

**SEM Analysis of Cultured Cells**

For SEM analyses, cells were grown on glass coverslips, and treated as indicated. After incubation, cells were fixed by replacing the medium with 2.5% glutaraldehyde (in PBS). Dehydration, desiccation, etc were performed as described above.

**Analysis of Serum Lipid Profiles**

Lipoprotein profiles were analyzed by FPLC using 2 Superose-6 columns (Amersham) connected in series as described elsewhere.

**Statistical Analysis**

Where indicated primary data were tested for a Gaussian distribution and equality of variances. Further analyses were performed using ANOVA (Bonferroni correction for multiple comparisons), followed by pair-wise comparisons.

**Results**

**Elevated Serum Cadmium Levels Are Associated With an Increased Risk for High Intima Media Thickness in Healthy Young Adults**

Distribution of CCA and ICA IMT in the 195 young healthy females (ARFY Study) is shown in supplemental Figure II. A total of 33 participants (16.9%) formed the “high IMT” group. Of the various metals measured, cadmium yielded an independent significant association with high IMT after adjustment for a broad array of vascular risk factors (multivariable odds ratio per standard deviation unit increment [95%CI] 1.6 [1.1–2.3], P=0.016). Risk steadily increased over tertile groups for cadmium concentration (multivariable OR [95%CI] 1.0, 5.2[1.2 to 22.4] and 6.4[1.2 to 33.4],
Of note, Zn appeared to abrogate Cd-mediated effects on IMT (Figure 1, \( P < 0.052 \)). No further interactions with other metals were observed. Serum Cd concentration was not associated with standard cardiovascular risk conditions. In a sensitivity analysis of the nonsmoking subpopulation (\( n = 121 \)), the association between Cd and high IMT remained robust: multivariable OR per standard deviation unit increment \([95\% \text{ CI}] 1.9 \) (1.1 to 3.1), \( P = 0.014 \) and for tertile groups (multivariable OR \([95\% \text{ CI}] 1.0, 5.6\) (0.9 to 33.9) and 14.3 (1.7 to 120.7), \( P = 0.013 \) for linear trend).

**Physiological Doses of Cadmium Increase Endothelial Permeability In Vitro: Inhibition by Zinc**

To determine the impact of Cd on vascular endothelial permeability, a new transwell-based test system was set up (see supplemental Methods), and effects of Cd on endothelial permeability were analyzed after 1, 2, and 7 days, as well as after 3 weeks. The histogram in Figure 2 shows that already on a short-term basis (24 hours) endothelial permeability is significantly increased by Cd at 15 \( \mu \text{mol/L} \), but also that long-term application of 1.5 \( \mu \text{mol/L} \) of Cd significantly increases the permeability of a vascular endothelium in culture. The left part of the histogram in Figure 2 (addition of Cd to the luminal side) compared to the right (addition to the adhesion substrate site) shows that Cd affects endothelial permeability in a cell polarity-dependent fashion. (\( P = 0.01 \)).

Scanning electron microscopic analyses (images A through D) revealed that the integrity of the endothelium was dramatically affected by the addition of 15 \( \mu \text{mol/L} \) of Cd for 96 hours. Starting with 48 hours and in good agreement with the data from the permeability assay, a progressive loss of endothelial integrity could be observed (data not shown). Zn potently prevented Cd-induced endothelial permeability. Among many elements and compounds tested, eg, metal chelators, antioxidants, metal ions (eg, manganese), and polyprenols, Zn proved to be the most powerful agent in inhibiting Cd effects (data not shown).

**Cadmium Inhibits the Proliferation of Vascular ECs and Induces Cell Death**

To uncover the reasons for increased endothelial permeability in response to Cd treatment, we tested for potential changes in the number of viable cells and for the induction of cell death by Cd. XTT-based time course analyses (Figure 3A) revealed that Cd reduces the number of viable cells significantly already 24 hours after the addition of 15 \( \mu \text{mol/L} \) or 100 \( \mu \text{mol/L} \) of Cd, and reaches a maximal effect between after 48 and 72 hours. Coapplication of 60 \( \mu \text{mol/L} \) Zn (added 24 hours before Cd addition) potently inhibited the Cd effect. Because the XTT assay measures only the number of viable cells and cannot differentiate between the inhibition of proliferation and the induction of cell death, we also tested directly for the induction of cell death by annexinV staining and FACS analyses (apoptosis) as well as by LDH-release assays (necrosis). Figure 3B shows that Cd causes a significant time-dependent increase in annexinV-positive cells not starting before 72 hours, which was slightly preceded by LDH release, arguing for the induction of a necrotic form of cell death. Figure 3C shows that contracted round cells (dying
or dead cells) have holes in their membranes, suggestive of an early presence of necrotic cells in the cultures.

**Cadmium-Induced DNA Strand Breaks Precede Cellular DNA Damage Response, and Caspase-Independent, Partly Calpain-Dependent, and Bcl-xL–Inhibitable Endothelial Cell Death**

Figure 4A shows that the presence of Cd in the cultures significantly increases the number of cells with DNA strand breaks compared to the controls, Zn-treated cells, and Zn plus Cd–treated cells already after 12 hours of incubation. To determine the cellular response to these strand breaks, we performed Western blot–based analyses of DNA-damage response proteins ie, phospho-ATM, p53, and p21(WAF1/Cip1). Western blots (Figure 4B) show that the addition of Cd to ECs leads to the phosphorylation of ATM on serine 1981 already 24 hours after Cd addition, and to the accumulation of p53 starting at 48 hours. In addition, the p53 downstream target, cell cycle inhibitor p21(WAF1/Cip1), was also increased on the protein level after 24 and 48 hours. To test for the involvement of mitochondrial signaling in Cd-induced cell death, ECs were infected with retroviruses containing a Bcl-xL expression vector. Figure 4C shows that the overexpression of Bcl-xL significantly inhibited Cd-induced cell death, arguing for the opening of the permeability transition pore complex (PTPC) in the course of Cd-induced cell death. Although the final outcome of Cd-induced cell death was necrosis (Figure 3B and 3C), the data in Figure 4D and 4E show that apoptosis-executing enzymes ie, caspases are cleaved to their active forms, and show a small increase in activity compared to the control in response to the treatment of ECs with 15 μmol/L Cd, but also that high concentrations (100 μmol/L) inhibit the activation, and possibly also the activity of caspases. To functionally test for the relevance of caspases activity in Cd (15 μmol/L)-induced cell death, we inhibited caspases with the pan-caspase-inhibitor zVAD-fmk and performed Western blots of caspase target proteins. Figure 4C shows that the presence of zVAD had no effect at all on the extent of Cd-induced cell death. Similar results were obtained after 24, 48, 72, and 96 hours, with other caspase inhibitors (ie, DEVD-fmk, and LEHD-fmk), and with 15 and 100 μmol/L Cd (data not shown). In addition, Figure 4B shows that neither the caspase-3 target PARP, nor the capase-8 target BID are cleaved in cells treated with Cd (also note the downregulation of BID by Zn). Further inhibitor experiments (Figure 4C) revealed that the inhibition of calpains partially protects cells from Cd-induced cell death, that Zn not only inhibits the reduction in viable cell numbers by Cd (Figure 3), but also cell death induction, and that Cd-induced cell death is not dependent on PARP activity. The SEM image (Figure 4F) shows that contracting ECs have small membrane blebs with a rough surface. This finding may be interpreted as the presence of imperfect membrane blebbing.

**Cadmium Causes Endothelial Damage, Atherogenic Alterations of the Lipid Profiles, and Accelerates Atherosclerotic Plaque Formation in ApoE KO Mice**

Because increased endothelial permeability is a key component of our hypothesis on atherogenesis-initiation by Cd, we tested for the presence of morphological alterations of the aortic endothelium by Cd in ApoE KO mice in vivo. Figure 5 shows that the vascular endothelium of control mice (Figure 5A) has a flat surface, Cd treatment resulted in structural changes which may be interpreted as contraction and detachment of ECs ie, endothelial damage (Figure 5B). We also observed
holes between ECs (Figure 5C) which were probably caused by the contraction of dying ECs, and found signs of endothelial necrosis (Figure 5D). In controls or Zn-only treated animals, no contraction or necrotic cells could be detected. The endothelium of animals that were treated with Cd plus Zn showed essentially the same pattern as Cd-only treated animals. Along with the analysis of endothelial morphology, lipid profiles of ApoE KO mice with or without Cd and with or without Zn in drinking water were analyzed (Figure 5E), and the plaque area of mouse aortas was determined (Figure 5F). The addition of Cd to the drinking water of mice changed the lipid profiles of ApoE KO mice toward an even more atherogenic profile (increased VLDL particles; VLDL\(^{15}\); LDL\(^{18}\), HDL\(^{27}\)), and Zn reduced Cd effects. The plaque surface area increased significantly in Cd-treated mice compared to the controls (\(P<0.005\)). Although the coapplication of Zn reduced the median plaque area compared to Cd-only treatment, the changes did not reach significance. About 75% of control animals were free of atherosclerosis (AHA Grade 0), whereas about 90% of Cd-treated littermates already showed atherosclerotic wall changes (AHA Grade I through VIII) including atheroma formation (AHA Grade IV through VIII) in about 40% of the cases (\(P<0.001\)).

**Discussion**

Although Cd has long been known for its carcinogenic and toxic activities, the role of Cd in CVD is still not clear. To address this issue we conducted a study on 195 healthy young female subjects. Epidemiological data clearly indicated that high serum levels of Cd increases the risk of high IMT, a well-established marker for early atherosclerosis. Because the average age of the study population was 20.6 years, these results suggest that Cd may play an important role in the initiation of atherosclerosis. The generally accepted response-to-injury hypothesis postulates a disruption of endothelial barrier function as the initial step in atherogenesis.\(^26\) This hypothesis is supported by our finding that Cd causes endothelial damage in vitro and in animals in vivo, where also accelerated plaque formation could be observed. Analysis of serum lipid profiles revealed an increase in VLDL particles in response to Cd treatment. Based on the relation between alterations in lipid profiles and their impact on plaque...
formation we calculated that in our model the altered lipid profile by Cd could only be made responsible for 20% of the Cd effect on plaque formation. In addition, no evidence for an effect of Cd on the lipid profiles in our study on human subjects could be obtained. Based on the results of this study we hypothesize that Cd primarily exerts its atherogenic activity by causing endothelial damage.

In contrast to previous reports, serum Cd levels were not associated with the smoking status of the study participants. We speculate that the low exposure of smoking individuals to cigarette smoke may account for this finding (frequency of smokers with a cumulative exposure of 3.0 pack-years was 7.8%).

The final outcome of Cd exposure to ECs is necrosis (Figure 3), but a large number of intracellular signaling processes seem to be involved, the inhibition of which abrogates Cd-induced cell death. Our in vitro data suggest that Cd, which is taken up by ECs via solute carriers or ion channels clustering on the luminal side of ECs (see Figure 2), causes DNA strand brakes. An involvement of oxidative stress, as has been suggested by others seems unlikely in our model because we were not able to detect oxidative stress by various methods (eg, 123 Di-hydro-rhodamine, H2-DCF-DA staining, Oxyblotting, and no shift in the GSH:GSSG ratio toward GSSG; data not shown). The phosphorylation of ATM on serine 1981 clearly indicates that ECs sense DNA damage and react by upregulation and stabilization of p53 and its downstream target, cell cycle inhibitor p21/WAF1/Cip1 (note the drop in the number of viable cells in the absence of cell death after 24 and 48 hours in response to Cd treatment; Figure 3A and 3B). The fact that viral overexpression of Bcl-xL potently inhibited Cd-induced cell death argues for the essential involvement of mitochondrial signaling in Cd-induced cell death. However, other classical apoptotic processes like caspase-activation and –activity (Figure 4D and 4E) as well as surface changes that may be interpreted as membrane blebbing (Figure 4F), although present, were not completed or are not relevant in Cd-mediated cell death. Because of the strict dependence of Cd-induced cell death on cell signaling, we hypothesize that Cd-induced cell death rather represents an atypical form of apoptosis that has the necrotic rupture of the plasma membrane as end point, than classical necrosis.

Because necrotic endothelial cells could also be found in vivo, we speculate that this process may also contribute to fatty streak and plaque formation in humans. Necrosis is known to cause inflammation and the attraction and activation of macrophages, both well known contributors to the atherosclerotic process, In addition, disruption of the endothelial barrier function may enhance lipid deposition and infiltration of the vessel wall by macrophages.

The capacity of Zn to interfere with Cd toxicity in vitro is well established in the literature, and our in vitro and human data clearly support this view. However, the potential consequences of an altered balance between serum Cd and Zn levels (in favor of the former) in atherogenesis have not been reported. In the human study part, the association between Cd and high IMT was confined to individuals in the low and medium Zn tertile groups and showed a dose-response effect. In brief, odds ratios [95%CI] for the low (≦200 ng/kg), medium (201 to 300 ng/kg), and high (300 ng/kg) Cd tertile group in individuals with low and medium Zn level amounted at 1.0, 2.9[0.8 to 8.3], 7.7[2.1 to 28.0] in unadjusted and 1.0, 7.4[1.3 to 41.4], 19.6[2.2 to 173.2] in multivariable analysis (Figure 1). It must be kept in mind, however, that Zn supplementation showed only a nonsignificant tendency to reduce Cd-mediated plaque formation in ApoE KO mice. The fact that Zn administration via drinking water led only to a 12% to 32% increase in serum Zn levels may explain this finding. Changes in formulation or a different route of application may improve the Zn effect.

Cd is abundantly present in our environment. A potential proatherogenic effect—even if modest compared to other traditional risk factors—has a significant impact population wide. Possible pathogenic effects of Cd on the vascular endothelium have been described and could form the basis for medical interventions. Most interestingly Zn seems to abolish the deleterious effect of Cd and might—warranting results from further studies—prove to be an effective preventive measure for people exposed to Cd.

Acknowledgments

The authors thank Prof Dr Frank Thevenod for discussions, Dr Christian Ploner for help with virus-based expression of bclXL, Dr

Figure 5. Cadmium causes endothelial damage, alterations in lipid profiles, and accelerated plaque formation in ApoE knock-out mice in vivo. The figure shows electron microscopic images of the aortic endothelium of ApoE KO mice exposed to Cd or Zn via drinking water. A, Control-treated animals; B through D, Cd-treated animals. The lipid profiles of animals plus WT control animals is given in E. F, Analysis of the aortic atherosclerotic plaque area of the animals. For details see supplemental materials.
Martin Hermann for confocal microscopy, Rajam Csordas-Iyer for correction of the English language, and Angelika Flörli and Sandra Frotschnig for excellent technical assistance.

Sources of Funding
This project was supported by the Austrian National Bank (Project #12697 to D.B.).

Disclosures
None.

References
Cadmium Is a Novel and Independent Risk Factor for Early Atherosclerosis Mechanisms and In Vivo Relevance

Barbara Messner, Michael Knoflach, Andreas Seubert, Andreas Ritsch, Kristian Pfaller, Blair Henderson, Ying H. Shen, Iris Zeller, Johann Willeit, Günther Laufer, Georg Wick, Stefan Kiechl and David Bernhard

Arterioscler Thromb Vasc Biol. 2009;29:1392-1398; originally published online June 25, 2009; doi: 10.1161/ATVBAHA.109.190082

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/29/9/1392

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2009/08/21/ATVBAHA.109.190082.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Cadmium a Novel and Independent Risk Factor for Early Atherosclerosis

Mechanisms and *in vivo* Relevance

Barbara Messner, Michael Knoflach, Andreas Seubert, Andreas Ritsch, Kristian Pfäller, Blair Henderson, Ying H Shen, Iris Zeller, Johann Willeit, Günther Laufer, Georg Wick, Stefan Kiechl, and David Bernhard.

**Figures**

**ONLINE DATA SUPPLEMENT Figure 1:**

Maximum far wall intima media thickness (IMT) was measured at predefined segments the proximal 10 mm of the internal (ICA) and the distal 40 mm of the common carotid artery (CCA) of both sides.
ONLINE DATA SUPPLEMENT Figure 2: Distribution of IMT levels measured at the common (CCA) and proximal internal carotid artery (ICA) in the ARFY Study (mean maximum IMT of both sides).

METHODS

Reagents
All reagents used were purchased from Sigma-Aldrich (Vienna, Austria) unless stated otherwise, and were of analytical grade quality. Cd and Zn were applied to cells by the dilution of stock solutions; (Stock solution of CdCl₂ 15 mM (Sigma-Aldrich, Vienna, Austria) and ZnCl₂ 10 mM (Merck, Darmstadt, Germany), both in A. bd.).

Association between Intima Media Thickness and Serum Metal Concentrations in Healthy Young Females - the ARFY-Study
The ARFY-study included 205 female participants from the Innsbruck School of Nursing aged 18–22 years. Classic vascular risk factors, lifestyle behaviours and family history were assessed by validated and standardized procedures¹. Characteristics of study participants are
summarised in ONLINE DATA SUPPLEMENT Table 1. IMT, a marker of early atherosclerosis and fatty streak formation, was quantified by high resolution B-mode ultrasound (General Electrics, logiq 700, 10 MHz transducer) in two predefined segments. The maximum IMT was recorded on the far wall of the left and right distal 40mm of the common and proximal internal (10mm distal of the bifurcation) carotid arteries (see ONLINE DATA SUPPLEMENT Figure 1). Maximum IMT of each segment of both sides was averaged. High IMT was defined as an reading exceeding the 90th percentile of the distribution in one of the two examined segments1,2 (see ONLINE DATA SUPPLEMENT Figure 2). In addition, serum metal concentrations (aluminium, chrome, iron, nickel, copper, zinc, selenium, strontium, cadmium, barium and mercury) were measured by induced-coupled plasma mass spectrometric analyses (ICP-MS)3. Fasting serum samples from 195 of the 205 study participants were available for assessment of metal concentration; all were stored in metal-free tubes.

The association between Cd and high IMT was analysed by means of multivariate logistic regression analysis adjusted for systolic blood pressure, active and passive smoking, fasting plasma glucose, insulin resistance, LDL and HDL cholesterol, waist circumference, social status, family history for hypertension, GOT, lipoprotein(a), C-reactive protein, homocysteine, T-cell reactivity against human Hsp60, soluble Hsp60 in serum, asthma and oral contraceptives. Differential effects of Cd on high IMT according to Zn levels were tested by inclusion of an appropriate interaction term.

Cell Culture

The isolation and culture of human umbilical vein ECs (HUVECs) has been described elsewhere 4. In the long-term culture experiments culture medium (EBM 2, Lonza GmbH, Wuppertal, Germany) was replaced with fresh medium including the indicated compounds.
every second day. In all experiments using Zn, cells were pre-incubated with Zn for 24 hours before the addition of Cd.

Quantification of Cell Death

The detection and quantification of cell death with the Annexin/PI method and light scatter analyses were performed as previously described. For cell death analysis, 3 x 10^5 HUVECs per well were seeded into gelatine-coated 6-well plates (Greiner Bio One, Kremsmünster, Austria). Inhibitor concentrations used were zVAD (Alexis Biochemical, San Diego, USA), 30µM; 3-Aminobenzamide (3-ABA, Sigma-Aldrich, Vienna, Austria), 2mM; Calpain inhibitor III, 50µM (Sigma-Aldrich, Vienna, Austria). Retroviral infection and expression of Bcl-xL in HUVECs was performed as detailed previously.

Lactate dehydrogenase release assay

The amount of lactate dehydrogenase (LDH) released from cells was quantified using the LDH cytotoxicity kit II (Biovision, Mountain View, CA, USA) according to the manufacturer’s instructions.

Detection of DNA strand breaks

The detection and quantification DNA strand breaks was performed with the In situ cell death detection kit, POD (Roche, Vienna, Austria) according to the manufacturer’s instructions.

Monolayer Permeability Assay

For the analyses of endothelial monolayer permeability 1 x 10^5 HUVECs were seeded into the upper chamber of gelatine-coated 24-well Transwell plates (Greiner Bio One, Kremsmünster, Austria). The experiment was started, i.e. the indicated compounds were added two days after
the seeding of cells. To determine monolayer permeability the medium in the bottom well was replaced with PBS after the indicated time of treatment, and 32 µg of horseradish peroxidase (HRP (Sigma-Aldrich, Vienna, Austria) was added to each upper well chamber. After various times of incubation at cell culture conditions, HRP enzyme activity was determined in the bottom well. To do so, 100 µl of the PBS solution in the bottom well were transferred into a 96-well plate and 100 µl of ABTS buffer containing 0.32 mg ABTS/ml was added. After an additional incubation time of 45 min (RT°C, dark), absorption at 560nm was analysed on an ELISA reader (Anthos Labtec HT2 Salzburg, Austria).

**Western Blotting**

Western Blotting was performed as previously described \(^4\). Primary antibodies used were anti-phospho serine (1981)-ATM antibody (Cell Signaling, Danvers, MA, USA), anti-p53 antibody (BD Pharmingen, Rockville, MD, USA), anti-p21/CIP1/WAF1 antibody (BD Pharmingen, Rockville, MD, USA), anti-caspase-3 (BD Pharmingen, Rockville, MD, USA), anti-PARP antibody (Cell signalling, Danvers, MA, USA), and anti-BID antibody (Abcam, Cambridge, UK).

**Caspase 3 Activity Assay**

To analyse capase-3 activity, 1 x 10⁶ HUVECs per well were seeded in gelatine-coated petri dishes ((BD Pharmingen, Rockville, MD, USA)). Caspase-3 activity was performed as described elsewhere \(^6\).
Analysis of Cell Proliferation

$3 \times 10^3$ HUVECs were seeded in the 96-well plates (Greiner Bio One, Kremsmünster, Austria) and treated with Zn and various Cd concentration for the indicated times. Cell proliferation was determined using the XTT assay (Biomol GmbH, Hamburg, Germany).

Treatment of Animals and Assessment of Atherosclerotic Plaque Area

ApoE knockout (KO) mice were purchased from the Charles River Laboratories (USA) and kept in the Central Animal Testing Facility (ZVTA) of the Innsbruck Medical University at 24°C and a 12-h light/dark cycle. Animals received normal diet and drinking water ad libitum until the age of 8 weeks. Then, female ApoE KO mice were divided randomly into four groups. Group 1 received normal water; group 2, 100mg/l of CdCl$_2$ in drinking water; group 3, 400mg/l ZnCl$_2$; and group 4, 100mg/l CdCl$_2$ plus 400mg/l ZnCl$_2$. In addition to receiving the indicated compounds via drinking water, the mice were fed a western type diet (crude fat 21.2%; Ssniff, Soest, Germany). After 12 weeks of treatment, animals were anesthetized with xylasol/ketamine and weighed. Thereafter, the thorax was opened and blood samples were taken with a syringe from the vena cava, which were consequently subjected to serum preparation which was then stored at -80°C. In parallel to serum preparation, the aorta was excised between the aortic arch and the iliac bifurcation. The aorta was cleaned by removing connective tissue and fat, washed in PBS with 20µM BHT and 2µM EDTA (pH=7.4), opened longitudinally, pinned on silicone plates with acupuncture needles (0.20 x 15mm, asia-med, Suhl, Germany) and fixed overnight in 4% PFA, 5% sucrose, 20µM EDTA (pH 7.4). Fixed tissues were then incubated with Sudan IV (0.5% Sudan IV in 35% ethanol and 50% acetone) for 15 min to stain atherosclerotic plaques. After destaining of aortas with 75% ethanol, pictures were taken with a Samsung NV 10 camera, which were then subjected to image analysis using the Image ProPlus 5.1 software (plaque area).
A total of 18 sections of the aortic arch and descending aorta derived from 6 Cd-fed mice and 12 sections from 5 control mice were stained with haemotoxilin-eosin and Movat’s staining and graded according to AHA lesion types (I: initial change, II: minimal change, III: preatheroma, IV: atheroma, V: fibroatheroma, VI: hemorrhagic/thrombotic lesion, VII: calcific lesion, VIII: fibrotic lesion) by an external experienced rater blinded to mouse treatment and amount of atherosclerotic plaque area.

The treatment protocol has been approved by the Commission for Animal Testing of the Austrian Ministry for Science and Research.

*Fixation and Scanning Electron Microscopy of Mouse Aortas*

For scanning electron microscopic (SEM) analyses of the vascular endothelium of mouse aortas, animals were treated and anesthetized as described above. After anaesthesia the thorax was opened, the left heart ventricle was cannulated and the right atrium was perforated, followed by rinsing of the circulation with 0.9% NaCl for 2-3 min through the cannula in the left ventricle. Aortas were then fixed by changing the perfusate to 2.5% glutaraldehyde in PBS (pH = 7.4) solution and rinsing for 5-6 minutes. After careful removal of the aortas, tissues were dehydrated in a graded ethanol series (70%, 90%, 100%, 100%, and 100% aceton), desiccated by critical point drying (Balzers Union Med 030, Liechtenstein), mounted, sputtered with gold-palladium (Balzers Med 080; Liechtenstein), and examined with a Zeiss DSM 982 Gemini scanning electron microscope. The images were taken from the central non-plaque containing parts of the aortas. The *in vivo* fixation procedure has been approved by the Commission for Animal Testing of the Austrian Ministry for Science and Research.
SEM Analysis of Cultured Cells

For SEM analyses 1 x 10^5 HUVECs were grown on gelatine-coated round glass coverslips (Menzel Gläser, Thermo Fischer Scientific, USA) in 24-well plates, and treated as indicated. After the incubation, cells were fixed by replacing the medium with 2.5% glutaraldehyde (in PBS). Dehydration, desiccation etc. were performed as described above.

Analysis of Serum Lipid Profiles

Lipoprotein profiles were analysed by FPLC employing two Superose-6 columns (Amersham, Braunschweig, Germany) connected in series as described elsewhere \(^7\).

Statistical analysis

In cell culture and animal experiments (where applicable) the distribution of values of all groups were tested for a Gaussian distribution (Kolmogorov-Smirnov test) and equality of variances (Levene’s test). Further analyses were performed using ANOVA / Bonferroni adjustment, followed by t-test comparisons of the groups.

Reference List


FIGURE LEGENDS

Figure 1 – The balance between serum cadmium and zinc levels defines the risk for increased intima-media thickness in healthy young adults.
The histogram in figure 1 shows an analysis of the risk for increased IMT at the internal and common carotid arteries in relation to serum Zn and Cd levels. The study population was split into tertiles according to serum Cd and Zn concentrations (i.e. low (1), medium (2) and high (3) levels) and the odds ratio (OR) for increased IMT of the groups was determined. Since the subgroups with low and medium Zn concentrations showed comparable findings the groups were pooled to increase group sizes and statistical power.

**Figure 2 – Cadmium increases the permeability of vascular endothelial monolayers in vitro – protection by zinc.**

The left histogram of figure 2 shows an analysis of time- and dose-dependent changes of endothelial monolayer permeability in vitro with or without the application of the indicated concentrations of Cd and/or 60 µM Zn to the luminal side of the cells. The right histogram shows the effects of an abluminal addition of Cd to the monolayers for 48 hours. Mean values + S.D. of a representative experiment performed in quadruplicates are shown. Asterisks indicate significant differences (i.e. p < 0.05) compared to the corresponding controls.

The images in figure 2 show scanning electron microscopic analyses of vascular endothelial cells in vitro. The cells were treated for 96 hours as follows: A…control; B…15 µM Cd; C…60 µM Zn; D… 60 µM Zn plus 15 µM Cd. Representative images are shown. In all experiments, Zn was added 24 hours prior to the addition of Cd.

**Figure 3 – Cadmium inhibits the proliferation of endothelial cells and causes a necrotic form of cell death**

Figure 3A shows the effects of an application of 15 µM and 100 µM of Cd on endothelial cells +/- the addition of 60 µM of Zn on the number of viable cells in vitro determined by the XTT assay. Mean values +/- S.D. of a representative experiment performed in quadruplicates
are shown. Asterisks indicate significant differences (i.e. p < 0.05) compared to the corresponding controls.

In Figure 3B, Cd effects on cell death induction, analysed by annexinV-FITC staining and FACS analyses as well as by lactate dehydrogenase release, are depicted. Mean values +/- S.D. of a representative experiment performed in triplicates (annexinV staining) and quadruplicates (LDH assay) are shown. Asterisks indicate significant differences (i.e. p < 0.05) compared to the corresponding controls.

§…specific LDH release = % of LDH released by the treatment of total LDH present in cultures – % of released LDH of controls of total LDH present in controls. §§…% of cell death induced by treatment - % of cell death in controls.

Figure 3C shows a scanning electron microscopic analysis of a cell treated with 15 µM of Cd for 96 hours. A representative image is shown.

Figure 4 – DNA strand breaks and DNA damage response precede caspase-independent, BclXL-inhibitable cadmium-induced cell death.

In order to detect potential DNA strand breaks in response to Cd treatment of endothelial cells in vitro and the interference of Zn with Cd effects TUNEL assays were performed. Figure 4A shows mean value ratios of TUNEL-positive cells/total cells +/- S.D. of a representative experiment performed in triplicates. Asterisks indicate significant differences (p< 0.05) compared to the control. The Western blots in Figure 4B show a time course expression analysis of classical DNA damage response proteins and caspase-target proteins in the presence or absence of Cd and Zn. Representative blots are shown. p-ATM…phosphor-ATM (on serine 1981). Figure 4C summarises the results of experiments where the effect of different inhibitors of cell signalling processes were studied for their potential to inhibit Cd-induced cell death. All experiments were performed in triplicates and were repeated at least
three times. Mean values +/- S.D. are shown. Asterisks indicate significant differences (p < 0.05) compared to the controls. The Western blot in Figure 4D shows an analysis of caspase-3 activation (pro-enzyme at 32 kD; active fragments at 20 and 17 kD) 72 hours after Cd addition (Zn was added 24 hours prior to Cd addition). A representative blot is shown. An effector-caspase activity assay (caspases 3, 6, and 7) is depicted in Figure 4E. Means +/- S.D. of a representative experiment performed in triplicates is shown. All groups, except the Zn and the 15µM Cd + Zn groups differed significantly. Figure 4F shows the surface of Cd-treated endothelial cells at 5000 x magnification (treatment 96 hours). A representative image is shown.

Figure 5 – Cadmium causes endothelial damage, alterations in lipid profiles, and accelerated plaque formation in Apo E knockout mice in vivo.

After the administration of Cd and/or Zn via drinking water (control group, no additives in drinking water) to Apo E KO mice for 12 weeks, scanning electron microscopic images of the aortic endothelium were taken. Representative images are shown for control-treated animals (A) and Cd-treated animals (B, C, D). In image C, arrows indicate contracted endothelial cells at different stages of contraction. The number (1, 2, 3, 4) indicates the potential sequence of events. Intercellular gaps (C) and necrotic endothelial cells (D) which were not present in the controls are also indicted by arrows. The aortic endothelium of Zn-treated animals did not differ from the controls, and the endothelium of Zn plus Cd-treated animals did not differ from Cd-only treated animals. An analysis of the lipid profiles of the above treatment groups and WT control animals is given in Figure 5E. In this experiment VLDL particles had an EV of ~ 15; LDL ~ 18, and HDL ~ 27. An analysis of the atherosclerotic plaque area of the aorta of the animals is given in Figure 5 F. (n per group = 7).
Table 1: Characteristics of the ARFY population (all-female) expressed as mean ± standard deviation, median (interquartile range) or number (percentage).

<table>
<thead>
<tr>
<th>VARIABLES</th>
<th>mean±SD, Median(IQR), n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age – years</td>
<td>20.6±1.0</td>
</tr>
<tr>
<td>Body-mass index – kg/m²</td>
<td>21.9±2.9</td>
</tr>
<tr>
<td>Waist – cm</td>
<td>84.8±6.9</td>
</tr>
<tr>
<td>Systolic blood pressure – mmHg</td>
<td>112.2±8.7</td>
</tr>
<tr>
<td>Diastolic blood pressure – mmHg</td>
<td>68.9±7.2</td>
</tr>
<tr>
<td>Hypertension (office) – no.(%)*</td>
<td>10(4.9)</td>
</tr>
<tr>
<td>Family history, hypertension – no.(%)</td>
<td>52(25.4)</td>
</tr>
<tr>
<td>Fasting glucose – mmol/l</td>
<td>4.6±0.4</td>
</tr>
<tr>
<td>Total cholesterol – mmol/l</td>
<td>4.7±0.9</td>
</tr>
<tr>
<td>HDL-cholesterol – mmol/l</td>
<td>1.9±0.4</td>
</tr>
<tr>
<td>LDL-cholesterol – mmol/l</td>
<td>2.7±0.8</td>
</tr>
<tr>
<td>Triglycerides – mmol/l‡</td>
<td>1.1(0.7-1.5)</td>
</tr>
<tr>
<td>HOMA – Insulin resistance-index</td>
<td>1.9±1.0</td>
</tr>
<tr>
<td>AST – U/l</td>
<td>20.4±6.3</td>
</tr>
<tr>
<td>Lipoprotein (a) – mmol/l‡</td>
<td>0.4(0.1-1.0)</td>
</tr>
<tr>
<td>Homocysteine – μmol/l‡</td>
<td>8.7 (7.5-10.1)</td>
</tr>
<tr>
<td>C-reactive protein – mg/l‡</td>
<td>2.0(1.0-4.0)</td>
</tr>
<tr>
<td>Anti-mHsp60 antibody – titer</td>
<td>2.9±1.2</td>
</tr>
<tr>
<td>Soluble hHsp60 – μg/μl‡</td>
<td>3.3 (0.2-21.7)</td>
</tr>
<tr>
<td>hHsp60 stimulation-index‡</td>
<td>28.7(8.1-73.8)</td>
</tr>
<tr>
<td>Asthma – no.(%)</td>
<td>13(6.3)</td>
</tr>
<tr>
<td>Smoking – no.(%)</td>
<td>82(38.4)</td>
</tr>
<tr>
<td>Pack-years of smoking‡</td>
<td>0.0 (0.0-0.8)</td>
</tr>
<tr>
<td>ETS (calculated cumulative exposure)‡‡</td>
<td>0.6 (0.0-5.0)</td>
</tr>
<tr>
<td>Oral contraceptive intake – no.(%)</td>
<td>154(75.1)</td>
</tr>
<tr>
<td>Low social status – no.(%)</td>
<td>99(48.3)</td>
</tr>
<tr>
<td>IMT internal carotid arteries – mm</td>
<td>0.52±0.12</td>
</tr>
<tr>
<td>IMT common carotid arteries – mm</td>
<td>0.40±0.08</td>
</tr>
</tbody>
</table>


* Hypertension was defined as systolic ≥140, diastolic blood pressure ≥90 mmHg or intake of antihypertensive drugs.
‡‡ Due to a non-Gaussian distribution variable levels are expressed as medians (interquartile range).
§ Environmental tobacco smoke (ETS): in analogy to the pack-years of smoking, cumulative exposure was calculated with the formula, ‘hours of exposure per day’ x ‘years of exposure’.
Online supplement: Assessment of Vascular Risk Factors in the ARFY Study

Participants' risk factors were assessed according to standardised protocols validated and used previously in the Bruneck\textsuperscript{1} and ARMY\textsuperscript{2} studies.

Waist was measured at the narrowest point between the costal margin and the iliac crest (on the naked abdomen) and hip circumference was recorded over the widest diameter of the buttocks (with underwear). Subjects were categorized as ‘smokers’ if they reported regular consumption of at least 1 cigarette per week and lifetime consumption exceeded 40 cigarettes. Cigarette pack-years were calculated by multiplying the years of smoking by the packs smoked per day.

Subjects were given an automated blood pressure monitor (Omron, Mannheim, Germany) and advised to measure BPs at 7 a.m. and 7 p.m (on both arms). Hypertension was defined as a mean systolic BP $\geq 140$ and/or a mean diastolic BP $\geq 90$ mmHg. No regular use of anti-hypertensive drugs was reported. A family history of hypertension was considered positive if at least one first-degree relative was taking or took hypertension and BP-lowering medication.

Insulin resistance was estimated according to the HOMA-Index\textsuperscript{4}. Social status was deduced from the parents' occupation, i.e. rated low for an unskilled worker and high for academics and skilled or supervisory workers. Asthma was self-reported physician-diagnosed. In analogy to pack-years of active smoking, cumulative exposure to environmental tobacco smoke (ETS) was computed by multiplying the ‘hours per day in ETS’ and the ‘years of exposure’.

Blood samples were drawn after an overnight fast and abstention from smoking and glucose, triglycerides, total, LDL- and HDL-cholesterol determined with standard colorimetric assays (ModularP, Roche Diagnostics, Mannheim, Germany), C-reactive protein (CRP) with a latex-enhanced immunologic assay (ModularP, Roche Diagnostics, Mannheim, Germany), lipoprotein(a) (Lp(a)) with immunoturbidimetry (ModularP, Roche Diagnostics, Mannheim,
Germany), insulin with an enzyme-linked chemiluminescent immunosorbent-assay (ModularE170, Roche Diagnostics, Mannheim, Germany), homocysteine with an automated fluorescence-polarization immunoassay (Axsym, Abbott, Wiesbaden, Germany) and AST with a UV-test standardised according to the International Federation of Clinical Chemistry (ModularP, Roche Diagnostics, Mannheim, Germany). All samples (except those for lymphocyte proliferation) were immediately cooled to 4°C and centrifuged within 90 min. An enzyme-linked immunosorbent assay was used to determine antibody titers to recombinant mycobacterial and human HSP60 and the serum concentration of soluble human Hsp60 (sHsp60). The use of peripheral blood mononuclear cell (PBMC) proliferation assays to determine T-lymphocyte reactivity to various antigens in vitro has also been described before1.

Reference List

