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Cadmium induces vascular permeability via activation of the p38 MAPK pathway

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ABSTRACT

The vasculature of various organs is a targeted by the environmental toxin, cadmium (Cd). However, mechanisms leading to pathological conditions are poorly understood. In the present study, we examined the effect of cadmium chloride ($CdCl_2$) on human umbilical vein endothelial cells (HUVECs). At 4 μM, $CdCl_2$ induced a hyper-permeability defect in HUVECs, but not the inhibition of cell growth up to 24 h. This effect of $CdCl_2$ was dependent on the activation of the p38 mitogen-activated protein kinase (MAPK) pathway. The p38 MAPK inhibitor SB203850 suppressed the $CdCl_2$-induced alteration in trans-endothelial electrical resistance in HUVEC monolayers, a model measurement of vascular endothelial barrier integrity. SB203850 also inhibited the Cd-induced membrane dissociation of vascular endothelial (VE) cadherin and β-catenin, the important components of the adherens junctional complex. In addition, SB203850 reduces the Cd-induced expression and secretion of tumor necrosis factor α (TNF-α). Taken together, our findings suggest that Cd induces vascular hyper-permeability and disruption of endothelial barrier integrity through stimulation of p38 MAPK signaling.

1. Introduction

Cadmium (Cd) is an environmental toxin produced by the burning of fossil fuels and by multiple industrial applications [1]. In recent years, Cd uptake by human populations has increased dramatically. Uptake can be through contaminated water and food, cigarette smoking or through occupational exposure [2]. Following absorption, Cd travels in the bloodstream as free ions or bounded with albumin or metallothioneins, and distributes into tissues all over the body [3]. Accumulation of Cd can damage multiple organs including the lung, liver, testis, and kidney with the extent of tissue damage depending on the dose, route and duration of exposure [1,4]. One of the primary targets of Cd-induced toxicity is thought to be the vascular endothelium [3].

The vascular endothelium is the inner cell lining of blood vessels. The major role of the vascular endothelium is to control the exchange of substances and immune cells between the bloodstream and the various tissues of the body [5]. The vascular endothelium thus acts as a barrier. This barrier function is dependent on the integrity of tight- and adherens-junction complexes [6] and any damage to the endothelial barrier results in pathological microvascular hyper-permeability [7]. It has been suggested that Cd exposure may directly damage endothelial cells lining blood vessels. The subsequent increase in endothelial barrier permeability could induce the various types of secondary, organ-specific pathological changes seen with Cd exposure [8]. Previous studies have provided evidence that the effect of Cd on microvascular permeability results from direct action of Cd on endothelial cells [3]. In particular, Cd causes the dissociation of vascular endothelial (VE)-cadherin and β-catenin from adherens junctions in endothelial cells [9]. The mechanism by which this dissociation can be induced is not clear.

In endothelial cells, Cd induces oxidative stress [10], which contributes to Cd-induced toxicity in the vascular system [11]. Oxidative stress can activate mitogen-activated protein kinase

Abbreviations: $CdCl_2$, cadmium chloride; HUVEC, human umbilical vein endothelial cell; EC, endothelial cell; MAPK, mitogen-activated protein kinase; DMSO, dimethyl sulfoxide; EGM, endothelial growth media; TNF-α, tumor necrosis factor-α; VE-cadherin, vascular endothelial-cadherin; ECIS, electric cell-substrate impedance sensing.

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(MAPK) pathways, which regulate a vast array of physiological processes [12,13]. In the endothelium, the p38 MAPK pathway plays a role in regulating cell proliferation, migration and thus permeability, during various pathological insults [14,15]. It has been shown that TNF-α can induce p38 MAPK activation in endothelial cells, thereby leading to formation of stress fibers and vascular hyper-permeability [16,17]. Cd directly activates p38 MAPK in a variety of cell types, including endothelial cells [18,19]. However, a connection between p38 MAPK and Cd-induced endothelial barrier hyper-permeability has not been demonstrated.

In this study, the functional effect of a low concentration of Cd on the growth and permeability of HUVECs has been examined. Using real-time intercellular resistance analysis, we demonstrate that Cd induces an increase in endothelial cell permeability concomitant with activation of the p38 MAPK pathway. We used the p38 MAPK inhibitor SB203850 to demonstrate that p38 activation is required for Cd-induced changes in endothelial barrier function. SB203850 also blocked Cd-induced re-localization of VE-cadherin and β-catenin, suggesting disruption of adherens junctions contributes to Cd-induced hyper-permeability. In addition, SB203850 suppressed Cd induced expression and secretion of TNF-α. This study definitively demonstrates a role for p38 MAPK in mediating Cd-induced endothelial barrier hyper-permeability.

2. Materials and methods

2.1. Cell culture

HUVECs were purchased from ATCC (Manassas, VA) and maintained in endothelial growth media (EGM-2) supplemented with EGM-2-MV bullet kit (Lonza, Basel, Switzerland) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin), in humidified air at 37 °C with 5% CO_2. $CdCl_2$ was purchased from Sigma Aldrich (St. Louis, MO).

2.2. Cell viability and growth assay

Cell viability was assessed after 24 and 48 h of $CdCl_2$ treatment. Cultures were washed and incubated in 0.05% Trypsin for 2 min at 37 °C. After disaggregation, cell suspensions were diluted 1:1 in 0.4% Trypan blue (w/v in 0.9% NaCl) (Santa Cruz Technology, Santa Cruz, CA) and the percentage of dye-free cells was calculated. Cell proliferation was evaluated by a MTT assay kit (Cayman Chemical, Ann Arbor, Michigan). Briefly, cells were seeded at a density of 5×10^3 cells/well in a 96-well plate. The next day, media with various concentrations of $CdCl_2$ (Sigma, St. Louis, MO) was added to the cell culture. At each time point, the culture was washed with PBS, and 10 μl of MTT solution (5 mg/ml) was added to each well for 2 h. Formazan crystals were then solubilized and colorimetric intensity was analyzed in a 96-well format plate reader (Molecular Device, Sunnyvale, CA) at a wavelength of 570 nm. Each experiment was repeated in triplicate.

2.3. FITC-dextran transwell assay

HUVEC monolayers were plated on the transwell insert and cultured until confluent. The chambers were washed with Hepes medium, and FITC-dextran (Invitrogen, Calsbard, CA) was added to the top chamber. After 12 h, samples were removed from the bottom chamber and read in a fluorometer (Molecular Device) at an excitation of 485 nm and an emission of 520 nm. Data represents the mean of three experiments.

2.4. Western blot analysis

Western blot were performed as previous described [20]. Briefly, cells were lysated in ice cold RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 50 mM NaF, 1% NP40, 0.1% DOC, 0.1% SDS, 1 mM EDTA and supplemented with 1 mM PMSF and 1 μg/ml leupeptin). Equal amounts of protein, as determined by BCA assay, were separated by SDS–PAGE (8% acrylamide gel) and transferred to PVDF membrane. Membranes were blocked with 2% non-fat milk, and incubated with the primary antibodies at 4 °C overnight in PBS-T. Primary antibodies included rabbit anti-p38 and rabbit anti-phospho p38 antibody (Cell Signaling Technology, Beverly, MA). Immunoreactivity was visualized with HRP-linked secondary antibodies and chemiluminescence.

2.5. Electric cell-substrate impedance sensing (ECIS) analysis

Intercellular resistance across a monolayer of HUVECs measured using the ECIS technique (ECIS model 1600; Applied BioPhysics, Troy, NY). Briefly, 8-well ECIS arrays (8W10E+) were first coated with fibronectin (Invitrogen). Then, HUVECs were plated at a density that would allow formation of confluent monolayers directly on top of the electrodes. After treatment with media containing $CdCl_2$ alone or $CdCl_2$ and SB203850 (Cell Signaling Technology) in combination, alternating current was applied across the electrodes and electrical resistance was recorded. Data plots are representative of triplicate experiments, with each graph showing resistance readings from a separate well. Each well reading is an average derived from the 40 distinct electrodes that record from each well.

2.6. Immunofluorescence

HUVEC monolayers grown on fibronectin-coated glass chamber slides were exposed to either $CdCl_2$ alone or $CdCl_2$ with SB203850 in combination. After 12 h, the media was aspirated and the monolayers were washed with PBS containing 100 mM L-glycine, fixed with 4% paraformaldehyde, and washed with PBS for 10 min three times. Immunofluorescence was performed using a primary antibody against human VE-cadherin (1:200; Abcam, Cambridge, MA) or β-catenin (1:500; Abcam) and an Alexa Fluor® 546 Anti-Rabbit secondary antibody (1:200; Life Technologies, Calsbard, CA). The slides were photographed using an Olympus LCX100 Imaging System (Olympus Corporation, Tokyo, Japan) with an excitation wavelength of 546 nm.

2.7. ELISA

HUVECs were grown into confluence and applied with serum/growth factor-free media containing 4 μM $CdCl_2$. After 12 h exposure, the media were collected for detection of TNF-α protein level using human TNF-α Quantikine ELISA kit (R&D systems, Minneapolis, MN, USA). The measurements were performed 4 times ($n = 4$).

2.8. Quantitative real-time PCR (qRT-PCR)

RNA isolation and cDNA synthesis were performed using the RNeasy Mini kit (Qiagen). qRT-PCR was performed using a ViiA7 Real-Time PCR System (Applied Biosystems). All PCR reactions were repeated in triplicate. Relative expression was calculated using GAPDH as an endogenous internal control. The primer sequences were as follows:

TNF-α forward primer, 5′-GAGTGACAAGCCTGTAGCCCATGTTG TAGCA-3′

TNF-α reverse primer, 5′-GCAATGATCCCAAAGTAGACCTGCC-CAGACT-3′
GAPDH forward primer, 5′-TGATGACATCAAGAAGGTGGTGAAG-3′
GAPDH reverse primer, 5′-TCCTTGGAGGCCATGTGGGCCAT-3′

2.9. Statistical analysis

Statistical significance was assessed using paired-sample *t*-tests. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Low dose $CdCl_2$ induces hyper-permeability in HUVEC monolayers

Cd induces apoptosis in multiple cell types. This depends on Cd concentration and the length of exposure [21]. After 24 and 48-h exposure to $CdCl_2$, a dose-dependent decrease of cell viability was found in cultured HUVECs (Fig. 1A). At a concentration of 4 μM, $CdCl_2$ did not significantly affect cell viability at both time points (Fig. 1A; 24 h, $p = 0.32$; 48 h, $p = 0.19$). In addition, 4 μM $CdCl_2$ did not show a significant inhibition on HUVEC cell density during the first 24 h of culture as examined by MTT assay (Fig. 1B). However, the same concentration of $CdCl_2$ induced a remarkable increase in HUVEC monolayer permeability through trans-well FITC-dextran assay as early as 12 h after initiating exposure (Fig. 1C; $p < 0.01$). Thus, we conclude that low-dose $CdCl_2$ increased endothelial cell permeability without inhibiting cell proliferation or viability.

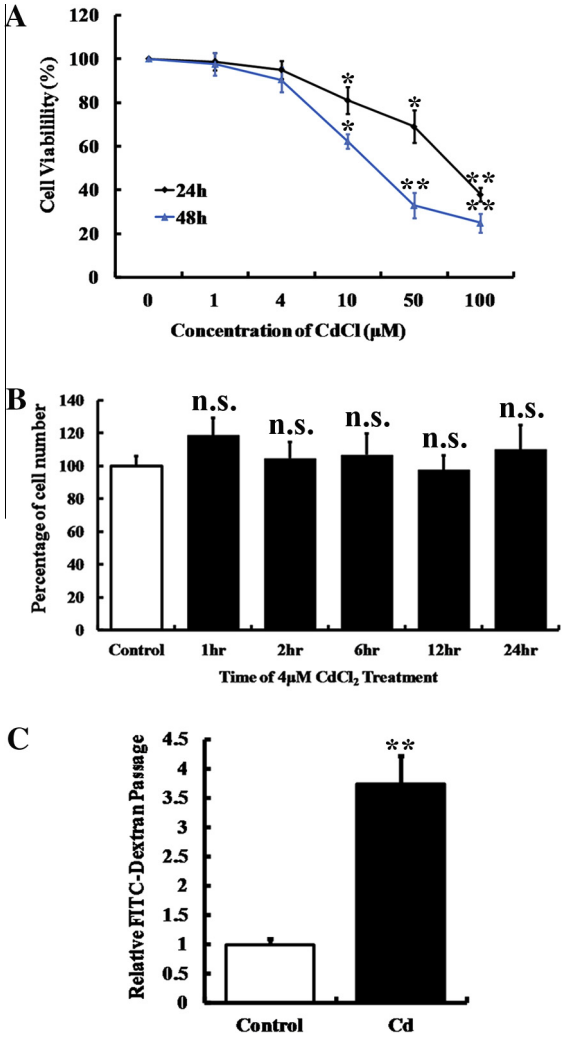

Fig. 1. The effects of cadmium on HUVEC proliferation and permeability. (A) Trypan blue viability assay of HUVECs treated with different concentrations of $CdCl_2$ for 24 and 48 h. $n = 4$; $^{*}p < 0.05$; $^{**}p < 0.01$; (B) MTT assay of HUVECs treated with 4 μM $CdCl_2$ at different time points; The OD reading was converted to cell number. $n = 4$; n.s., non-significant; (C) FITC-dextran transwell assay of HUVEC monolayers in the presence and absence of 4 μM $CdCl_2$. $n = 6$; $^{**}p < 0.01$.

3.2. p38 MAPK signaling mediates Cd-induced hyper-permeability

In response to environmental stresses, p38 MAPK is activated by phosphorylation of a conserved Thr180-X-Tyr182 motif by the upstream kinases MKK3 and MKK6 [13]. Previous studies have shown that Cd can activate p38 MAPK in various cell lines [15,18]. We examined the phosphorylation of p38 MAPK protein in HUVECs treated with a low dose (4 μM) of $CdCl_2$ by Western blotting. An increase in p38 MAPK phosphorylation was observed at all time points after low-dose $CdCl_2$ exposure, whereas the total levels of p38 MAPK protein remained unchanged.

SB203850 is a pyridinyl imidazole inhibitor that inhibits the activity of the all p38 MAPK isoforms [22]. We examined the effect of SB203580 on $CdCl_2$-induced hyper-permeability. To definitively measure any subtle alterations in permeability that accompany Cd exposure, we utilized the ECIS system, which allows for real-time measurements of electrical resistance across a monolayer of endothelial cells [17]. In the ECIS circuit, current flows across the cell monolayer and the gaps between cells function as resistors. Using this assay we found that transendothelial resistance was reduced significantly by 4 μM $CdCl_2$ (Fig. 2B and C; $p < 0.01$). It was not altered by 10 μM SB203580 alone, but pre-treatment with 10 μM SB203580 for 1 h suppressed the reduction of transendothelial resistance induced by $CdCl_2$. In addition, SB203580 significantly repressed $CdCl_2$-induced permeability in trans-well FITC-dextran assay (Fig. 2D; $p < 0.01$). This suggests that the Cd induced hyper-permeability requires activation of p38 MAPK pathway.

3.3. p38 MAPK inhibition represses $CdCl_2$-induced VE-cadherin and β-catenin re-distribution

Vascular permeability is regulated in part by the endothelial cell–cell adherens junctions which are largely composed of vascular endothelial cadherin (VE-cadherin), an endothelial-specific adhesion protein, and its binding partner β-catenin [23]. We hypothesized that Cd may increase vascular permeability via a p38 MAPK-induced disruption of VE-cadherin/β-catenin localization to endothelial cell junctions. The localization of VE-cadherin was examined in HUVEC monolayers by immunoflouresence. As expected, in control cells VE-cadherin was highly localized to the cell–cell contacts throughout the monolayer (Fig. 3A). Upon treatment with 4 μM $CdCl_2$, lower levels of VE-cadherin were found associated with adherens junctions. However, pretreatment with the p38 inhibitor SB203850 (10 μM) for 1 h prior to $CdCl_2$ blocked the re-localization of VE-cadherin (Fig. 3A). β-catenin binds to the intracellular C-terminal portion of VE-cadherin and bridges VE-cadherin with α-catenin that tightly controls the actin skeleton. By immunoflouresence, we found that β-catenin showed a similar distribution pattern to VE-cadherin (Fig. 3B). The membrane associated β-catenin was reduced with Cd exposure and this reduction was partially prevented by SB203850 pretreatment. Therefore, Cd-induced VE-cadherin re-distribution required activation of p38 MAPK.

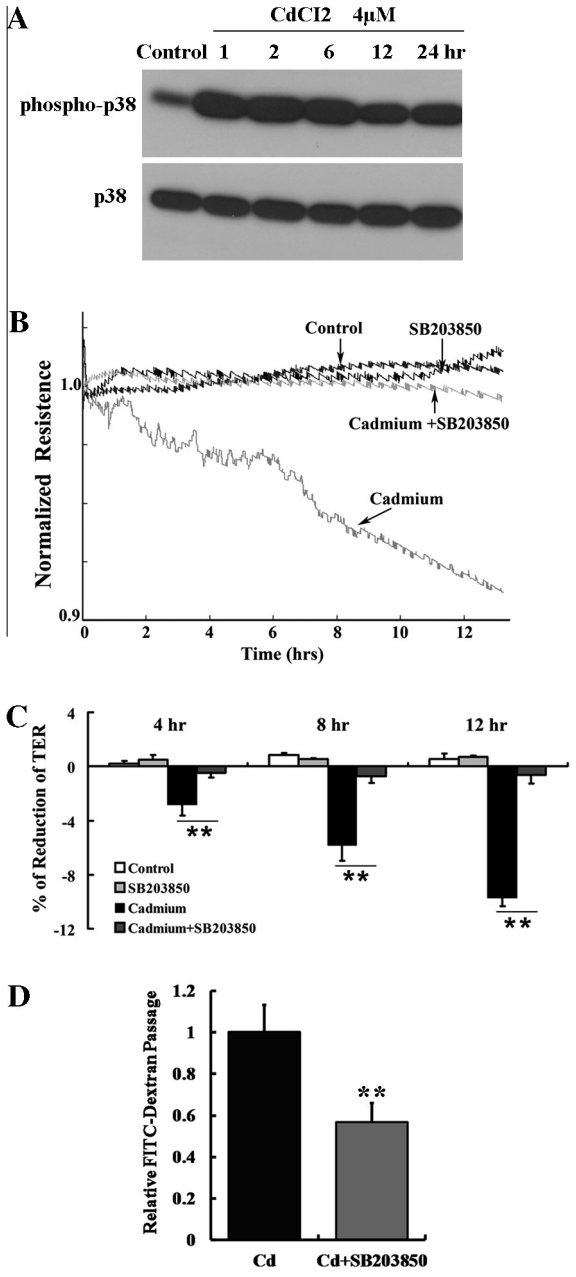

Fig. 2. p38 MAPK mediates cadmium induced permeability. (A) Representative blots from total p38 MAPK and phospho-p38 MAPK from protein samples of HUVECs treated with 4 μM $CdCl_2$ at different time points. (B) Real time transendothelial electrical resistance (TER) measurement of HUVEC monolayer treated with 4 μM $CdCl_2$, 10 μM SB203850, and $CdCl_2$ with pretreatment of SB203850. (C) Bar graph of the mean percentage of TER. Error bars represent the SEM ($n = 4$). $^{**}p < 0.01$; (D) FITC-dextran transwell assay of HUVEC monolayers treated with $CdCl_2$ in the absence or presence of SB203850. $n = 6$; $^{**}p < 0.01$.

3.4. Suppression of p38 MAPK signaling blocks $CdCl_2$-induced TNF-α secretion and expression

TNF-α is a major proinflammatory cytokine that can increase vascular permeability via disruption of intercellular junctions. We examined the influence of $CdCl_2$ on the production of TNF-α in HUVECs by ELISA. TNF-α level in the culture media was remarkably increased after 24-h of Cd exposure (7.75-fold) (Fig. 4A; $p < 0.01$). In addition, the level of TNF-α mRNA was significantly increased, suggesting transcriptional upregulation contributed to increased secretion (Fig. 4B; $p < 0.01$). With SB203850 pretreatment, Cd-induced increase of TNF-α secretion and expression was significantly reduced (Fig. 4A and B; $p < 0.01$). Our data indicate that low doses of Cd can increase TNF-α gene expression and secretion in endothelial cells. Activation of p38 MAPK pathway is upstream of this activity.

4. Discussion

Epidemiological studies have demonstrated that serum levels of Cd correlate with risks for cardiovascular disease and inflammation. These pathological manifestations are thought to result from Cd-induced vascular toxicity [24]. When the vascular endothelium is exposed to Cd, the result can be loss of endothelial barrier integrity and thus an increase in vascular permeability. However, the mechanism that underlies this effect has yet to be fully elucidated. This is the first study that links the p38 MAP kinase pathway to Cd-induced vascular hyper-permeability.

We demonstrate an increase in permeability of HUVEC monolayers in response to 4 μM of $CdCl_2$. In human embryo lung fibroblasts, Cd induces significant increase of cell proliferation at low concentrations and inhibits cell growth at high concentrations [21]. In HUVECs, the Cd-induced hyper-permeability was independent to any changes in cell proliferation or viability. Consistently, others also have found that low concentrations of Cd (less than 5 μM) did not induce a significant amount of cell death in several cell types [25]. Cd has also been shown to disrupt endothelial cell junctions at a concentration as low as 0.1 μM [9]. This effect of Cd is hypothesized to be causative in the loss of barrier integrity in tissue capillaries and ultimately could result in edema and hemorrhaging. Environmental intake of Cd by human populations usually results in an increased but relatively low concentration of Cd in blood serum [24]. Therefore, Cd induced hyper-permeability, rather than cell death, is more than likely the primary effector of Cd-induced pathophysiology.

It is well known that MAPK pathways are involved in the stress response to environmental stimuli [26]. Activation of the p38 MAPKs can promote cell migration [27], and Cd activates p38 MAPKs in some cell lines [18,28]. With respect to HUVECs, it has been reported that all three main MAPK branches are sensitive to Cd [29]. Cd also activated p38 MAPK in cerebrovascular endothelial cells [30]. In the present work, we performed a time course study and demonstrated that 4 μM of $CdCl_2$ continuously phosphorylated p38 MAPK up to 24 h, suggesting that this pathway is consistently activated in endothelial cells by Cd exposure. TNF-α, which is the primary inflammation cytokine and induces vascular permeability, also activated p38 MAPK in a persistent fashion [16]. The activation of p38 MAPK might be induced by oxidative stress, possibly through protein tyrosine phosphorylation [14]. This is in line with the fact that Cd and TNF-α both induce ROS in endothelial cells [31].

In our hands, the ECIS system provided a highly sensitive method to analyze the electrical resistance of cell monolayers cultured on small electrodes [17]. We found that $CdCl_2$ decreased the trans-endothelial resistance of HUVEC monolayers, suggesting a disruption of barrier integrity induced by Cd. The $CdCl_2$ induced decline in resistance was suppressed by pretreatment with SB203580. Therefore, p38 MAPK mediates the effect of Cd on endothelial cell barrier function. Our findings with low-dose Cd mimic the effect of TNF-α on vascular permeability. The TNF-α induced effect is also blocked by SB203580 [16,17]. Therefore, high levels of TNF-α and low concentrations of Cd both aberrantly activate p38 MAPK signaling to disrupt endothelial barrier integrity. Adherens junctions are found ubiquitously in the vascular endothelium and are required to maintain endothelial integrity [23]. VE-cadherin is a principal constituent of endothelial adherens junctions [32]. The intracellular domain of VE-cadherin is bound to β-catenin, which in turn, is bound to α-catenin. The complex

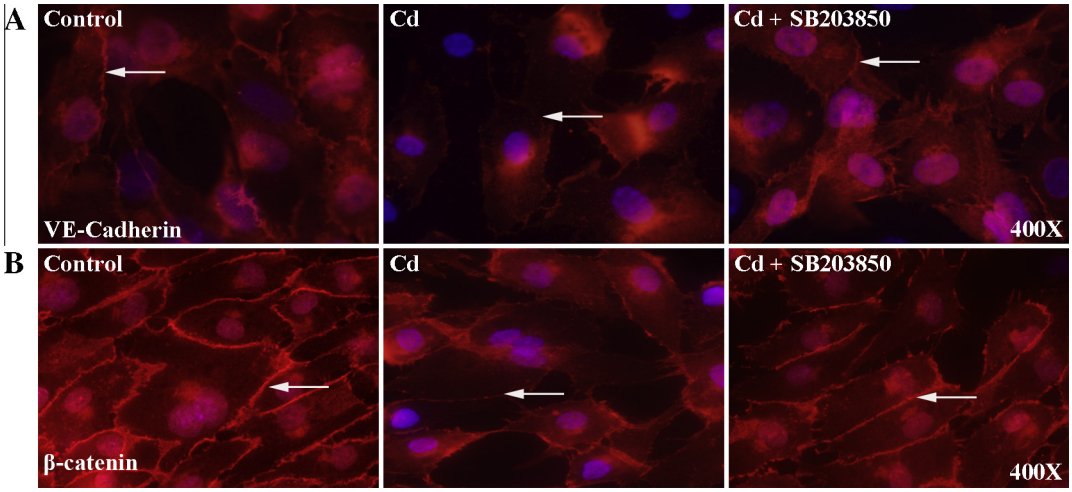

Fig. 3. The effects of cadmium and SB203850 on adherens junction-associated protein distribution in HUVECs. (A) Immunofluoresence staining of VE-cadherin on HUVEC monolayer, and HUVECs monolayer treated with 4 μM $CdCl_2$ in the absence or presence of SB203850; (B) Immunofluoresence staining of β-catenin on HUVEC monolayer, and HUVEC monolayer treated with 4 μM $CdCl_2$ in the absence or presence of SB203850; Arrows refer to the cell–cell junctions of the HUVEC monolayer.

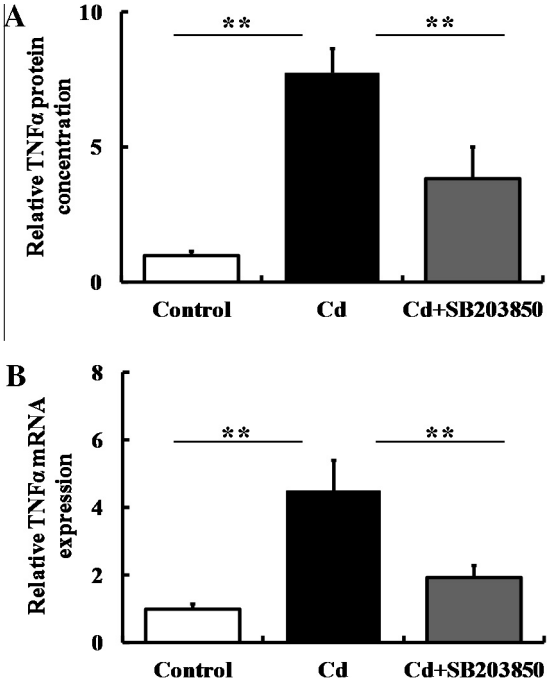

Fig. 4. The effects of cadmium and SB203850 on TNF-α secretion and expression in HUVECs. (A) Relative concentration of TNF-α measured by ELISA in HUVEC culture media treated with 4 μM $CdCl_2$ in the absence or presence of SB203850. $n = 6$; $^{**}p < 0.01$. (B) Relative *TNF-α* mRNA expression measured by RT-PCR in HUVECs treated with 4 μM $CdCl_2$ in the absence or presence of SB203850. $n = 6$; $^{**}p < 0.01$.

serves as a key structural component of adherens-type junctions [23,32]. In our study, low doses of Cd were able to induce the dispersion of VE-cadherin. This could be suppressed by the p38 MAPK inhibitor SB203850, suggesting a regulatory role for p38 MAPKs in VE-cadherin dissociation from adherens junctions. The detailed mechanism by which p38 MAPK regulates VE-cadherin complex stability is still unclear, but it may be associated with the phosphorylation of Hsp27, which is activated downstream of p38 MAPK [33].

Cd can induce inflammation *in vivo*, and rats injected intraperitoneally with $CdCl_2$ present with elevated activity of the pro-inflammatory cytokines including TNF-α [34]. Our study demonstrates that Cd increases the secretion and transcription of TNF-α, implicating this cytokine in the Cd induced hyper-permeability phenotype. Similar to Cd, TNF-α induces VE-cadherin dissociation from adherens junctions which could be blocked by p38 MAPK inhibition [16,17]. In cultured human peripheral blood mononuclear cells, Cd has been shown to induce the expression of TNF-α, IL-1β, IFN-γ, and IL-6 [35]. In addition, is has previously been demonstrated that TNF-α is secreted from bovine aorta endothelial cells in response to low concentration of Cd [36]. We found that Cd induced TNF-α production was partially inhibited by SB203850, suggesting that p38 MAPK might coordinate with other signaling pathways in regulating TNF-α secretion and expression.

Taken together, our results implicate a central role for p38 MAPK in the Cd-induced stress response that leads to disruption of endothelial barrier integrity. Targeted therapeutics that block p38 MAPK signaling may prove to be valuable therapeutically as agents that counters the toxic response to Cd exposure and other environmental toxins that modulate endothelial barrier permeability.

Conflict of interest

All authors have no conflict of interest to declare.

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