Cadmium reduces nitric oxide production by impairing phosphorylation of endothelial nitric oxide synthase

Syamantak Majumder, Ajit Muley, Gopi Krishna Kolluru, Samir Saurabh, K.P. Tamilarasan, Sidhharth Chandrasekhar, Hima Bindu Reddy, Sharad Purohit, and Suvro Chatterjee

Abstract: Cadmium (Cd) perturbs vascular health and interferes with endothelial function. However, the effects of exposing endothelial cells to low doses of Cd on the production of nitric oxide (NO) are largely unknown. The objective of the present study was to evaluate these effects by using low levels of $CdCl_2$ concentrations, ranging from 10 to 1000 nmol/L. Cd perturbations in endothelial function were studied by employing wound-healing and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays. The results suggest that a $CdCl_2$ concentration of 100 nmol/L maximally attenuated NO production, cellular migration, and energy metabolism in endothelial cells. An egg yolk angiogenesis model was employed to study the effect of Cd exposure on angiogenesis. The results demonstrate that NO supplementation restored Cd-attenuated angiogenesis. Immunofluorescence, Western blot, and immuno-detection studies showed that low levels of Cd inhibit NO production in endothelial cells by blocking eNOS phosphorylation, which is possibly linked to processes involving endothelial function and dysfunction, including angiogenesis.

Key words: cadmium, endothelial cells, angiogenesis, nitric oxide.

Résumé : Le cadmium (Cd) perturbe la santé vasculaire et interfère avec la fonction endothéliale. Cependant, le modus operandi des effets d'une exposition de l'endothélium à de faibles doses de Cd en relation avec l'oxyde nitrique est essentiellement inconnu. L'objectif de cette étude était d'évaluer les effets d'une exposition des cellules endothéliales à de faibles doses de Cd sur la production d'oxyde nitrique. Les concentrations de Cd allant de 10 à 1000 nmol/L ont été utilisées pour investiguer les effets du Cd sur la production d'oxyde nitrique. La perturbation des fonctions endothéliales par le Cd ont été étudiées à l'aide d'essais de cicatrisation et d'un essai MTT (bromure de 3-(4,5-dimethylthiazol-2-yl)-2,5-diphényltétrazolium). Les résultats suggèrent qu'une concentration de 100 nmol/L de Cd atténue la production d'oxyde nitrique, la migration cellulaire et le métabolisme énergétique des cellules endothéliales. Un modèle d'angiogenèse vitelline a été utilisé pour étudier l'effet d'une exposition au Cd sur l'angiogenèse. Les résultats démontrent que, alors que de faibles concentrations de Cd atténuent l'angiogenèse, une supplémentation en oxyde nitrique la rétablit. Des études en immunofluorescence, en buvardage Western et en immuno-détection ont montré que le Cd empêche la phosphorylation de l'eNOS pour bloquer la production d'oxyde nitrique dans les cellules endothéliales. En conclusion, les résultats de notre étude indiquent que de faibles concentrations de Cd bloquent la production d'oxyde nitrique en bloquant la phosphorylation de l'eNOS, ce qui est possiblement lié à des processus impliquant la fonction et la dysfonction endothéliales, y compris l'angiogenèse.

Mots-clés : cadmium, cellules endothéliales, angiogenèse, oxyde nitrique.

[Traduit par la Rédaction]

Introduction

Cadmium (Cd) is a naturally available metallic component in the earth's crust and is implicated in the lives of plants, animals, and humans (Bokori and Fekete 1995). Although it has many uses in consumer products, such as batteries, metal coatings, and some metal alloys, it has also been declared a human carcinogen (Waalkes 2003). One of the most serious causes of Cd poisoning is tobacco smoking

Received 5 June 2007. Revision received 10 October 2007. Accepted 18 October 2007. Published on the NRC Research Press Web site at bcb.nrc.ca on 25 January 2008.

Abbreviations: Cd, cadmium; DEAN, diethylamine NONOate; DMEM, Dulbecco's modified Eagle's medium; ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; 5-FU, 5-fluorouracil; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate-buffered saline.

S. Majumder, A. Muley, G.K. Kolluru, S. Saurabh, K. Tamilarasan, S. Chandrasekhar, H.B. Reddy, and S. Chatterjee.[1] Vascular Biology Lab, Life Sciences, AU-KBC Research Centre, MIT Campus, Anna University, Chennai, Tamil Nadu 600044, India.
S. Purohit. Center for Biotechnology and Genomic Medicine, Medical College of Georgia, Augusta, GA 30912, USA.

[1]Corresponding author (e-mail: soovro@yahoo.ca).

 doi:10.1139/O07-146 © 2008 NRC Canada

Fig. 1. (*a*) Assessment of the toxic dose of cadmium (Cd) in endothelial cells (ECs). A cell viability assay with trypan blue was performed to determine the LD_{50} of Cd (in the form of $CdCl_2$). Blue staining indicates dead cells. Cells were treated for 8 h with concentrations of $CdCl_2$ (ranging from 0 to 1000 μmol/L), after which they were stained with 0.4 mg/mL trypan blue and incubated for 10–15 min. Once staining was complete, the stained cells for each $CdCl_2$ treatment were counted and the numbers plotted as percentages of dead cells. The observations suggested that 100 μmol/L is the LD_{50} of Cd for ECs. Each point on the graph represents 300 cells. *, Significantly different from control (cells not treated with Cd) ($p \leq 0.05$). (*b*) Measurement of nitric oxide (NO) production by ECs following Cd treatment. NO production by ECs was measured with a Griess assay. ECs were treated with different doses of $CdCl_2$ ranging from 0 to 1000 nmol/L. NO generation reduced when the cells were treated with ≥100 nmol/L $CdCl_2$. The results suggest that Cd affects endothelial NO production in a dose-dependent manner. *, Significantly different from control cells ($p < 0.05$). (*c*) Temporal effect of Cd on EC NO production. NO production was measured with a Griess assay. ECs were treated with 100 nmol/L $CdCl_2$ for different time periods. NO generation reduced when the cells were treated with 100 nmol/L $CdCl_2$ for 4–8 h. The results suggest that Cd affects endothelial NO production in a time-dependent manner, particularly within 4–6 h. **, Significantly different from control ($p < 0.001$).

(Zawadzka et al. 1989). A tobacco addict smoking 20 cigarettes daily absorbs 2–4 μg Cd each day and accumulates 0.5 mg Cd in a year (Massadeh et al. 2005; Oldereid et al. 1994).

Efforts have been made to understand the mechanisms of action of Cd toxicity, which causes multiple respiratory disorders, acute pneumonitis, emphysema (Nemery 1990; Oberdorster 1986), and impaired endothelium-dependent vasodilation of different vessels (Barua et al. 2001). Although a score of population studies and experimental works shows that Cd impairs cardiovascular health, the cellular and molecular mechanisms of Cd-mediated cardiovascular abnormalities have not yet been explored. Cd is known to affect the vascular tone of blood vessels and blood pressure (Baranski et al. 1983), and it is evident that Cd exposure produces hypertension in rats (Puri 1999). One of the biophysical explanations of Cd-associated hypertension is that Cd replaces zinc, thereby contributing to the brittleness and hardness of arteries, which in turn causes hypertension (Chisolm and Handorf 1985). The work of Demontis et al. (1998) demonstrated that Cd affects mean arterial blood pressure in hypertensive rats by increasing both the alpha and beta isoforms of PKC (protein kinase C) in both SHR and WKY vascular smooth muscle cells. Kolluru et al. (2006) found that micromolar concentrations of Cd led to endothelial dysfunction by blocking eNOS activity. In this context, there is clear evidence that low and chronic exposure of vascular systems to Cd impairs vascular health (Navas-Acien et al. 2004). We hypothesize that low-level exposure to Cd attenuates eNOS activation by disrupting the phosphorylation pattern of eNOS. In this work, we partially dissect the mechanism of Cd-dependent eNOS inactivation.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Hi-Media (Mumbai, India). FBS was purchased from Invitrogen Life technologies (www.invitrogen.com). Diethylamine NONOate (DEAN) was purchased from EMD Biosciences, Inc. (www.emdbiosciences.com). Antibodies were purchased from Calbiochem, EMD Chemicals Inc. $CdCl_2$ was purchased from Hi-Media, and used as the source of Cd. All other chemicals were of reagent grade and were obtained commercially.

Cell culture

An immortalized endothelial hybrid cell line, EAhy926, was obtained from Dr. C.J.S. Edgell (the University of North. Carolina at Chapel Hill, Chapel Hill, N.C., USA), and was cultured in DMEM supplemented with 10% FBS (*v*/*v*), 1% penicillin–streptomycin (*w*/*v*).

NO estimation

Endothelial cells (ECs) were incubated for 4 h with $CdCl_2$ at 37 °C and 5% CO_2. NO was measured with a Griess assay protocol, described elsewhere (Nims et al. 1996).

Wound healing assay

We used the wound healing method to assess cell migration. EAhy926 cells (10^6) in 2 mL DMEM – 10% FBS were seeded in a 35 mm dish. When the cells reached confluence 24 h later, scratching the monolayer with a 1 mm wide sterile plastic scraper made a linear wound. As per the experimental protocol described elsewhere (Staton et al. 2004), cells were washed with PBS and treated with Cd (in the form of $CdCl_2$) at concentrations ranging from 0 to 1 μmol $CdCl_2$/L. and incubated for 8 h. Bright-field images were taken at 4× magnification under an inverted bright field microscope. The rate of wound healing was quantified from the images using Scion Image alpha release 4.0 3.2 and Adobe Photoshop 6.0.

MTT assay

EAhy926 cells are grown to about 70% confluence in 24 well plates. The cells were then subjected to Cd treatment for 8 h. After an 8 h incubation period, MTT was added to the media for a final concentration of 0.2 μg/mL. After 2 h incubation with MTT, cells were subjected to isopropyl alcohol treatment. Isopropyl alcohol lysed the cells to release MTT-based colour products from the cells. The OD of the colour products was measured at 575 nm by using a Varian Cary 4000 UV-Vis spectrophotometer.

Egg yolk angiogenesis assay

Fourth day incubated eggs were collected from the Poultry Research Station, (Nandanam, Chennai, India). Eggs were broken and gently plated on a cellophane bed in Petri dishes under sterile conditions. $CdCl_2$ discs (0–5 μmol/L) were then placed on the egg yolks, which were incubated for another 12 h. Images were taken at a 20× magnification using a Nikon CoolPix camera adapted to a

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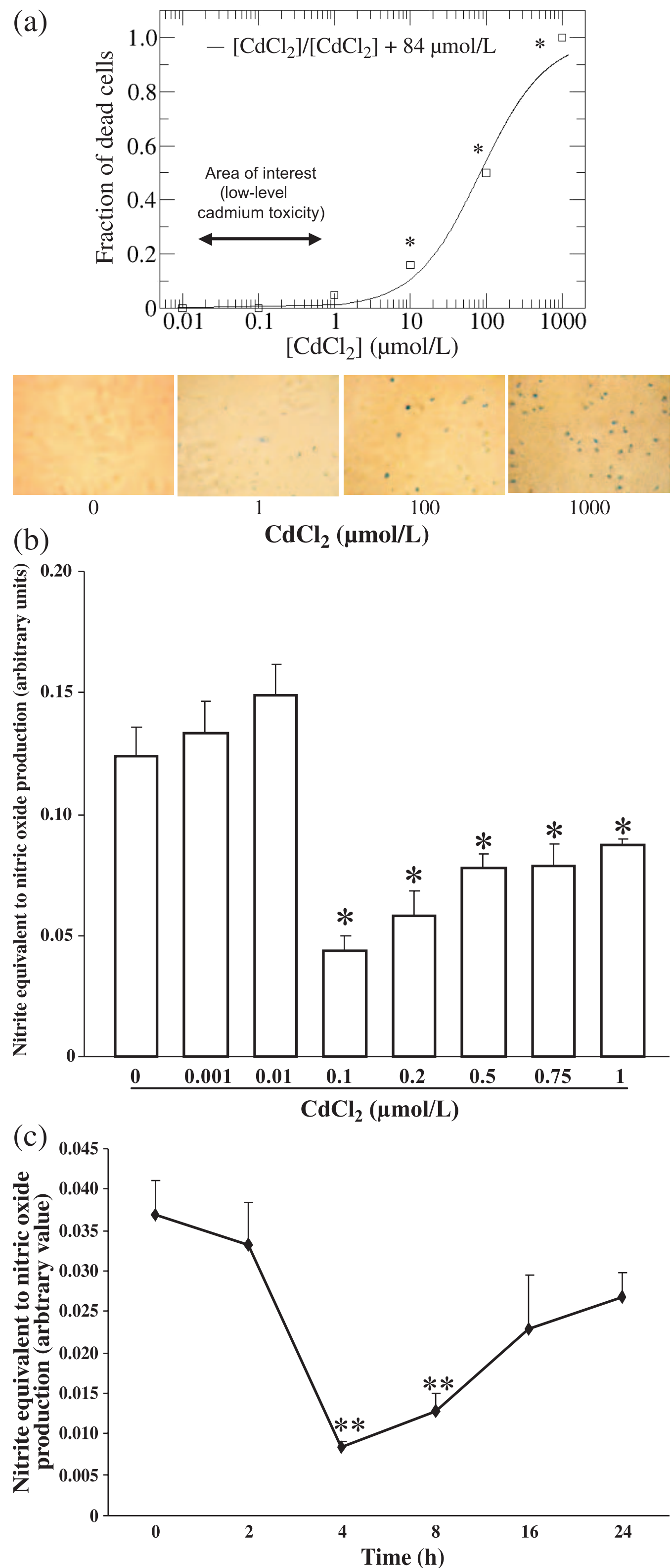

stereo-microscope after 0, 6, and 12 h of incubation. The quantification of angiogenesis was performed by using Scion Image alpha release 4.0 3.2 and Adobe Photoshop 7.0.

Cell viability assay

EAhy926 cells were grown to about 60% confluence in a 24 well plate. The cells were then subjected to Cd treatment for 4 h. Trypan blue (0.4 mg/mL) was added to the media, and the cells were incubated for another 15 min. After this incubation period, the media was removed and 1× PBS was added to all the wells. The number of cells with a blue nucleus were counted under a microscope.

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Fig. 2. (*a*) Cadmium (Cd) inhibits wound healing of endothelial cells (ECs). EC monolayers were wounded by scratching the monolayer surfaces, and then treated with $CdCl_2$ for 8 h. ECs treated with either 100 or 200 nmol/L $CdCl_2$ healed more slowly compared with the control cells. The result suggest that Cd blocks EC wound healing. *, Significantly different from control ($p < 0.05$). (*b*) Withdrawal of Cd from the media reverses Cd-mediated effects on ECs. EC monolayers were wounded by scratching the monolayer surfaces, and then subjected to Cd treatment for 4 h. ECs treated with either 100 or 200 nmol/L $CdCl_2$ for up to 4 h healed more slowly compared with the control cells. Cells were then washed with PBS and further incubated with fresh DMEM for 6 h. The inhibitory effect of Cd on EC wound healing was reversedwithin 4 h of washing post-treatment. *, Significantly different from control cells ($p < 0.05$).

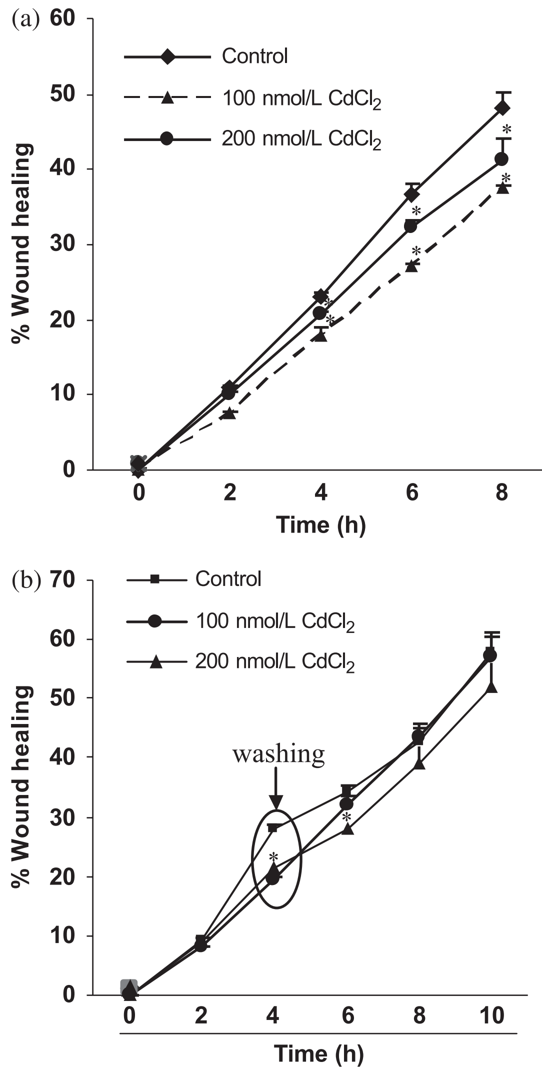
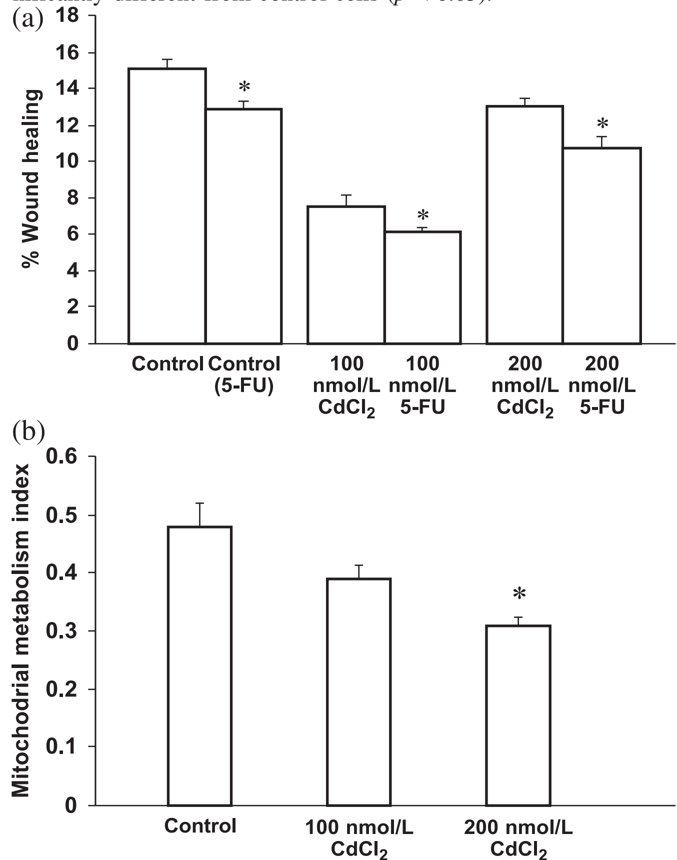


Fig. 3. (*a*) Cadmium (Cd) blocks migration of endothelial cells (ECs). EC monolayers were wounded by scratching the monolayer surfaces, and then treated with $CdCl_2$ and 10 µg/mL 5-fluoro uracil (5-FU) for 4 h. Control sets both with and without 5-FU showed a significant difference in wound healing after 4 h, and the same result was observed for cells that had been treated with $CdCl_2$ (with and without 5-FU). The results suggest that Cd inhibits EC wound healing by blocking EC migration rather than proliferation. *, Significantly different between control 5-FU treated cells and control cells, between cells treated with 100 nmol/L 5-FU and 100 nmol/L $CdCl_2$ alone, and between cells treated with 200 nmol/L 5-FU and 200 nmol/L $CdCl_2$ alone ($p < 0.05$). (*b*) MTT assay for mitochondrial succinate dehydrogenase activity of ECs treated with $CdCl_2$. EC mitochondrial succinate dehydrogenase activity was determined with an MTT assay. Cells were subjected to Cd treatment for 4 h and then incubated with MTT for 2 h. The OD of the colour metabolite was measured at 575 nm. There was a significant decrease in MTT-based colour product formation by $CdCl_2$-treated ECs. *, Significantly different from control cells ($p < 0.05$).



Immunofluorescence

These studies were carried out on EAhy926 cells in 12 well plates using the cold paraformaldehyde – Triton X-100 procedure (Mukhopadhyay et al. 2006; Shah et al. 2005). Cells were incubated at 4 °C overnight with rabbit polyclonal antibodies (dilution, 1:1000) against phospho-eNOS. Next, corresponding goat anti-rabbit secondary antibodies (dilution, 1:2000), tagged with fluorescein isothiocyanate, was used. Images were collected using a Nikon epifluorescence microscopy system equipped with a CCD camera loaded with black and white film.

Western blot analyses of proteins

Protein sample electrophoresis was performed by SDS–PAGE using 10% polyacrylamide gels, as described elsewhere (Nakashima et al. 1999; Sehgal et al. 2002; Shah et al. 2002), and transferred onto a nitrocellulose membrane

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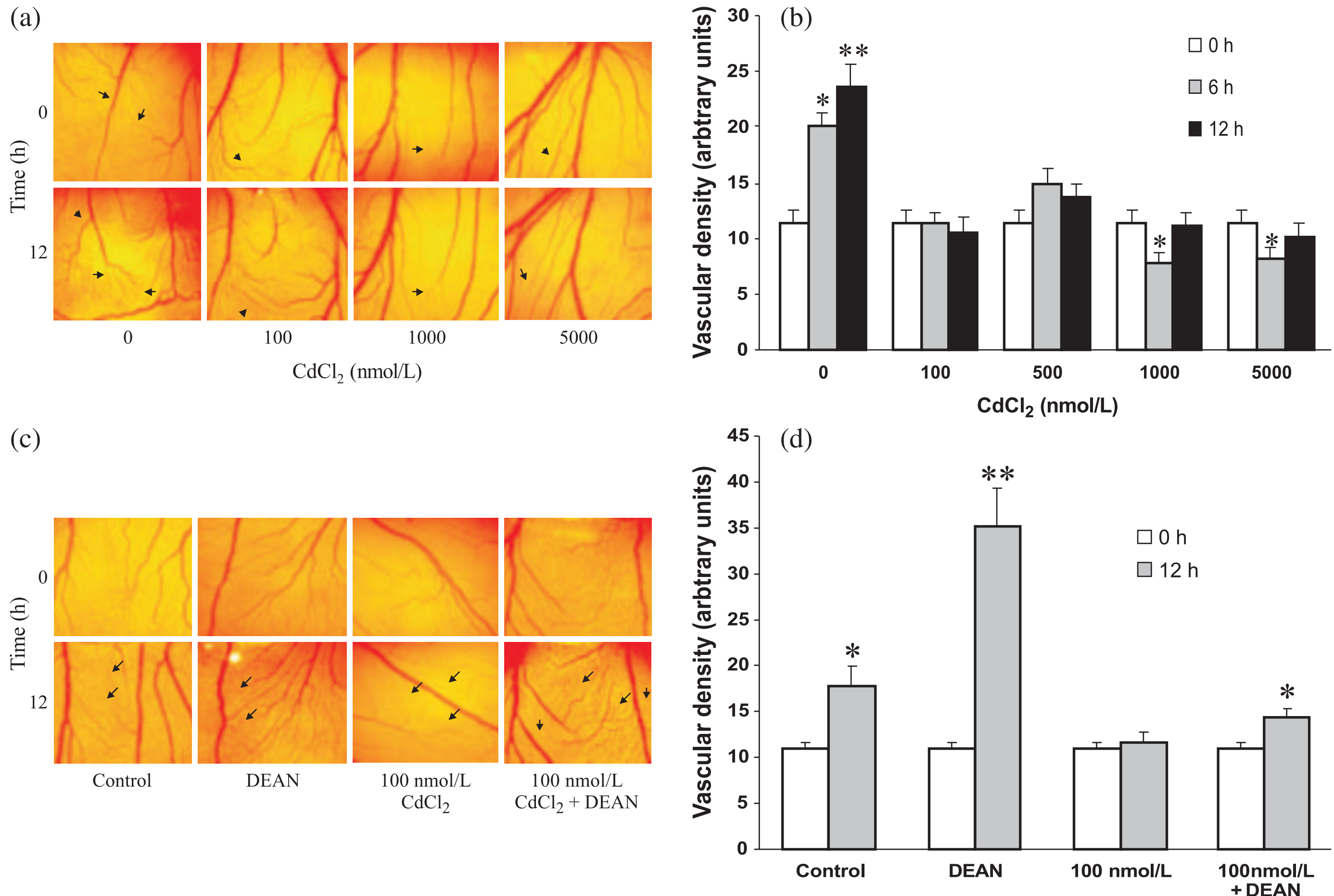
Fig. 4. (*a*) Cadmium (Cd) blocks angiogenesis in the egg yolk model in a dose-dependent manner. Fourth day incubated chicken eggs were broken and the entire contents transferred into a sterile Petri dish. Next, the vascular bed was incubated with different concentrations of $CdCl_2$ soaked in sterilized paper discs and placed on the vascular bed for 12 h. It is evident from the representative images that up to 500 nmol/L $CdCl_2$ inhibited angiogenesis, and higher concentrations of $CdCl_2$ not only inhibited angiogenesis, but also destroyed preformed blood vessels. (*b*) The inhibition of blood vessel formation was estimated by counting the number of red pixels using an analytical module of Image J software. The red pixel count for cells treated with 100 and 500 nmol/L $CdCl_2$ remained unchanged, whereas the count decreased in cells treated with higher concentrations of $CdCl_2$. *, Significantly different from cells at 0 h of treatment ($p < 0.05$). (*c*) Cd inhibited angiogenesis, whereas DEAN (diethylamine NONOate) alone induced angiogenesis (black arrows). DEAN administration, along with Cd, restored the inhibitory effect of Cd on angiogenesis in the egg yolk vascular bed model. (*d*) The recovery of blood vessel formation was estimated by counting the number of red pixels using an analytical module of Image J software. The red pixel count for cells treated with Cd remained unchanged, whereas that of cells treated with DEAN + $CdCl_2$ was significantly higher. *, Significantly different from cells at 0 h of treatment ($p < 0.05$).



(0.45 µm, MDI) by a wet blotting method described previously. Following transfer, the membrane was probed with anti-eNOS and anti-phospho-eNOS antibodies (dilution, 1:1000). The anti-phospho eNOS antibody was targeted against phosphorylated Ser1179. Thereafter, secondary antibodies tagged with horseradish peroxidase were used at 1:2000 dilutions. Finally, the membranes were developed using 3′,3′,5′,5′-tetramethylbenzidine – H_2O_2 substrate.

Statistical analysis

All experiments were performed in triplicate ($n = 3$) unless otherwise specified. The data are presented as means ± SE. The data were analyzed using a one-way ANOVA test, Student's *t* test, and the Tukey post-hoc test, as appropriate. $p \leq 0.05$ was considered statistically significant.

Results

Assessment of Cd toxicity in ECs

Our interest is to study the effect of a low dose of Cd on the control of vascular function, particularly in ECs. To ensure that the dose of Cd used in this study was within an apparently safe range, i.e., a range of concentrations that do not produce any adverse effect on cells and cellular metabolism, we performed a viability assay to determine the LD_{50} of Cd in ECs. Cells were treated with concentrations ranging from 0 to 1000 µmol/L $CdCl_2$ for 8 h followed by staining with 0.4 mg/mL trypan blue. The fraction of dead cells was obtained by counting the number of cells with a blue coloured nucleus. A plot of the percentage of dead cells vs $CdCl_2$ concentration is shown in Fig. 1*a*. We did not observe a significant number of dead cells in the culture me-

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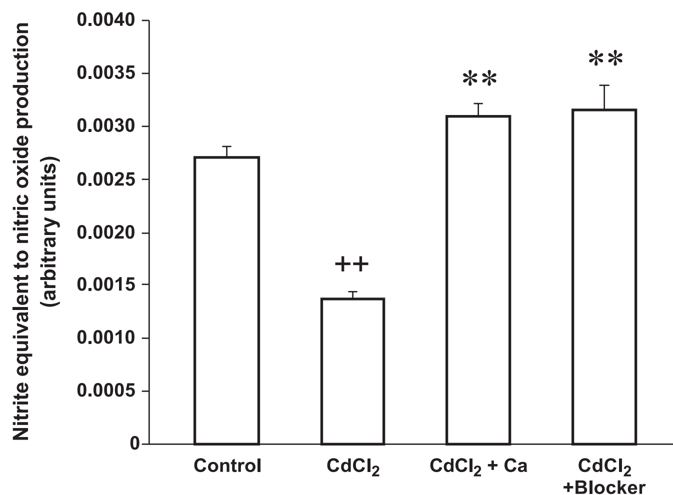
Fig. 5. Cadmium (Cd) inhibits nitric oxide (NO) production in a calcium-independent manner. Endothelial cells (ECs) were treated with $CdCl_2$ alone, with a combination of $CdCl_2$ + calcium (500 μmol/L), with a combination of Cd + the calcium channel blocker verapamil (100 sμmol/L). After 4 h, NO production by ECs was measured with a Griess assay. The results show that treatment with $CdCl_2$ alone inhibited NO production, whereas treatment with the combinations $CdCl_2$ + calcium or $CdCl_2$ + verapamil did not alter NO production by treated ECs compared with that by control cells. **, Significantly different from cells treated with $CdCl_2$ ($p <$ 0.001); ++, significantly different from control cells ($p < 0.01$).



dium containing 0, 0.01, or 0.1 μmol/L $CdCl_2$. There were marginal increases of 5% and 16% in dead cells when the culture contained 1 and 10 μmol/L $CdCl_2$, respectively. Fifty percent of the total cell population was viable after treatment with 100 μmol/L $CdCl_2$, whereas the cell population was completely unviable after treatment with 1000 μmol/L $CdCl_2$. These observations suggest that 100 μmol/L is the LD_{50} of Cd for ECs (Fig. 1*a*). The data fit quite well (solid line) with a K_d of 84 μmol/L and a Hill coefficient of 1. This value of K_d is consistent with that for $CdCl_2$ used as a calcium channel block and is compatible with data on the effects of $CdCl_2$ on the integrity of other endothelial parameters (Weidner and Sillman 1997). Regardless of of this value of LD_{50} for Cd, the results of our assessment of Cd toxicity suggested that the concentration we used in our subsequent experiments was well within the apparent safe range, because it was almost 1000-fold lower than the LD_{50} for Cd.

Effects of low Cd concentration on EC generation of NO

We determined EAhy926 cell NO production in response to different doses of $CdCl_2$ ranging from 0 to 1000 nmol/L. No effect on NO production was observed when cells were treated with 1 and 10 nmol/L $CdCl_2$. Treatment of ECs with 100 and 200 nmol/L $CdCl_2$ caused a reduction in the production of NO by 70% and 57%, respectively (Fig. 1*b*). Interestingly, increasing $CdCl_2$ concentrations above 500 nmol/L reduced the impact of Cd on the inhibition of NO production to 30%. We did not investigate this phenomenon further, but used 100 nmol/L $CdCl_2$ to further investigate the effects of ultra-low concentrations of Cd on endothelial cell NO production.

To elucidate further the temporal effect of Cd on NO production by ECs, EAhy926 cells were treated with 100 nmol/L of $CdCl_2$, and NO production was monitored for 24 h. Figure 1*c* shows that a 100 nmol/L treatment of EC ensured significant ($p < 0.001$) reductions in NO of 72% and 61% after 4 and 8 h, respectively, whereas no significant ($p >$ 0.05) inhibition resulted after shorter (2 h) or longer (16 and 24 h) exposures to Cd (Fig. 1*c*). These results suggest that Cd exerts its effect on NO production in a time-dependent manner, and specifically, within 4–16 h.

Effects of low concentrations of Cd on NO-mediated physiological function

NO is one of the key regulators of migration and proliferation of ECs. Therefore, to determine the effect of Cd on endothelial function in relation to low NO production, a wound-healing assay was performed. Scratch wounds were created on monolayer cultures of EC. The rate of wound healing under different conditions was monitored at regular intervals for the next 8 h. Marked drops in the rate of wound healing, 23% and 15%, were observed in the cells treated with 100 and 200 nmol/L $CdCl_2$, respectively (Fig. 2*a*). No significant ($p > 0.05$) difference from the control (untreated) cells was observed after 2 h of treatment, although after 4 h, a significant ($p < 0.05$) inhibition of wound healing was noted in cells exposed to 200 nmol/L $CdCl_2$. Further incubations of 6 and 8 h had significant ($p < 0.05$) effects on wound healing in cells treated with 100 and 200 nmol/L $CdCl_2$.

To determine whether Cd withdrawn from the media is able to reverse the effect of Cd on ECs, we washed Cd-treated cells (for 4 h) 3 times with 1× PBS, and incubated the cells with fresh Cd-free media for another 6 h. The results (Fig. 2*b*) indicate that Cd removal restored the normal rate of wound healing in ECs within 2 and 4 h of washing post-treatment for 100 and 200 nmol/L $CdCl_2$, respectively.

Low Cd concentrations inhibit EC migration

The recovery of cell function in the wound healing experiments was due to a combined effect of EC migration and proliferation. To determine whether Cd-mediated inhibition of wound healing was due to the inhibition of migration or proliferation, we performed wound-healing experiments in the presence of 5-fluro uracil (5-FU), an anti-proliferative agent. The results of these experiments (Fig. 3*a*) show that 5-FU-dependent inhibition of wound healing is insensitive to Cd treatment. Control cells showed 14% inhibition of wound healing with 5-FU treatment. This was similar to that of cells exposed to 100 and 200 nmol/L Cd for 8 h, which showed 19% and 16% inhibition of wound healing, respectively, with 5-FU. This observation suggests that Cd-mediated inhibition of wound healing is based on inhibition of cellular migration rather than that of proliferation.

Low Cd concentrations inhibit mitochondrial succinate dehydrogenase activity

An MTT assay was performed to assess the activity of succinate dehydrogenase, an indicator of proliferation, in EC cells treated with $CdCl_2$. MTT assays produce a colored product, whose intensity depends on mitochondrial dehydrogenase activity. EC cells were treated with 100 and

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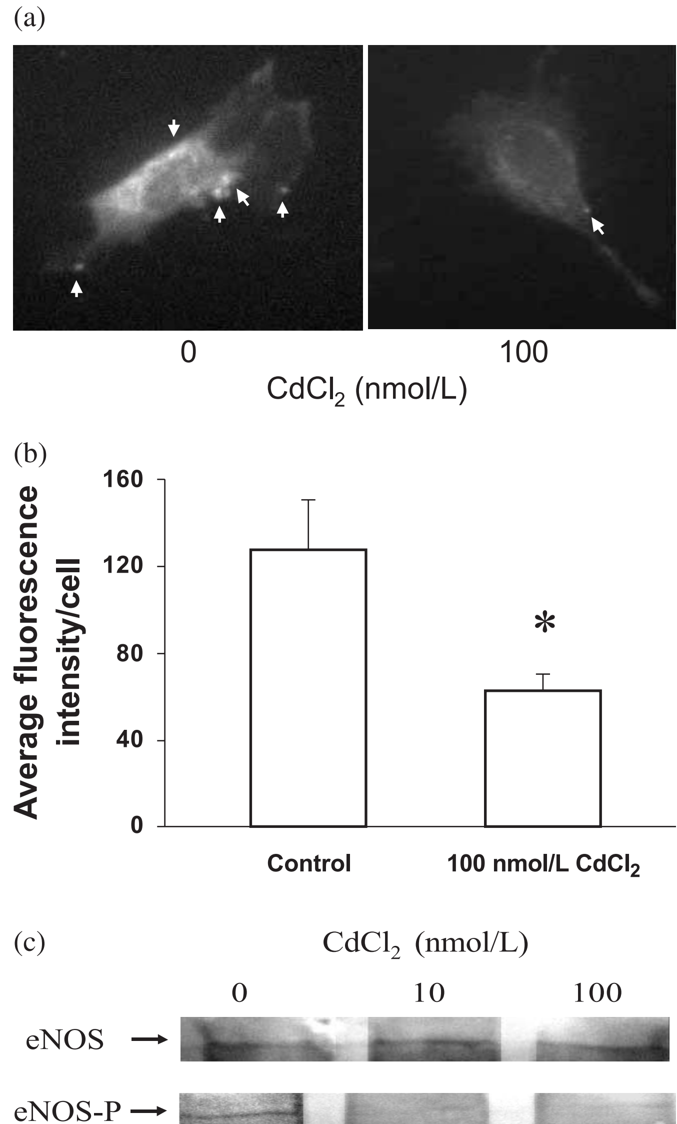

Fig. 6. Cadmium (Cd) inhibits eNOS by blocking phosphorylation of eNOS. (*a*) Immunofluoroscence was carried out on endothelial cells (ECs) in 12-well plates using the cold paraformaldehyde – Triton X-100 protocol (Mukhopadhyay et al. 2006; Shah et al. 2005). Cells were incubated at 4 °C overnight with rabbit polyclonal antibodies (dilution, 1:1000) against phospho-eNOS. Next, corresponding goat anti-rabit secondary antibodies (dilution, 1:2000), tagged with FITC (fluorescein isothiocyanate) was used to immunodetect the phosphorylated eNOS population. Images were acquired with a Nikon epifluorescence microscopy system equipped with a CCD camera loaded with black and white film. The total fluorescence intensity observed for the Cd-treated cells was lower than that for control cells. Further, the number of FITC-positive spots originating from antibodies bound to phophorylated spots was lower in Cd-treated cells compared with that in control cells. The results suggest that Cd blocks eNOS phosphorylation. (*b*) Images in *a* were analyzed with an analytical module of Image J software. The data suggest that Cd-treated cells fluoresced less than the control cells. *, Significantly different from the control cells ($p < 0.05$). (*c*) Protein samples were prepared from cells that were either treated or not treated with $CdCl_2$. A Western blot analysis to determine eNOS expression and phosphorylation was performed using prepared protein samples. Immunodetection of phosphorylated eNOS proteins in the Western blot reveals that Cd blocks phosphorylation of eNOS, while total eNOS expression wasunchanged by Cd treatment.

200 nmol/L $CdCl_2$ in a culture medium for 8 h, and MTT was added to determine mitochondrial metabolism. Cd treatments of 100 and 200 nmol/L $CdCl_2$ resulted in 20% and 40% reductions in enzyme activity, respectively (Fig. 3*b*) The results indicate that succinate dehydrogenase was inhibited in mitochondria as a result of Cd challenge.

Cd blocks angiogenesis in the egg yolk model in a dose-dependent manner

The egg yolk vascular bed model is an angiogenesis model with which one can track and observe the formation of blood vessels. Fourth day fertilized eggs with semideveloped vascular beds were plated on Petri dishes and treated with different concentrations of $CdCl_2$ ranging from 0–5000 µmol/L. Real-time tracking of the developing vascular beds demonstrated that Cd inhibited blood vessel formation in a dose-dependent manner (Fig. 4*a*). Figure 4*b* summarizes the results for the area of vascular coverage after exposure to various $CdCl_2$ concentrations at 6 and 12 h. We note that $CdCl_2$ concentrations up to 500 nmol/L specifically attenuated the formation of terminal capillaries, whereas higher concentrations, i.e., ≥1000 nmol/L, destroyed blood vessels after 6 h. This observation is supported by the results from cell-based assays, in which the maximum effect of Cd was seen within 4–8 h of treatment. In another set of experiments with the egg yolk model, a combination treatment of Cd and DEAN restored the effect of Cd on angiogenesis (Figs. 4*c* and 4*d*), whereas Cd alone maintained its effect on angiogenesis.

Cd inhibits NO production in a calcium-independent manner

To determine whether the effect of Cd is dependent on calcium, we measured NO after Cd treatment, along with verapamil and calcium. For the calcium treatment, cells were first exposed to a calcium ionophore (1 µmol/L), and after 5 min, 500 µmol/L was added. In all cases, the cells were treated with $CdCl_2$ and then incubated for 4 h. The results show that Cd-mediated inhibition of NO production is a calcium-independent phenomenon. We observed that Cd in nanomolar concentrations attenuated NO production in ECs. When the cells were treated with the calcium channel blocker verapamil (100 µmol/L) along with $CdCl_2$, we did not observe any further inhibition of NO production (Fig. 5). Thus, we infer that blocking calcium channels restricted Cd entry into the cells, which ultimately blunted Cd-dependent inhibition of NO production in ECs.

Cd inhibits NO production by blocking eNOS phosphorylation

We used immunofluroscence techniques to study the effect of Cd on eNOS phosphorylation. Cells were fixed using 2% paraformaldehyde and then probed with a primary antibody specific for phosphorylated eNOS. Overall, less fluo-

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rescence was observed in Cd-treated cells compared with that in control (untreated) cells. In control cells, the focal points of phosphorylated eNOS were observed under UV illumination, whereas Cd treatments abolished the phosphorylated eNOS population from both the perinulclear and plasma membrane regions (Figs. 6*a* and 6*b*).

To semiquantify eNOS phosphorylation after Cd treatment, protein samples from cells either treated or not treated with $CdCl_2$ were prepared, subjected to SDS–PAGE, and subsequently immunoblotted. The results suggest that Cd attenuates eNOS phosphorylation, even though the expression level of total eNOS remained unchanged after Cd treatment (Fig. 6*c*).

Discussion

The results of this study show that Cd blocks NO production via inactivation of eNOS (Figs. 6*a*, 6*b,* and 6*c*). The work of Abu-Hayyeh et al. (2001) has shown that ECs respond to lower Cd concentrations compared with smooth muscle cells (Abu-Hayyeh et al. 2001). Their calculations revealed that the average Cd concentration in the medial layer of blood vessel is 7 μmol/L (Abu-Hayyeh et al. 2001). Cd is known to replace zinc and contributes to the brittleness and hardness of the arteries that is believed to cause hypertension (Chisolm and Handorf 1985). Skoczynska and Martynowicz (2005) demonstrated that rats poisoned with hypertensive doses of Cd showed diminished vascular response to L-NOARG, an inhibitor of NOS, by shifting the dose–response curve to the right. From these observations, we surmise that deposited Cd, which remains 30 years after deposition in the blood vessel walls, may leach out of the wall in a low but in a chronic pattern. This assumption led us to test endothelial function under nanomolar doses of Cd. It should be noted that the Cd concentrations we used here are about 10 000 times lower than the LD_{50} of Cd for ECs (Fig. 1*a*). Our study shows that these ultra-low concentrations of Cd significantly reduce NO production from endothelial monolayers, and thereby impair endothelial functions (Figs. 1*b* and 1*c*). Another important point is that Cd maximally attenuated NO production at the 4th h of treatments compared with that in the 8th h (Fig. 1*c*). Earlier studies showed that Cd is capable of metallothionein induction and gives peaks at the fourth h of induction (Caperna and Failla 1984; Margarita et al. 2001). Based on this information, we speculate that Cd is available for blocking NO production until metallothionein relocates active Cd from the cytosol to less active subcellular compartments. Subcellular trafficking of NOS also can be a determinate of Cd-based NOS inactivation, which is associated with calcium–calmodulin interaction. It has been shown that Cd interferes with calcium–calmodulin complexation (Cheung 1984; Yuan et al. 2004). Therefore, subcellular relocalization of NOS modulates NOS–calmodulin–Cd interaction by placing NOS in less or more active compartments.

Earlier studies demonstrated that Cd introduces profound time- and dose-dependent alterations of phospholipid metabolism in bovine pulmonary artery ECs (Nelson et al. 1991), which in turn supports our results showing that Cd has a dose- and time-dependent effect on endothelial function, including angiogenesis (Figs. 4*a* and 4*b*). A quantitative analysis of the effect of Cd on EC proliferation and migration by Kishimoto et al. (2005) suggests that EC migration is susceptible to Cd cytotoxicity and that EC outgrowths are inhibited by Cd, which in turn supports our observation that low concentrations of Cd block EC wound healing by blocking migration (Fig. 2*a*). However, the wound-healing process can be a summation of the effects of EC migration and proliferation. To determine the exact nature of Cd interference in EC monolayer wound healing, we used 5-FU to block cell proliferation. The results show that Cd-mediated inhibition of wound healing is independent of proliferation (Fig. 3*a*). We proceeded to confirm this observation with an MTT assay to ensure that Cd did not impair proliferation. However, contrary to our expectation, the MTT assay showed a lower level of mitochondrial succinate dehydrogenase activity after Cd treatment (Fig. 3*b*). This observation led us to speculate that Cd, at least at low concentrations, failed to reduce the number of viable cells by blocking succinate dehydrogenase; rather, it seems to have enhanced the number of viable cells by antagonizing apoptosis. In support of our observations, a previous finding by Gunawardana et al. (2006) demonstrated that Cd suppressed DNA laddering, nuclear condensation, procaspase-3 cleavage, caspase-8, caspase-9, and the release of cytochrome *c* from mitochondria in rat mesangial cells. They concluded that Cd inhibits both intrinsic and extrinsic apoptotic pathways in renal mesangial cells (Gunawardana et al. 2006).

It has already been noted that ≤100 nmol/L Cd can increase the incorporation of [^{3}H]thymidine into the acid-insoluble fraction of growing bovine and rabbit aortic smooth muscle cells, but not into that of growing bovine aortic ECs (Fujiwara et al. 1998). Peereboom-Stegeman and Jongstra-Spaapen (1979) showed that the effects of injected Cd can be reversed even after prolonged incubation on the microcirculation in rat uterus. To determine whether the effects of low Cd concentration on ECs can be reversed, we performed wound-healing assays by washing cells after 4 h of Cd treatment. The results (Fig. 2*b*) suggest that the effects of Cd can indeed be reversed within 4 h of withdrawing Cd from the cells.

The involvement of NO in angiogenesis has been well documented (Ekmekcioglu et al. 2005). A recent study by Yu et al. (2005) using eNOS knockout mice suggests that endogenous eNOS-derived NO exerts direct effects on the preservation ofblood flow, thereby promoting arteriogenesis, angiogenesis, and mural cell recruitment to immature angiogenic sprouts. A study by Michaud et al. (2003) demonstrated that cigarette smoke, a source of Cd toxicity, impaired hypoxia-induced angiogenesis. Vascular endothelial growth factor (VEGF) is a dimeric protein that induces angiogenesis through binding to VEGF-receptor-2 tyrosine kinase (VEGFR2 TK) on the surface of ECs. Cd is also implicated in angiogenesis by inhibiting VEGFR2 TK (Parast et al. 1998). The present work is consistent with these earlier data, in that it shows that 100 nmol/L Cd attenuates egg yolk angiogenesis at the capillary ends. However, a higher dose of Cd (in the millimolar range) possibly caused necrotic elimination of preformed blood vessels, as observed by trypan blue inclusion in the affected blood vessels (data not shown). The results of the endothelial cell viability assay

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with trypan blue also revealed that, in higher doses, Cd induced cell death in endothelial monolayers (Fig. 1*a*). These observations raise the question of whether Cd-induced cell death in blood vessels is due to apoptosis. Recent work by Liu and Templeton (2007) highlights the observation that, at 10–20 μmol/L, Cd initiates apoptosis in mesangial cells via CaMK-II dependent signaling. A score of earlier works have also showed that Cd induces apoptosis in various cell types, such as lymphoblastoid cells and osteoblasts (Coutant et al. 2006; Coonse et al. 2007).

Previous work by various groups has already shown that eNOS can be activated via 2 different pathways, the calcium–calmodulin pathway (Jagnandan et al. 2005) and the eNOS phosphorylation pathway (Sessa 2004; Fulton et al. 2001). In this study, we hypothesized that eNOS inactivation under conditions of low Cd stress is associated with eNOS phosphorylation, and thereby leads to endothelial dysfunction. In support of this hypothesis, the results in this work demonstrate that 100 nmol/L of Cd inactivates eNOS by blocking eNOS phosphorylation (Figs. 6*a*, 6*b*, and 6*c*). At the same time, the results from Western blots show that eNOS expression is similar in both Cd-treated and untreated ECs (Fig. 6*c*). Further supporting this hypothesis is the inhibitory effect of Cd uptake on basal NO production by ECs (Fig. 5). Interestingly, the co-application of verapamil or calcium along with $CdCl_2$ maintained the level of basal NO production. It has been shown that calcium hinders Cd uptake by cells (Hinkle et al. 1987; Reeves et al. 2005). Thus, the effects of Cd on NO production are apparently at least partially due to transmembrane influx of Cd and enhanced subcellular Cd pool. Overall, the data suggest that Cd-mediated inhibition of NO production results from the blocking of eNOS phosphorylation.

In summary, this work partially elucidated the mechanism of eNOS inactivation by low levels of Cd by showing that the effects of Cd are due to a decrease in NO production by ECs. This study indicates that low levels of Cd induce endothelial dysfunction, and we attribute this effect to the blocking of eNOS phosphorylation. A further probe into the site-specific phosphorylation patterns of eNOS and their interplay with Cd, which is presently underway, will unravel the mechanism of low-level Cd perturbations in vascular diseases.

Acknowledgements

This study was financially supported by an Indian Council of Medical Research (ICMR) grant to S.C. (5/8/4/9(Env)/2005-NCD-I). S.P. is a recipient of a JDRF Advanced Postdoctoral fellowship (JDRF No. 10–2006–792).This work was partially supported by a Wellcome Trust Grant (No. 070069).

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