Increased Serum Cadmium and Strontium Levels in Young Smokers
Effects on Arterial Endothelial Cell Gene Transcription

David Bernhard, Andrea Rossmann, Blair Henderson, Michaela Kind, Andreas Seubert, Georg Wick

Objective—Metal constituents of tobacco have long been suspected to contribute to cardiovascular diseases. In this study, we determined the serum concentrations of aluminum, cadmium (Cd), cobalt, copper, iron, manganese, nickel, lead, strontium (Sr), and zinc of young nonsmokers, passive smokers, and smokers.

Methods and Results—Cd and Sr were found to be significantly increased in smokers compared with nonsmokers. The effects of these metals on primary arterial endothelial cells were then assessed using microarray technology and real-time polymerase chain reaction (RT-PCR). The data showed that Sr does not interfere with endothelial cell transcription. In contrast, the effects of Cd in amounts delivered to the human body by smoking were dramatic.

Conclusions—Arterial endothelial cells responded to Cd exposure by massively upregulating metal and oxidant defense genes (metallothioneins) and by downregulating a number of transcription factors. In addition, the mRNA of the intermediate filament protein vimentin, crucial for the maintenance of cellular shape, was reduced. Surprisingly, a number of pro-inflammatory genes were downregulated in response to Cd. The present data suggest that by delivering Cd to the human body, smoking deregulates transcription, exerts stress, and damages the structure of the vascular endothelium; furthermore, in contrast to the effects of cigarette smoke as a whole, Cd seems to possess anti-inflammatory properties. (Arterioscler Thromb Vasc Biol. 2006;26:833-838.)

Key Words: atherosclerosis ■ cigarette smoking ■ endothelial ■ metals ■ microarray ■ osteoporosis

Cigarette smoking is a well-established risk factor for cardiovascular diseases. We have previously shown that among young males, smoking was the most important risk factor for early pro-atherogenic vessel wall changes (increased intima-media thickness). However, the mechanisms by which cigarette smoke constituents contribute to atherogenesis are poorly understood. Therefore, we performed a number of in vitro analyses and could show that out of 4800 different compounds in cigarette smoke, the mixture of metals and oxidants constitutes the crucial endothelium-damaging noxa. This combination leads to a chain reaction of protein oxidation, functional impairment of the microtubule system, contraction of endothelial cells, endothelial dysfunction, and, finally, to denudation of the inner vascular surface. According to the “response to injury” hypothesis of atherosclerosis, endothelial dysfunction and vessel denudation play the central role in the atherogenic process.

Cigarette smoke contains a number of different metals, including aluminum (Al), cadmium, chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn). Smoking leads to an increase of serum Cd, Cr, and Pb, and increased serum Cd levels have been shown to contribute to smoking-induced peripheral arterial disease. Abu-Hayyeh et al (2001) reported that along with increased serum levels, Cd also accumulates in the aortic vessel walls of smokers at concentrations of up to 7 µmol/L. Furthermore, there is epidemiological evidence that smoking-increased serum levels of Cd and Pb, and smoking-reduced serum levels of selenium (Se), result in hypertension, cardiovascular diseases, and myocardial infarction. The contribution to atherogenesis of most other metal constituents of cigarette smoke has received little attention. Moreover, the mechanisms by which metals like Cd, Pb, and Se contribute to cardiovascular diseases are not yet clear.

This study was designed to investigate the effect of smoking on serum metal concentrations of young active and passive smokers in comparison to nonsmokers, and to elucidate the effects of the altered metal concentrations of these on arterial endothelial cell transcription.

Materials and Methods

Subjects
After obtaining written informed consent, blood samples were collected during practical coursework from 56 students of the
Innsbruck Medical University. The subjects were also asked to complete a questionnaire. The whole procedure was made anonymous and the study was approved by the Ethics Committee of the Innsbruck Medical University. All participants were informed about the purposes and scope of the study, and signed appropriate consent forms. Data assessment was complete for all participants. Four subjects were excluded from the study either because they were using medication or had acute or chronic diseases.

Assessment of Smoking Habits
All participants were requested to complete an anonymous questionnaire designed to assess their full spectrum of active and passive smoking habits, including active and passive cigarette smoking, pipe and cigar smoking, duration of smoking, brand smoked, and former smoking history. Study subjects were then grouped into 4 subgroups. Group 1 comprised nonsmokers who never smoked a cigarette but reported exposure to passive smoking up to 3.5 hours per week. Group 2 included passive or second hand smokers who, like group 1, had never smoked a cigarette but reported exposure to passive smoking of 3.5 hours per week. Group 3 comprised active smokers smoking 3 cigarettes per day. Group 4 included active smokers smoking 3 cigarettes per day.

Furthermore, the subjects were asked about their present and past medical history of acute or chronic diseases, and present and past history of medication intake.

Serum Preparation and Induced Coupled Plasma–Mass Spectrometric Analyses
For details see http://atvb.ahajournals.org.

Statistics
For details see http://atvb.ahajournals.org.

Analysis of the Sr Content of Cigarette Smoke Extracts
For details see http://atvb.ahajournals.org.

Arterial Endothelial Cell Isolation and Culture
For details see http://atvb.ahajournals.org.

Results
Smoking Habits of a Sample of Medical Students in Austria
Table 1 summarizes the smoking habits of a sample of third-year medical students in Austria. Noteworthy is that the proportion of smokers is extremely high (35%), and nearly as dramatic as the situation in the average adult Austrian population (47%; 2004 Austrian General Population Survey; http://data.euro.who.int/?TabID=2404). In addition, 71% of nonactive smokers are regularly exposed to passive smoke, and only 3 individuals reported not being exposed to any secondhand smoke at all. Among the 56 subjects entering the study, 4 subjects were former smokers. Two of these were excluded from further analyses because they also had disease. The remaining 2 ex-smoking subjects were excluded from statistical analysis of serum metal concentrations because the study group was too small. Furthermore, 1 pipe smoker and 1 cigar smoker were also excluded from metal analyses to keep a pure cigarette smoking group for statistical examination. Thus, in all data on smoking habits 52 subjects are included, whereas 48 subjects are included in the final analysis of serum metal concentrations (Table 1 and Figure 1).

Metal Content of Serum in Young Adults in Relation to Their Smoking Status
Metal concentrations of sera from the students were determined by induced coupled plasma–mass spectrometric (ICP-MS) analyses. No significant changes in concentrations in relation to the smoking status could be observed for the
compared with passive smokers or smokers smoking
serum metal concentrations of both Cd and Sr of nonsmokers

cant differences (*Signifi-

increased Sr and Cd levels compared with nonsmokers, but

smoking 

significantly increased in smokers compared with nonsmok-

2). However, the serum concentrations of Cd and Sr were

rettes per day showed a trend to increase but failed to reach

significance. N indicates number of subjects.

Sr Contents of Cigarette Smoke

It is well known that tobacco and cigarette smoke contain
high levels of Cd,

but because no data were available for the
Sr content, we analyzed the Sr concentrations of smoke extracts of 12 different commercially available cigarette brands, as outlined in the Methods section. None of the tested brands increased the Sr concentration of medium solutions (data not shown).

Microarray Analysis of the Effects of Increased Serum Cd and Sr Levels on Arterial Endothelial Cell Transcription

To study the biological consequences of vascular endothelial cell exposure to the metals Sr and Cd, human arterial endothelial cells were incubated with 1.5 μmol/L Cd (the rough concentration found in the intima of arterial vessel walls of smokers) for 6 and 24 hours, as well as with 1.36 μmol/L Sr for 24 hours. Three independent experiments were performed for each condition. Up to 19 genes were found to be regulated after Sr exposure in single experiments. However, no identical gene was regulated in ≥2 microarray experiments, although the concentration of Sr used represents 3-times the concentration found in smokers’ blood (40 μg/L). This concentration was used to get an idea of the effects of a high Sr concentration on endothelial cell transcription.

In contrast to Sr, Cd regulated a number of genes after 6 as well as after 24 hours. The number of genes regulated, and the overlapping genes between the time points, are depicted in Figure 2. Details of the genes found to be regulated (acces-

sion numbers, names, function, expression levels, and fold regulation) are given in Table 3. After 6 hours, a total of 11 genes were regulated (3 upregulated and 8 downregulated), and after 24 hours 21 genes were regulated (8 upregulated and 13 downregulated). Among the 11 genes regulated after 6 hours, 9 were also regulated in the same direction after 24 hours, only chemokine (C-X-C motif) ligand 2 was no longer regulated, and annexin A1 was regulated in the opposite direction after 24 hours. Six genes regulated by Cd (MT1K, MT1X, MT1E, MT1G, COX II, and annexin A1) were also analyzed by RT-PCR (Table 3) and found to correlate well with the microarray data.

Discussion

Cigarette smoke is known to alter various physiological processes toward a pathophysiological state. Smoking is established to be one of the foremost top risk factors for atherosclerosis. However, it is not clear how smoking initiates and/or enhances atherogenesis. We have previously

![Figure 1. Cigarette smoking increases serum Cd and Sr levels of young smokers. The left panel shows serum Cd concentra-
tions of young nonsmokers (non), passive smokers (passive), smokers smoking <3 cigarettes per day (<3c/d), and smokers smoking ≥3 cigarettes per day (≥3c/d). The right panel shows the serum Sr concentrations of the same study groups. *Signifi-
cant differences (P<0.05; Mann-Whitney test). The difference in serum metal concentrations of both Cd and Sr of nonsmokers compared with passive smokers or smokers smoking <3 cigarettes per day showed a trend to increase but failed to reach significance. N indicates number of subjects.](http://atvb.ahajournals.org/)

![Table 2. Mean Serum Metal Concentrations in Young Nonsmokers, Passive Smokers, and Smokers](http://atvb.ahajournals.org/)
smokers smoking universities). Although forbidden, smoking is even tolerated in virtually all (eg, smoking is allowed in all bars and restaurants and, especially the Austrian government, are very slow in adapting legislation to protect nonsmokers from cigarette smoke quitting, are rare. Also pertinent is the high number of general population from starting to smoke, or helping them smoke, and that education to prevent medical students and the fact that it is not unusual for an Austrian physician to smoke constituents found to be important affect vascular sclerosis. In the present study, we address the question of how smoking causes atherosclerosis. In the present study, we address the question of how smoke constituents found to be important affect vascular endothelial physiology.

First, one of the most obvious results from this study is the fact that the number of smokers among medical students in Austria is very high. An important reason for this might be the fact that it is not unusual for an Austrian physician to smoke, and that education to prevent medical students and the general population from starting to smoke, or helping them quit smoking, are rare. Also pertinent is the high number of passive smokers among students. European governments, and especially the Austrian government, are very slow in adapting the legislation to protect nonsmokers from cigarette smoke (eg, smoking is allowed in all bars and restaurants and, although forbidden, smoking is even tolerated in virtually all public and government buildings and offices, including the universities).

Analyses of the serum metal concentrations revealed that smokers smoking >=3 cigarettes per day had significantly increased serum levels of Cd and Sr compared with nonsmokers. Although not significant, passive smokers and study subjects smoking <3 cigarettes per day also showed increases in the concentration of both metals. Analyses of the Sr content of cigarette smoke revealed that cigarette smoke is not able to significantly increase the Sr concentrations of a hydrophilic solution. This result makes it clear that increased serum Sr levels are not the result of Sr delivery to the body, but rather suggest that the body’s Sr physiology is changed by smoking. Based on the fact that the biological and chemical properties of Sr are quite similar to those of Ca, we suggest 2 potential mechanisms that may lead to an increase of Sr in the serum of smokers. First, it is epidemiologically well-established that smokers are at a higher risk for osteoporosis. It is assumed that smoking elutes bone Ca (and with that also Sr) by interfering with estrogen receptor function and/or by inducing estrogen metabolizing enzymes. Because Sr has a larger atomic radius than Ca, the absorption of Sr in biological systems compared with Ca is low. Therefore, we hypothesize that renal Sr secretion might also be reduced resulting in increased serum Sr levels. Second, cigarette smoke, especially the metal in Cd cigarette smoke, is suspected to reduce or damage renal function. Because renal secretion is an important regulatory mechanism in the serum homeostasis of Ca and Sr, impaired renal function by smoking might account for the increased serum Sr levels observed in smokers. Accordingly, increased Sr and Ca levels in smokers may contribute to, or be a consequence of, smoking caused osteoporosis and/or vessel calcification.

To address the question how increased serum levels of Cd and Sr might affect vascular endothelial cells, we performed microarray analyses. In the case of Sr, no reproducible changes in cellular transcription could be observed. Based on this finding, we suggest that increased levels of Sr alone are unlikely to constitute an important player in smoking-generated endothelium-dependent disorders, but, in conjunction with other constituents of cigarette smoke, they may do so. In addition, effects of increased Sr levels on cell types other than arterial endothelial cells cannot be excluded.

In contrast to Sr, the data presented in Table 3 demonstrate that, at certain doses, endothelial cells respond to Cd in cigarette smoke by regulating a number of genes. Basically, the genes regulated by Cd can be classified as: (1) metallothioneins; (2) genes involved in inflammatory processes; (3) transcription factors; and (4) other genes.

Table 3 shows that a number of metallothionein 1 (MT1) subtypes, namely MT1K, MT1X, MT1F, MT1E, MT1G, and MT1H, were upregulated by Cd. The roles of the individual MT1 subtypes are unknown, but MT1s are important cellular tools in the general defense against toxic metals and oxidants. There is evidence that MTs might contribute to an oxidant-scavenging mechanism in atherosclerotic plaques and that they also interfere with Zn-mediated nitric oxide (NO) metabolism in the vascular wall. Our data indicate that Cd induces cellular stress, which endothelial cells try to counteract by the induction of MTs. Interestingly, endothelial cells seem to respond to much lower Cd concentrations compared with, eg, smooth muscle cells. This difference may be explained by the assumption that the endothelium constitutes a cell layer that not only regulates cellular and chemical transport but also serves as a protective barrier for the vascular wall.

The next important observation was that Cd downregulated a number of pro-inflammatory genes. Cd was found to repress prostaglandin (PG)-endoperoxide synthase 2 (cyclo-oxygenase 2 (COX 2)) transcription, which is the first rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins. The contribution of prostaglandins to inflammatory processes has been extensively studied. Most anti-inflammatory drugs are COX inhibitors. Furthermore, chemokine CXC-motif ligand 2 (CXCL2), a member of the GRO family of chemokines that facilitates the arrest of monocytes on endothelial cells, a crucial step for infiltration of the vessel wall, was also downregulated by Cd. The observation that Cd downregulates COX 2 and CXCL2 suggests that in contrast to the sum of chemicals in cigarette smoke, Cd seems to suppress inflammation and to inhibit infiltration into the vessel wall. Cd has dual effects on annexin A1 (ANXA1)
**TABLE 3. Cd-Induced Changes in the Gene Expression of Primary Human Arterial Endothelial Cells**

<table>
<thead>
<tr>
<th>Genbank Unigene</th>
<th>Function</th>
<th>Array Regulation (log)</th>
<th>RT PCR Regulation (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6 h 24 h</td>
<td>6 h 24 h</td>
</tr>
<tr>
<td><em>R06655</em></td>
<td>Metal detoxification</td>
<td>1.84</td>
<td>4.37</td>
</tr>
<tr>
<td><em>NM_002450</em></td>
<td>Metal detoxification</td>
<td>n.r.</td>
<td>2.25</td>
</tr>
<tr>
<td><em>AI125646</em></td>
<td>Not clear</td>
<td>1.43</td>
<td>1.16</td>
</tr>
<tr>
<td><em>M10943</em></td>
<td>Metal detoxification</td>
<td>n.r.</td>
<td>1.30</td>
</tr>
<tr>
<td><em>BF217861</em></td>
<td>Metal detoxification</td>
<td>n.r.</td>
<td>1.13</td>
</tr>
<tr>
<td><em>NM_005950</em></td>
<td>Metal detoxification</td>
<td>n.r.</td>
<td>1.09</td>
</tr>
<tr>
<td><em>NM_005951</em></td>
<td>Metal detoxification</td>
<td>n.r.</td>
<td>1.05</td>
</tr>
<tr>
<td><em>AL031602</em></td>
<td>Not clear</td>
<td>n.r.</td>
<td>1.01</td>
</tr>
<tr>
<td><em>M57731</em></td>
<td>Arrests monocytes</td>
<td>−1.64</td>
<td>n.r.</td>
</tr>
<tr>
<td><em>AA524053</em></td>
<td>RNA transport and splicing</td>
<td>n.r.</td>
<td>−1.01</td>
</tr>
<tr>
<td><em>AL068463</em></td>
<td>Immune stimulatory chaperone</td>
<td>n.r.</td>
<td>−1.19</td>
</tr>
<tr>
<td><em>NM_003407</em></td>
<td>Not clear</td>
<td>n.r.</td>
<td>−1.22</td>
</tr>
<tr>
<td><em>AY151286</em></td>
<td>Prostaglandin synthesis</td>
<td>−1.23</td>
<td>−1.29</td>
</tr>
<tr>
<td><em>AI520969</em></td>
<td>Structural protein</td>
<td>−1.59</td>
<td>−1.31</td>
</tr>
<tr>
<td><em>BF514079</em></td>
<td>Proliferation inhibitor</td>
<td>−1.38</td>
<td>−1.31</td>
</tr>
<tr>
<td><em>AI189753</em></td>
<td>Immune-stimulatory antigen, cell motility.</td>
<td>−1.52</td>
<td>−1.34</td>
</tr>
<tr>
<td><em>NM_006732</em></td>
<td>FosB murine osteosarcoma viral oncogene homolog B (fosB)</td>
<td>n.r.</td>
<td>−1.85</td>
</tr>
<tr>
<td><em>AL042660</em></td>
<td>Hypothetical protein DJ971N18.2</td>
<td>n.r.</td>
<td>−2.05</td>
</tr>
<tr>
<td><em>AU155094</em></td>
<td>Annexin A1 (ANXA1)</td>
<td>2.21</td>
<td>−2.03</td>
</tr>
<tr>
<td><em>AI459194</em></td>
<td>Tumour suppressor</td>
<td>−3.57</td>
<td>−2.85</td>
</tr>
<tr>
<td><em>BC004490</em></td>
<td>v-fos FBJ murine osteosarcoma viral oncogene homolog (fos)</td>
<td>n.r.</td>
<td>−3.17</td>
</tr>
</tbody>
</table>

*Regulation values below zero represent genes that were downregulated in response to Cd treatment of endothelial cells, whereas values above zero represent an upregulation of genes. Array data: A regulation value of 1 means that the indicated mRNA was increased 2-fold in response to the treatment.*

expression. ANXA1, which has been shown to suppress inflammatory processes, is upregulated after 6 hours and downregulated after 24 hours. The down regulation after 24 hours might be indicative of a return to basal ANXA1 levels. Nuclear receptor subfamily 4 group A 2 (NR4A2) has been linked to Parkinson disease and other neurological disorders, but the data are not clear. Interestingly, NR4A has recently been shown to be upregulated in macrophages in response to inflammatory stimuli. With the downregulation of tumor rejection antigen (gp96) 1 and transmembrane 4 superfamily member 1 (TM4SF1/L6 antigen), 2 potent immune stimulatory and cancer-relevant genes were found to be repressed by Cd after 24 hours.

Kruppel-like factor 4 (KLF4) and early growth response 1 (EGR1) inhibit cellular proliferation, and their downregulation has been linked to the genesis of cancers. This suggests a carcinogenic potential and mechanisms of Cd. In addition, Cd-mediated downregulation of KLF4 and EGR1 might increase smooth muscle cell proliferation and could thereby contribute to atherogenesis. However, EGR1 knockout mice show reduced inflammation and atherogenesis.

The prototypic immediate early fos genes together with jun isoforms constitute the AP-1 transcription factor. The downstream events of AP-1 activity are diverse, but FBJ murine osteosarcoma viral oncogene homolog B (fosB) has been shown to promote bone formation. Downregulation of fosB in osteoblasts, as we have seen in HAECs, might contribute to the osteoporosis causing activity of cigarette smoking and might interfere with Ca homeostasis, as suggested by our Sr data. This hypothesis is further underlined by the data for the v-fos FBJ murine osteosarcoma viral oncogene homolog (fos), which modulated bone metabolism, especially in combination with Cd. Further, an altered Ca metabolism might contribute to vascular calcification and also interfere with plaque stability.

Vimentin is part of the intermediate filaments, 1 of the 3 structural networks in cells. In recent publications of our group, we could show that vascular endothelial cell structure is disrupted by cigarette smoke constituents and by immunofluorescence analyses that all structural elements of the cells, including intermediate filaments, break down. Functional analyses revealed that breakdown of the microtubule system...
is the crucial event in this process, and that a combination of oxidants and metals is needed to cause collapse of the cells.\textsuperscript{2} The data of the present study now show that in addition, Cd contained in cigarette smoke also seems to weaken the cellular structure by reducing cellular vimentin levels.

The function of the zinc finger motif containing gene products, zinc finger 36 (ZFP36), zinc finger protein 207 (ZNF207), and IBR domain containing 3 (IBRDC3) are as yet unknown, but our data indicate they may play a role in the cellular response to Cd. Furthermore, the relevance of the downregulated genes splicing factor, arginine/serine-rich 7, 35 kDa, which is involved in RNA transport and alternative splicing, transmembrane 4 superfamily member 1, with relevance in carcinogenesis, as well as of the hypothetical protein DJ971N18.2 are not yet clear.

In summary, the data presented suggest that cigarette smoking changes the body’s metal homeostasis via 2 mechanisms. Increased levels of Cd are the result of direct delivery of Cd to the human body by cigarette smoke. Cd accumulates in the vasculature and changes arterial endothelial cell transcription. Apart from the effects of Cd on the transcriptome, interactions with other chemicals in smoke and with physiologically relevant ions are likely to occur, and these properties of Cd might potentiate the endothelium-damaging activity of cigarette smoke. The increased serum Sr levels in smokers is less well-understood but may be the consequence of previous pathophysiological changes in the human body induced by cigarette smoking.

Acknowledgments

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Material and Methods - DETAILS

Serum preparation and induced coupled plasma - mass spectrometric analyses (ICP-MS)

Blood samples were taken with Neutral S-Monovettes (no filling; Sarstedt, Wr. Neudorf, Austria) and allowed to agglutinate for 1.5 h at room temperature (RT). Blood clots were pelleted by centrifugation (2000g, 20 min, RT) and the serum transferred into reaction tubes. The samples were stored at -20°C and shipped on dry ice. For complete disintegration of the samples, 1 ml of concentrated suprapure HNO₃ was added to 0.5 ml of the samples, followed by incubation at 70°C. For metal analyses, the total sample weight was adjusted to 5g with water, and the samples were transferred to sample vials pre-leached to remove metal contaminants. Rhodium (Rh) was added to all samples as an internal standard. The addition of Rh allows compensation of differences in the matrix and the responding behaviour of the analyser. ICP-MS analysis was performed two times for ten elements using an ICP-MS VG Elemental PQII Turbo+; FIA sampling, and ICP-MS Agilent 7500ce, CETAC continuous sampling. Data analyses were performed with Metrohm IC-Net 2.3, ChemStation, and Microsoft Excel software. For calibration, aqueous elemental standard solutions of the metals with acid concentrations analogous to the samples were used. All solutions, tubes, plasticware etc. used were tested for potential metal contamination.

Statistics

The serum metal concentrations of the groups 1 – 4 were compared with either the two-sided Mann-Whitney test for independent samples, or in case of a Gaussian distribution (determined
via a Kolmogorov-Smirnov test) of the values, with ANOVA. Statistical analyses were performed using Microsoft Excel and SPSS 11.0 software.

**Analysis of the Sr content of cigarette smoke extracts**
The hydrophilic fraction of cigarette smoke from 12 commercially available cigarette brands was sampled by a technology that has previously been developed by our group allowing for a “physiological-like” gas-to-liquid-phase compound exchange (1). The extracts were prepared and the Sr amount was quantified by ICP-MS analysis as outlined above.

**Arterial endothelial cell isolation & culture**
Primary human arterial endothelial cells (HAECs) were isolated from discarded arterial tissues, provided by the Transplant Surgery Department of the Innsbruck Medical University, in accordance with the rules governing the use for research purposes of biological material obtained from patients during operations and which are generally discarded. (Ethics committee of the Innsbruck Medical University [http://www.uibk.ac.at/c/c1/c119](http://www.uibk.ac.at/c/c1/c119)). After washing the arteries with PBS, cells were detached by incubation with collagenase for 30 min at 37°C. Thereafter, the cells were collected by washing out with 10 ml of PBS (2), endothelial cells were separated from smooth muscle cells and fibroblasts by addition of endothelium-specific anti-CD31 antibodies to the cells, followed by antibody-mediated attachment of magnetic beads to the anti-CD31 antibodies (Dako), and magnetic separation. HAECs were grown for 2 passages to remove the magnetic beads, and purity of the cultures was checked by fluorescence activated cell sorter (FACS) analysis (acceptable purity was above 95 %). The cells were routinely passaged in 0.2% gelatine-coated (Sigma, Steinheim, Germany) polystyrene culture flasks (Becton Dickinson, Meylan Cedex, France) in Endothelial Cell Basal Medium (CC-3121, BioWhittaker, Inc., Walkersville, MD, USA) supplemented with endothelial growth medium (EGM) SingleQuots Supplements (including 2 % fetal bovine
serum (FBS), gentamicin, and amphotericin-B) and growth factors (CC-4133, BioWhittaker, Inc.) at 37°C in a humidified atmosphere containing 5% CO₂.

For microarray analyses, 3 x 10⁶ HAECs (passage 4) were seeded onto gelatine-coated petri dishes. The medium was replaced with fresh medium prior to the start of each experiment. Chemical reagents were purchased from Merck (Darmstadt, Germany) unless stated otherwise, and were of analytical grade quality.

**RNA isolation, microarray, and RT-PCR analyses**

After incubation of HAECs for 6 or 24 h with 1.5 µM Cd, or with 1.36 µM Sr for 24 h, cells were detached by trypsinisation, and collected by centrifugation (300g, RT, 5’). Cell pellets were stored at -20°C. For RNA preparation, the pellets were resuspended in TriReagent (Sigma) and RNA was isolated as previously described (3). Total RNA was further purified using the RNeasy kit (Qiagen, Hilden, Germany). RNA quantity and integrity were assessed by OD260/280 measurements and Agilent “lab-on-a-chip” technology (2100 Bioanalyzer, Agilent, Palo Alto, CA). Quality requirements were OD260/280 ratios of 1.8 to 2.2 and an 18S to 28S ratio of 1.8 to 2.2. Hybridization target preparations were performed according to protocols recommended by Affymetrix (Affymetrix Technical Manual). Briefly, 5µg (protocol 2) total RNA was reverse transcribed using an oligo(dT)-T7 promotor primer, before second strand synthesis by E.coli DNA polymerase (Affymetrix one cycle cDNA synthesis kit). After purification of double stranded cDNA with the Affymetrix GeneChip Sample Cleanup Module, biotin-labeled cRNA was produced by T7 polymerase (Affymetrix IVT Labeling kit). After lab on a chip-based quantification and integrity control, 20 µg cRNA was fragmented by alkaline treatment (Affymetrix GeneChip Sample Cleanup Module), and 15 µg fragmented cRNA was added to the Affymetrix hybridization cocktail (300 µl final volume). The arrays (Human genome U133 Plus 2.0; Affymetrix) were washed and stained according to the recommended Fluidics Station protocol (EukGE-WS2 version 5_450).
Fluorescence signal intensities from each feature on the microarrays were determined using the Affymetrix GeneChip Scanner 3000 and GCOS software (version 1.2), according to the manufacturer’s recommendations. The raw data from all arrays were normalized using the RMA package for “R” according to Irizarry et al. (4). Microarray analyses were performed on three independent experiments (biological replicates), and the genes reported to be regulated were found to be regulated in all experiments. Genes with an expression value $A$ below 5 were excluded from further analysis. Genes were considered to be regulated when the log$_2$ ratio of the expression values ($M$) was identical to or below -1 (two-fold down-regulation) or when $M$ was identical to or above 1 (two-fold up-regulation). Quantitative real time PCR of MT1K, MT1X, MT1E, MT1G, COX II, and annexin A1 was performed using Affymetrix primers based on Affymetrix probe sequences, and the LightCycler FastStart DNA Master SYBR Green kit (Roche) on the LightCycler 1.0 system (Roche).

Reference List


