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ORIGINAL ARTICLE

Cadmium exposure alters steroid receptors and proinflammatory cytokine levels in endothelial cells in vitro: a potential mechanism of endocrine disruptor atherogenic effect

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Abstract

Background Cadmium (Cd) is a widespread environmental pollutant that causes alterations in human health acting as endocrine disruptor. Recent data suggest that cardiovascular system might be a contamination target tissue, since Cd is found in atheromatic plaques. Thus, the purpose of this study was to evaluate the consequence of Cd exposure of endothelial cells in vitro to evaluate detrimental effect in vascular system by a potential sex-steroid hormone receptor-dependent mechanism(s).
Methods To this aim, Human Umbilical Vein Endothelial Cells (HUVECs) were cultured and exposed to several concentrations of cadmium chloride ($CdCl_2$) for different interval times.
Results $CdCl_2$ exposure of HUVECs induced a significant increase of ERβ and Cyp19a1 at both mRNA and protein levels, while a drastic dose-dependent decrease of AR expression level was observed after 24 h of exposure. On the contrary, an increase of $PhAR^{ser308}$ as well as a reduction of $PhGSK\text{-}3\beta^{ser9}$ and $PhAKT^{ser473}$ was detected after 1 h treatment. This effect was consistently reduced by GSK inhibition. Furthermore, $CdCl_2$ abolished DHT-induced cell proliferation in HUVECs suggesting an antagonist-like effect of Cd on AR-mediated signaling. Remarkable, after 6 h $CdCl_2$—treatment, a relevant increase in TNF-α, IL-6 and IL-8 mRNA was observed and this effect was blocked by the presence of an ERβ-selective antagonist. Moreover, Cd-induced TxR1 overexpression, likely, correlated with the activation of p38 MAPK/NF-κB pathway.
Conclusion In conclusion, our study demonstrates for the first time that Cd alters sex-steroid hormone receptors level and activity likely affecting intracellular signaling linked to a proinflammatory state in endothelial cells. This alteration might possibly lead to endothelial cell injury and vascular dysfunction and could be a mechanism of gender-specific atherogenic damages induced by endocrine disruptors and, thus, induce atherogenic events with increased risk of cardiovascular diseases in individuals exposed to this endocrine disruptor.

Keywords Cadmium · Endocrine disruptor · Androgen receptor · Estrogen receptor and cardiovascular diseases · Cytokines · Gender

Contribution of E. A. Greco and S. Migliaccio must be considered equal.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s40618-018-0982-1) contains supplementary material, which is available to authorized users.

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Introduction

Cadmium (Cd) is a highly toxic heavy metal found in soil, water, rock sediments usually in the form of complexes oxide, sulphide and carbonate in zinc, lead, and copper ores [1], which acts as an endocrine disruptor, mimicking estrogenic activity and interfering with intracellular pathway [2].

This metal is released as a by-product of various industrial activities, since it is used in the manufacture of electroplating, alloy production, nickel–cadmium batteries, fertilizers, and paint pigments. Beside occupational exposure, individuals could also be exposed to Cd from non-occupational sources, such as food and water due to soil contamination [3, 4]. Moreover, cigarette smoking is an important source of Cd exposure and smokers have about two times higher levels of this pollutant in their bodies then non-smokers [5]. Cd is a potent cell poison, which causes different types of damage, including cell death, alteration in cell proliferation, and disruption of intracellular pathway in different endocrine target cell types [2, 6–8]. Depending on the dose, route, and duration, exposure to Cd can cause specific damages in various organs including kidney, liver, lung, bone, testis, and placenta [9].

Interestingly, the half-life of Cd ranges from 12 to 30 years [10], and since human body does not possess an active mechanism for Cd elimination, its levels increase over time due to its slow elimination [11]. Cd forms complexes with Metallothionein, released into the blood, and transported to various tissues and organs [12]. Indeed, experimental evidences suggest that Cd causes several disorders in different organs and tissues including kidney, lung, bone, muscle but also endothelial dysfunction leading to several vascular diseases, including atherosclerosis, myocardial infarction, and stroke [9, 13–20]. In fact, it has been recently suggested that vascular endothelium is an important target of Cd toxicity [21]. Indeed, blood Cd level appears associated with accumulation of this metal in atherosclerotic plaques [17], suggesting potential direct effects on the progression of atherosclerotic process [18].

It is worth to underline that Cd has been defined a metallohormone [22], since it interacts with sex-steroid hormone receptors, interfering with downstream signaling pathways and further confirming its activity as endocrine disruptor [23–27]. Moreover, several studies have demonstrated, in cellular in vitro model systems, that Cadmium Chloride ($CdCl_2$) binds with high affinity to the hormone-binding domain of estrogen receptor (ER)-α mimicking many of the biological effects of estradiol in breast cancer cells [2, 24, 28]. Studies performed in vivo have also shown estrogenic-like effects in uterus and mammalian glands induced by Cd [29], while other have demonstrated that Cd has androgen-like activity in prostate cancer cells binding with high affinity the hormone-binding domain of androgen receptor (AR) inducing transcriptional activity whereas a decrease in expression level [25, 27].

Since it is well known that in both gender sex, steroid hormones greatly influence vascular functions in different periods of life, it can be hypothesized that alteration in hormones or receptor levels might influence vascular function and atherogenic events [30–33]. Experiments on female rats have indicated an interdependence of Cd and E_2 for eliciting effects on the immune system, suggesting that females may be at a greater risk than males for Cd-induced immunomodulation [31]. Moreover, recent studies have strongly suggested that pollution and soil/water contamination might augment cardiovascular diseases, though there is a scarce knowledge on whether and how endocrine disruptors, including Cd, might influence sex-steroid receptors signaling in vascular endothelial cells (ECs) likely leading to increased risk of cardiovascular diseases.

Thus, aim of this study was to evaluate potential alterations induced in ECs by Cd exposure in sex-steroid hormones receptors, eventually in proinflammatory cytokines, and characterize the mechanisms involved to further understand weather this heavy metal might lead to vascular injury by gender-specific sex-steroid receptor-dependent mechanisms.

Materials and methods

Cell culture and treatments

Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Lonza (Basel, Switzerland) and were cultured in endothelial basal cell medium-2 (EMB-2) supplemented using the EGM-2-MV bullet kit (Lonza) and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). Cells were cultured in humidified air at 37 °C with 5% CO_2. In all experimental protocols, cells were seeded at a density of 3000 cells/cm^2 and allowed to grow for 24 h. Experiments were performed in EBM-2 medium supplemented with 2% charcoal-treated calf bovine serum (CBS). Cadmium chloride (CdCl2), purchased from Sigma-Aldrich, St. Louis, MO, was dissolved in water and stock solution sterilized by filtration. Dihydrotestosterone (DHT; Sigma-Aldrich) and 17β Estradiol (E_2; Sigma-Aldrich) were used at the final concentration of 0.01 µM. The selective estrogen ERβ receptor antagonist 4-[2-Phenyl-5,7-bis (trifluoromethyl) pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP; Santa Cruz Biotechnology) was used at the final concentration of 5 µM. The p38 inhibitor SB203580 (Sigma-Aldrich) and glycogen synthase kinase 3 specific inhibitor AR-A014418 (Sigma-Aldrich) were added to

the cell cultures to reach a final concentration of 10 μM. All inhibitors were added 1 h before $CdCl_2$ exposure and maintained for the length of treatments.

Cell proliferation assay

HUVEC cell viability was determined using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay kit (Promega), based on cell-mediated (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction to formazan, following the manufacturer's protocol. Cells were cultured in 96-well plates at the density mentioned above. Cell viability was evaluated in cell cultures treated for 24 and 48 h with $CdCl_2$ at concentrations ranging between 1 and 20 μM, dihydrotestosterone (DHT) and after co-treatment with $CdCl_2$ (10 μM) and DHT (10 nM). The absorbance was measured at 490 nm using a plate reader (680 Microplate Reader; Bio-Rad) and expressed as the optical density value. Three independent experiments were performed in triplicate.

Western blotting

Cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 50 mM NaF, 1% nonidet P-40, 0.1% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml leupeptin]. Protein concentrations of cleared lysates were determined using the bicinchoninic assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (10–50 μg) were separated by SDS–polyacrylamide gel (8–12% polyacrylamide gel) and transferred to a polyvinylidene fluoride (PVDF) membrane. PVDF membranes were blocked with 5% skimmed milk and incubated overnight with the primary antibody in PBS-Tween at 4 °C. Primary antibodies including total or phospho-p38-MAPK, PhGSK-3β^{ser9}, and GSK-3β antibodies were purchased from Cell Signaling Technology. The AR, PhAR^{ser308}, ERβ, Cyp19a1, TrxR1, Ph-IkBα, AKT, and PhAKT ^{ser473} antibodies were purchased from Santa Cruz Biotechnology. GAPDH antibody was purchased from Sigma-Aldrich. The immunoreactive protein bands were detected by incubation for 1 h with horseradish peroxidase-conjugated secondary goat anti-rabbit (1:10,000, millipore) or goat anti-mouse (1:20.000, Sigma-Aldrich) in blocking solution at room temperature, and they were visualized by enhanced chemiluminescence (Amersham Biosciences). The bands were acquired on an ImageQuant LAS 4000 (GEHC) and quantified by ImageJ software (137F5).

Measurement of mRNA levels by quantitative RT-PCR

Total cellular RNA was extracted using the TRI reagent (Sigma) according to the manufacturer's instructions and subjected to DNase digestion (Ambion). Real-time quantitative RT-PCR was conducted using one-step SYBR GreenER (Life Technologies) in a 7500 real-time PCR system (Applied Biosystems) according to the manufacturer's protocol. Each analysis was performed in triplicate. Relative expression levels were calculated using the comparative cycle threshold (ΔΔCt) method using cyclophilin A as internal control. The primer sequences are summarized in Table 1.

ELISA assays

At the end of different experiments, culture medium of cells exposed to specific treatments was removed after 48 h, centrifuged at 500 g for 10 min at 4 °C, and supernatants stored at − 80 °C until used for assays. E_2 was measured by Human Estradiol ELISA Kit (Cusabio Biotech Co., LTD). High-sensitivity ELISA kits (IBL-international) were used for IL-6 and IL-8 detection in the culture medium. Each test was performed, according to manufacture's protocol. The IL-6 assay detection range was 1.56–100 pg/ml, and the IL-8 assay detection range was 15.6–1000 pg/ml. Data from ELISA assays were normalized for number of viable cells at the end of each experiment and expressed in pg/ml.

Table 1 Human-specific primers pair sequence for real-time PCR

Gene	Primers pair sequence	Base pair
TNF-α	F: CTTTGGAGTGATCGGCCCCCC R: CCATTGGCCAGGAGGGCATT	107
IL-6	F: TTCGGTACATCCTCGACGGC R: TCTGCCAGTGCCTCTTTGCT	71
IL-8	F: TCCTGATTTCTGCAGCTCTGTG R: GTCCAGACAGAGCTCTCTTCCAT	177
Cox-2	F: GCACCCCGACATAGAGAGC R: CTGCGGAGTGCAGTGTTCT	99
Esr2	F: AGCACGGCTCCATATACATACC R: TGGACCACTAAAGGAGAAAGGT	177
Cyp19a1	F: ACTACAACCGGGTATATGGAGAA R: TCGAGAGCTGTAATGATTGTGC	119
AR	F: TACCAGCTCACCAAGCTCCT R: GATGGGCTTGACTTTCCCAG	154
ICAM-1	F: GGCCGGCCAGCTTATACAC R: TAGACACTTGAGCTCGGGCA	146
iNOS	F: ACCAGTACGTTTGGCAATGGAGA R: GAACCGAGGGTACATGCTGGA	114
Cyclophilin A	F: GTCAACCCCACCGTGTTCTT R: AAAGTTTTCTGCTGTTTTTGG AATC	104

Results

Preliminary experiments were performed to define optimal non-cytotoxic concentrations of $CdCl_2$ to use in the experimental protocol and concentrations range previously found not to be cytotoxic in HUVECs were chosen [20]. The effects on cell viability were evaluated after 24 and 48 h of exposure to $CdCl_2$, resulting in a loss of cellular viability. The concentration of 20 µM reduced cell viability in a time-dependent manner, inducing about 70% decrease after 48 h of $CdCl_2$ exposure as compared to untreated cells (Fig. 1). Therefore, a $CdCl_2$ concentration range between 1 and 10 µM was used for all subsequent experiments.

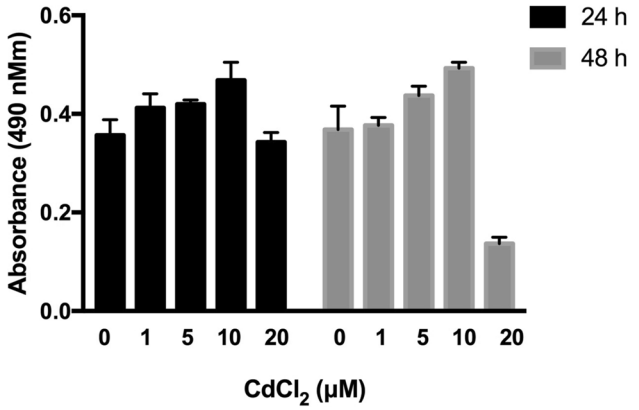

Fig. 1 Effects of Cadmium on HUVEC proliferation. HUVEC cells were incubated in growth medium in the absence or presence of 1–20 µM CdCl2 for 24 and 48 h. Number of viable cells was quantified using the MTS assay, as described in "Materials and methods"

Since our previous studies [2] have demonstrated that Cd can alter breast cancer cell homeostasis in vitro by an ER-mediated mechanism, experiments were performed to evaluate whether Cd could modify expression of estrogen receptor-β (esr2 or ERβ), androgen receptor (AR), and, also, aromatase isoenzyme, Cyp19a1. Interestingly, $CdCl_2$ exposure of HUVECs induced a significant increase in esr2 mRNA expression levels already after 6 h (Fig. 2a) with both concentrations used, returning towards basal levels after 24 h (Fig. 2d). Cyp19a1 mRNA expression levels were slightly higher after 6 h, showing a significant increased upon 24 h of $CdCl_2$ exposure vs untreated control (Fig. 2b, e). Moreover, Cd induced a significant dose-dependent decrease of AR mRNA expression levels in HUVECs already after 6 h (Fig. 2c) with a maximal effect after 24 h of treatment, as depicted in Fig. 2f. Accordingly, to gene expression data, a ~ twofold increase of ERβ protein level (Fig. 3a) and an evident dose-dependent decrease of AR protein expression were observed after 24 h of Cd exposure (Fig. 3b).

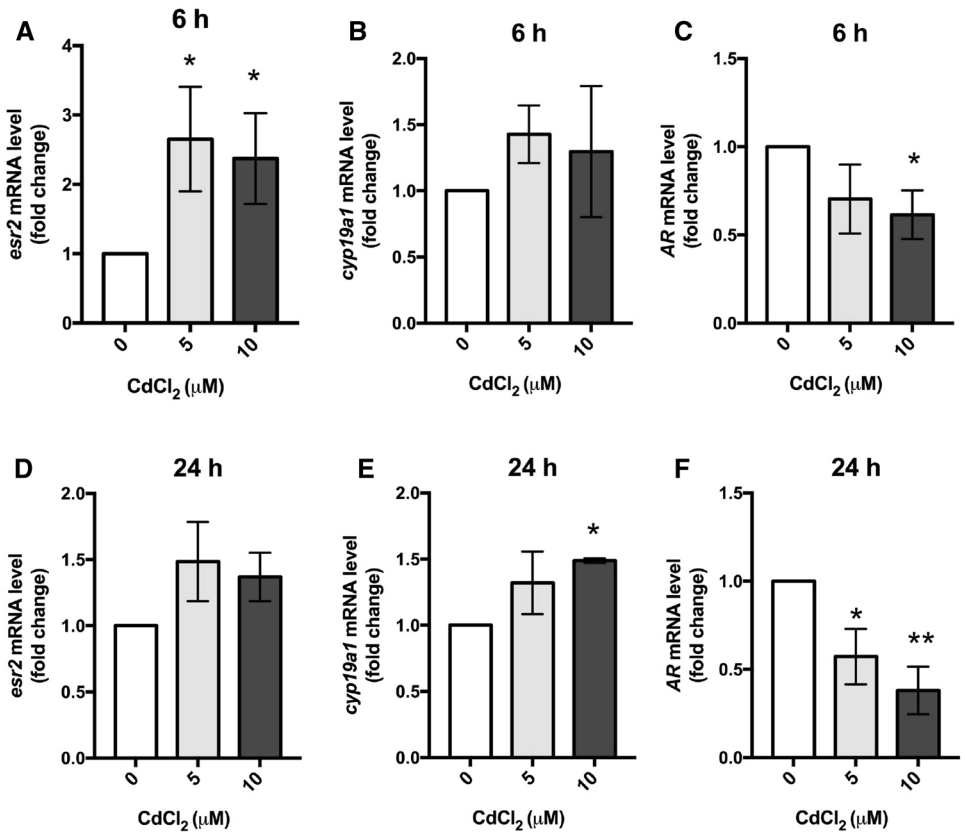

Fig. 2 Expression level of sex-steroid hormones receptors and aromatase in HUVECs. Cells were exposed to $CdCl_2$ for 6 h (**a**–**c**) or 24 h (**e**–**g**). Cyclophilin A mRNA was used to normalize the relative amount of mRNA. Results are presented as mean ± SD ($n = 3$) of three separate experiments. *$p \leq 0.05$ and **$p \leq 0.01$ vs vehicle-treated cells

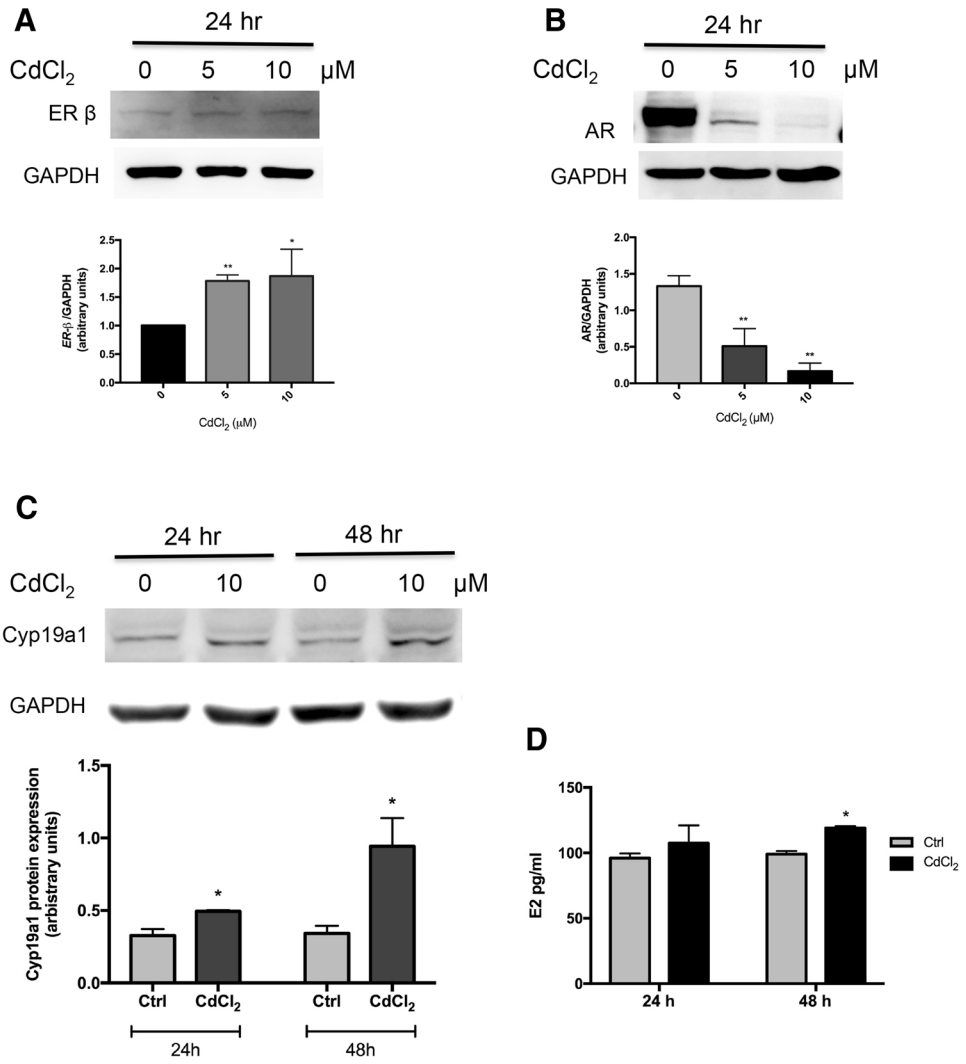

Fig. 3 Cadmium modulation of ERβ, AR, Cyp19a1 protein expression levels and E_2 release in culture medium. ERβ (**a**), AR (**b**), and Cyp19a1 (**c**) protein expression were analysed after 24 h of $CdCl_2$ exposure (5–10 μM). Protein expression was normalized by GAPDH expression level. E_2 level in cells' supernatant (**d**) was evaluated by ELISA method after 24 h and 48 h treatment with 10 μM $CdCl_2$. Number of viable cells was used for the normalization of E_2 amount. Results shown in the histograms represent the mean ± SD of three independent experiments. $^*p \leq 0.05$ $^{**}p \leq 0.01$ compared to vehicle-treated cells

In addition, Western blot analysis indicated a time-dependent increase of Cyp19a1 protein expression level (from 1.5- to 2.7-fold over control level) after 24 and 48 h exposure to 10 μM $CdCl_2$ (Fig. 3c). In accordance with the higher increase of Cyp19a1 protein expression level, $CdCl_2$ exposure of HUVECs for 48 h determined a ~ 20% increase in E_2 concentrations in the supernatant (Fig. 3d), indicating also an increase in Cyp19a1 activity.

Since AR is subjected to regulation by several kinases through post-translational modifications on serine, threonine, and tyrosine residues [34], experiments were performed to evaluate putative effects of Cd on modulation of AR activation. In particular, phosphorylation of AR on ser308 and GSK-3β on ser-9 (an inactive form of GSK-3β) was evaluated, since phosphorylation of the AR on ser308 by CDK11P58 represses its function [35, 36], indicating that an activation of Glycogen synthase kinase-3beta (GSK-3β) suppresses AR-mediated transcription [36]. Thus, HUVECs were exposed for 1 h to $CdCl_2$ which induced a dose-dependent increase of AR phosphorylation at ser308 as well as a reduction of GSK-3β phosphorylation at ser9 (Fig. 4a, b), indicating a decreased function of the receptor. To verify a putative involvement of GSK3β activity in AR phosphorylation, AR-A014418 was used to inhibit GSK3β. HUVEC cells treated for 1 h with 10 μM $CdCl_2$, in the presence of AR-A014418, showed a ~ 40% decrease of $CdCl_2$-mediated AR phosphorylation on ser308 (Fig. 4c, d). Since GSK-3β phosphorylation is mediated by AKT activation, AKT phosphorylation on ser473 was also analysed in the same experimental condition. After 1 h, $CdCl_2$ induced a reduction of AKT phosphorylation on ser473 (Fig. 4c, d); on the contrary, the presence of GSK-3β inhibitor caused an increased AKT phosphorylation (Fig. 4c, d). To test AR functionality, DHT was use to stimulate cell, since the previous studies indicated that DHT induces endothelial cell proliferation via an AR-mediated mechanism [37]. As depicted in Fig. 4e, the results obtained in our experimental model confirmed that DHT (0.01 μM) induced HUVECs

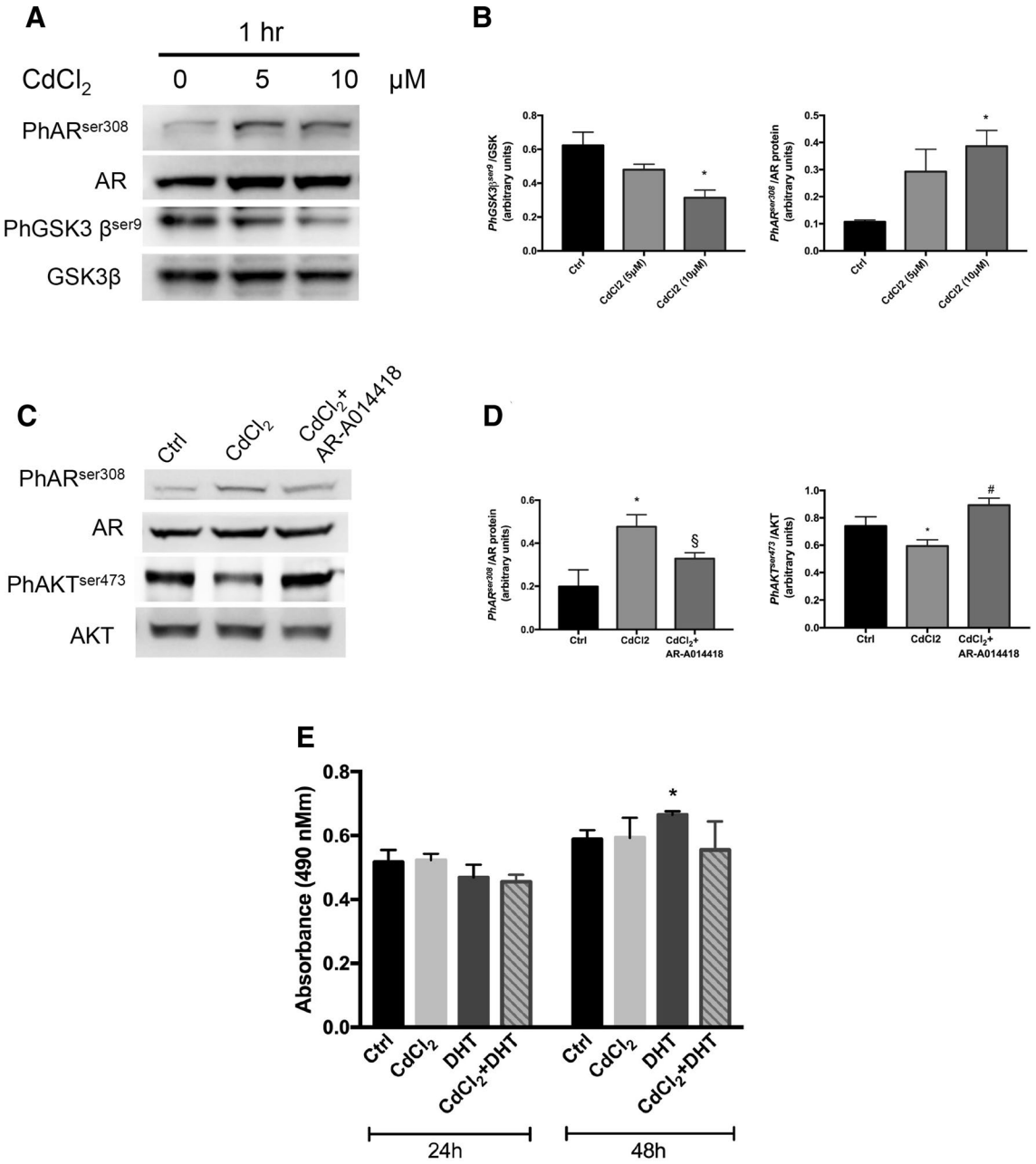

Fig. 4 Cadmium modulation of AR-mediated signaling pathway. Histograms depict mean value of ERβ and AR protein levels normalized to GAPDH level (**a**, **b**). Representative blot which depicts AR and its phosphorylation at serine 308 ($PhAR^{ser308}$), GSK-3β, and its phosphorylation at serine 9 ($PhGSK\text{-}3\beta^{ser9}$) after 1 h of Cd exposure (**c**). Histograms represent the mean values of ratio of $PhAR^{ser308}$ normalized over AR total protein level and the $PhGSK\text{-}3\beta^{ser9}$ level normalized to GSK-3β total protein content (**d**). AR phosphorylation status in HUVECs after 1 h $CdCl_2$ (10 μM) treatment, in the presence of GSK-3β inhibitor, AR-A014418 (10 μM) (**e**). Effect of $CdCl_2$ with or without DHT on HUVECs proliferation (**f**). Cells were incubated with or without $CdCl_2$ (10 μM), DHT (0.01 μM), or $CdCl_2$ in the presence of DHT for 24 and 48 h. Number of viable cells was quantified using the MTS assay. Results are presented as mean ± SD of three separate experiments. $*p \leq 0.05$ and $**p \leq 0.01$ vs vehicle-treated cells; $\S p \leq 0.05$ vs 10 μM $CdCl_2$-treated cells

proliferation after 48 h treatment, but DHT-induced cell proliferation was blocked by the presence in the culture medium of $CdCl_2$ suggesting an antagonist-like effect of Cd on AR-mediated signaling in endothelial cells.

Next, since systemic low chronic inflammation appears to play a role in atherogenic processes, and Cd exposure might increase vascular disease risk, likely inducing low-grade inflammation [38, 39], other experiments were performed

to evaluate whether Cd might stimulate markers of inflammation, such as proinflammatory cytokines.

In particular, Interleukin-6 (IL-6), IL-8, and tumour necrosis factor-α (TNF-α) were evaluated, since they can amplify inflammatory response by activating release of nitric oxide (NO) and reactive oxygen species and promoting tissue injury [40].

Exposure of HUVECs to CdCl2 (5–10 μM) for 6 h, induced a ~ threefold increase in TNF-α (Fig. 5a), a fivefold increase in IL-6 (Fig. 5c), and more then tenfold increase in IL-8 mRNA expression levels compared to vehicle-treated cells (Fig. 5e). Moreover, in the same experimental condition, it was also observed a twofold increase in Cyclooxygenase-2 (Cox-2, Fig. 5g), a key enzyme for inflammatory cytokine-induced angiogenesis [41]. After 24 h exposure HUVECs showed TNF-α—increased expression levels only after exposure to 10 μM $CdCl_2$ (Fig. 5b), whereas IL-6 (Fig. 5d) and IL-8 mRNA (Fig. 4f) demonstrated increased expression levels with both $CdCl_2$ doses (three- and eightfold increase vs control cells, respectively). To further dissect the events described above, and to evaluate whether the effect of Cd on ERβ-dependent signaling could affect modulation of proinflammatory cytokines, HUVECs were treated for 6 h with $CdCl_2$ or the natural ligand 17β-Estradiol (E_2) in the presence or absence of the selective inhibitor of ER β, PHTPP. At the end of Cd-exposure TNF-α, IL-6 and IL-8 mRNA level were evaluated. Whereas E_2 stimulation of HUVEC cells in the presence or absence of PHTPP did not modify cytokines mRNA expression level (Fig. 6), the presence in the medium of PHTPP completely blocked the Cd-induced increase of TNF-α and IL-6 (Fig. 6a, b) mRNA expression level and decreased ~ fivefold IL8 mRNA expression level (Fig. 6c), suggesting, at least in part, an ERβ-mediated mechanism. These data were confirmed by the increase of cytokines levels in the supernatant after 48 h $CdCl_2$ exposure. Cd exposure of HUVECs in the presence of PHTTP resulted in a blockade of both IL-6 and IL8 increase (data not shown), while TNF-α levels in HUVEC supernatant cells were not detectable, likely due to the very low levels, which were below the ELISA kits sensitivity (data not shown).

To further characterize the intracellular mechanism(s) involved in these events, p38 MAPK pathway was studied, since it has been demonstrated that $CdCl_2$ (4 μM)-induced effects in HUVEC cells could be partially blocked by pretreatment with a p38 inhibitor [38]. Thus, after confirmation of p38 MAPK activation induced by Cd in HUVECs (Fig. 7a), cells were exposed to $CdCl_2$ in presence or absence of the p38 inhibitor SB203580. Remarkably, the addition of p38 inhibitor SB203580 fully blocked the $CdCl_2$ augmentation of TNF-α, IL-6, and IL-8 mRNA expression after 24 h treatment (Fig. 7b), strongly suggesting a p38 involvement in the described events.

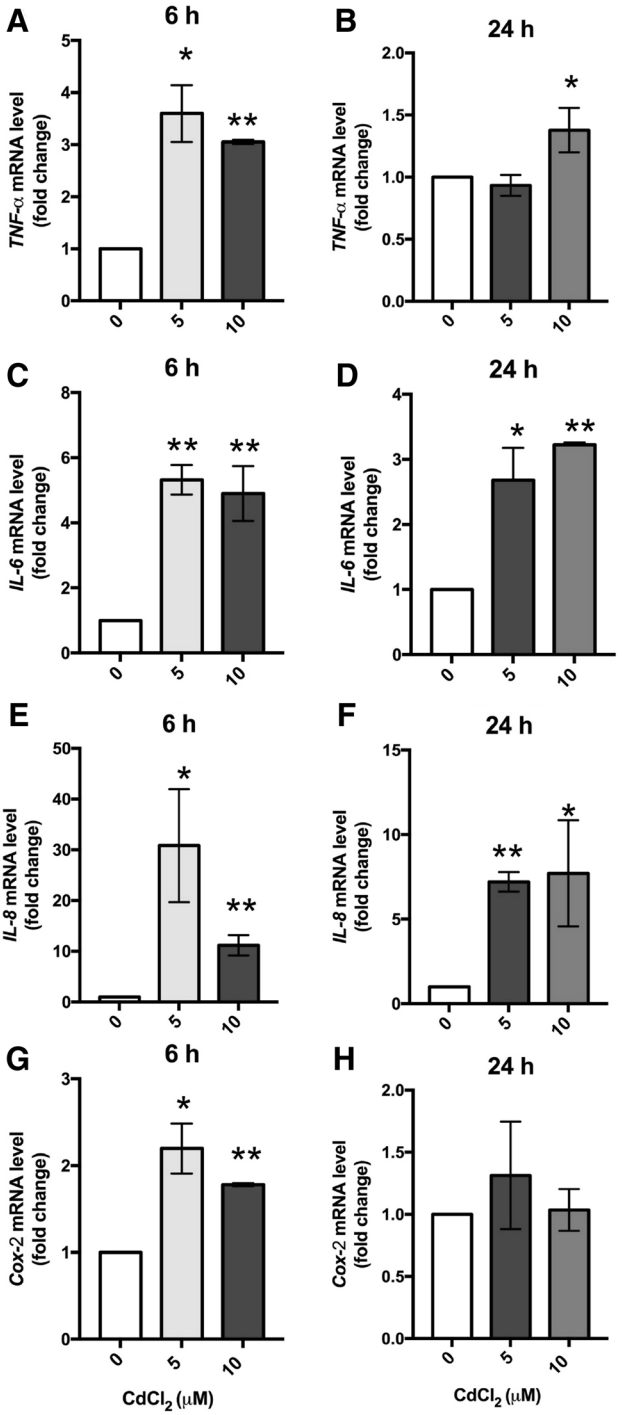

Fig. 5 Effects of Cadmium exposure on mRNA expression levels of inflammatory cytokine in HUVEC cells. RT-qPCR showing TNFα (**a**, **b**), IL-6 (**c**, **d**), and Cox-2 (**e**, **f**) mRNA levels of HUVECs' cells grown in the presence or absence of $CdCl_2$ (5–10 μM). Cyclophilin for 6 and 24 h. Cyclophilin A mRNA was used to normalize the relative amount of mRNA. Results are presented as mean ± SD ($n = 3$) of three separate experiments. *$p \leq 0.05$ and **$p \leq 0.01$ vs vehicle-treated cells

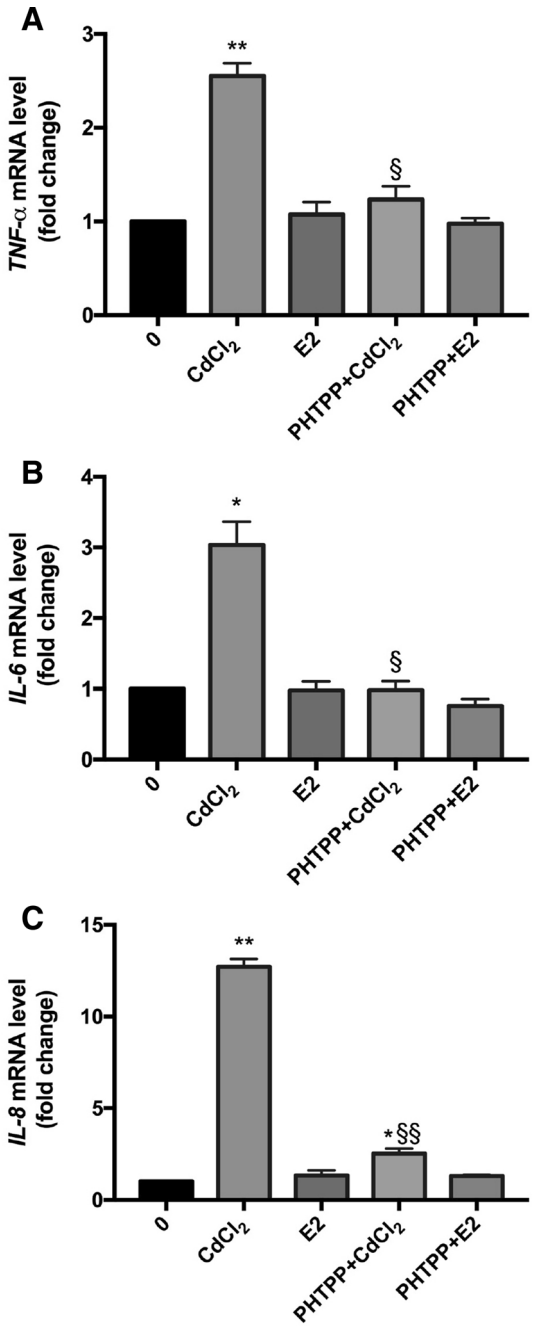

Fig. 6 Effect of ERβ blockage on proinflammatory cytokines expression. RT-qPCR showing TNFα (**a**), IL-6 (**b**), and IL-8 (**c**) mRNA levels of HUVECs cells exposed to $CdCl_2$ (10 µM) or E_2 (0.01 µM) in the presence or absence of PHTPP (5 µM), a selective inhibitor of ERβ

Moreover, Thioredoxin reductase 1 (TrxR1), an intracellular redox sensor and antioxidant enzyme, inducible by Cd trough activation of Nrf2 transcription factor and its binding to AU-rich elements (AREs) in TrxR1 gene promoter [42] was analysed. To evaluate potential involvement of TrxR1 protein in the Cd-induced HUVEC cell homeostasis alteration, TrxR1 protein expression was evaluated. After 24 h of $CdCl_2$ exposure, HUVEC cells showed a twofold increase of TrxR1 protein expression that was blunted by the co-incubation of the p38 inhibitor SB203580 (Fig. 7c). In addition, $CdCl_2$ exposure (30 min) induced a relevant increase of ph-IkBα protein level that was blunted by SB203580 (Fig. 7d), suggesting that the Cd overexpression of TrxR1, could be modulated by NF-κB pathway trough p38 MAPK activation. Furthermore, since atheromatic process is also linked to alterations of adhesion molecules and to iNOS, additional experiments were performed to evaluate whether Cd could alter the expression of these molecules. As depicted in Fig. 8, 24 h Cd exposure induced a dose-dependent increase in ICAM-1 as well as, in iNOS expression ((Fig. 8a, b) strongly indicating a disruption of the homeostasis of these molecules as well.

Discussion

Cadmium is a potent environmental pollutant that acts as endocrine disruptor leading to altered homeostasis of many tissues and organs [22]. Herein, we demonstrate for the first time that Cd can alter sex hormone receptors, AR and ERβ, expression levels in human vascular endothelial cells, which might be in part responsible for this pollutant toxic atherogenic effect. Indeed, our results confirm previous data indicating that non-cytotoxic concentrations of Cd can induce a deregulation of endothelial intracellular signaling pathways, which play key roles in vascular cells functions [6, 38], resulting in an important inflammatory state.

Interestingly, our novel results show that low doses of Cd induced a relevant down-regulation of AR expression level as well as an increased ERβ and Cyp19a1 expression levels, as well as an increased activity of Cyp19a1 as depicted by the modest increase in E_2 concentration in the supernatant of Cd-treated HUVECs. The mechanisms by which Cd affects AR signaling in endothelial cells processes, associated with proliferation and angiogenesis/repair, as well as with pathogenic processes, such as atherosclerosis and neoplasia, have not been fully characterized before [43]. The tolerable cadmium exposure was set at 2.5 µg/kg body weight per month [4] and a urinary threshold of 5.24 µg/g creatinine [3], which could be easily reached by smoking cigarettes and by industrial pollution. HUVECs provide a classic in vitro model system to study many aspects of endothelial functions and inflammation-related pathways in endothelium under normal and pathological conditions such as cardiovascular-related atherogenic complications.

The results of our study strongly suggest that Cd can interfere with both AR and ERβ expression and function and this interference appears to increase levels of proinflammatory

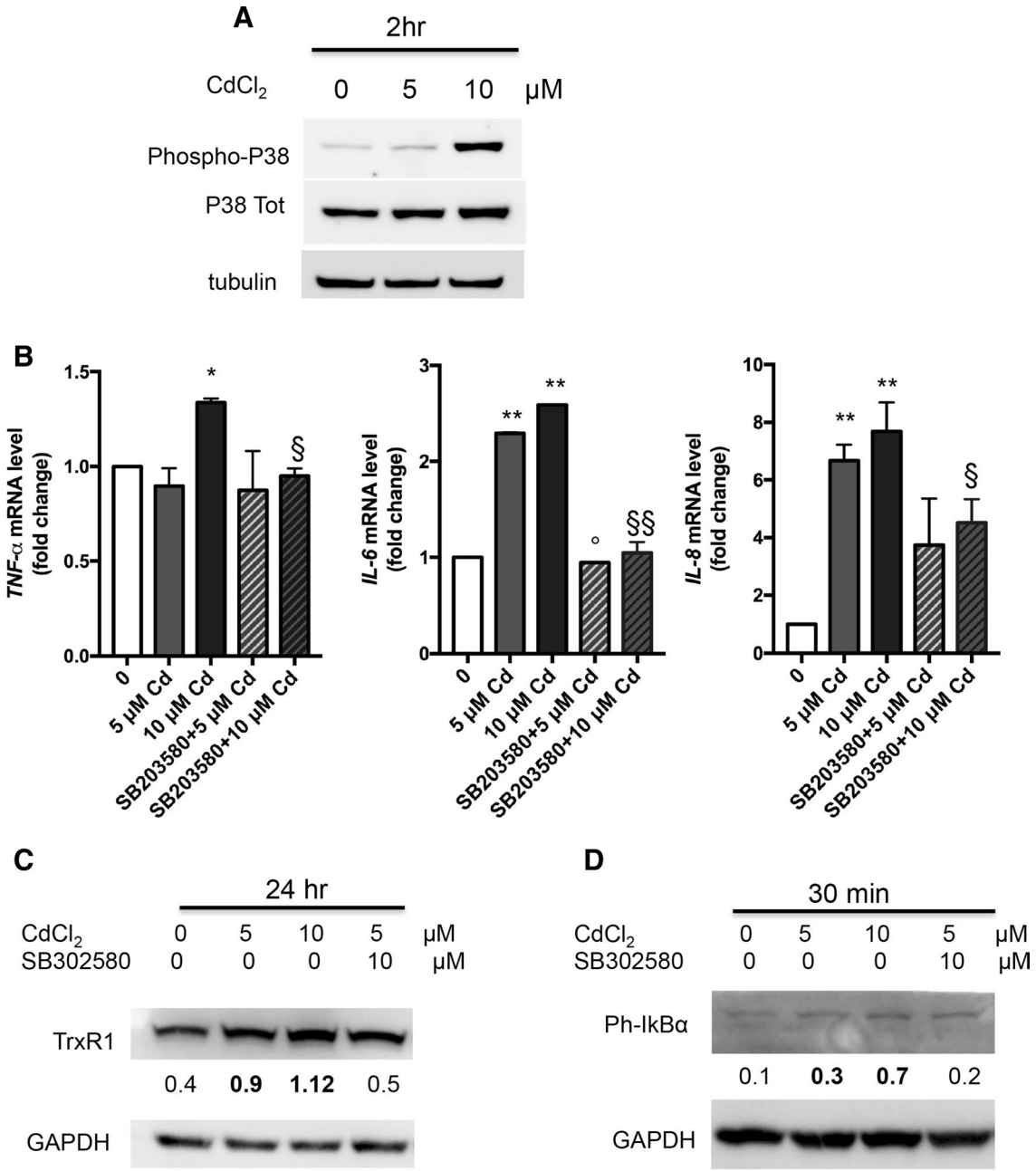

Fig. 7 Effects of p38 inhibitor on CdCl2-dependent inflammation and TrxR1 overexpression. Representative blots from phospho-p38 MAPK and total p38 MAPK, from protein samples of HUVECs exposed to 5–10 μM $CdCl_2$ for 2 h (**a**). Cells were pretreated for 30 min with p38 MAPK inhibitor SB203580 (10 μM) and then exposed to $CdCl_2$ (5-10 μM) in the presence or absence of the inhibitor. Histograms represent RT-qPCR showing TNFα, IL-6, IL-8 and (**b**) mRNA levels of $CdCl_2$-exposed cells in the presence or absence of the inhibitor for 24 h. Representative image of the Western blot analysis showing protein expression of TrxR1 (**c**) and Phospho-IkBα (**d**). Amount of each protein was calculated by normalizing to GAPDH protein levels. Results are shown as mean ± SD of at least three different experiments. $*p \leq 0.05$ and $**p \leq 0.01$ vs vehicle-treated cells; $°p \leq 0.05$ vs 5 μM $CdCl_2$-treated cells; $§§p \leq 0.01$ and $§p \leq 0.05$ vs 10 μM CdCl2-treated cells

cytokines explaining, at least in part, the atherogenic effect which have been deputed to this endocrine disruptor.

Interestingly, several mechanisms have been suggested to explain the role of this endocrine disruptor in promoting vascular ECs dysfunctions. Among all, it has been suggested a potential role-played by an interaction between cytokines and AR expression in human endothelial cells [43, 44]. Interestingly, Wang et al. [44] indicated that IL-6 secreted by ECs appears to be a key mediator for AR down-regulation in ECs–prostate cancer (PC) cells inhibiting AR functions and

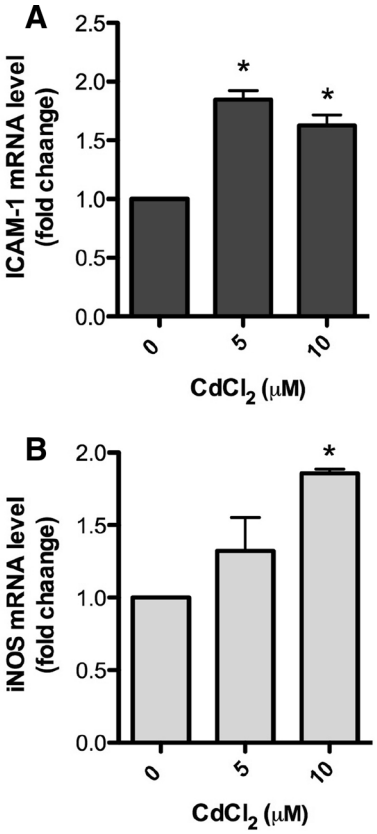

Fig. 8 Effects of 24 h Cadmium exposure on ICAM-1 (**a**) and iNOS (**b**) expression in HUVEC cells. Cyclophilin A mRNA was used to normalize the relative amount of mRNA. Results are presented as mean ± SD ($n = 3$) of three separate experiments. *$p \leq 0.05$ and **$p \leq 0.01$ vs vehicle-treated cells

inducing PC metastasis. We found an increase of AR phosphorylation on ser308, associated with a blockade of AR activity [35]. In addition, 1 h Cd exposure caused a reduction of GSK-3β phosphorylation. The strong reduction in AR phosphorylation in the presence of a GSK-3β inhibitor confirmed that this event was, at least in part, related to an increase in GSK-3β activation.

GSK-3β is a serine/threonine kinase with a key role in angiogenesis regulation [45] and in the inflammatory response in vascular ECs [46, 47]. Furthermore, its activation has been correlated with a suppression of AR activity in prostate cells [36]. GSK-3β is a known substrate of PI3 K/Akt signaling pathway, which usually inhibits GSK-3 by serine phosphorylation [47]. Consistent with Cd-induced GSK-3β activation, our results depict a reduction of Akt phosphorylation at ser473 in Cd-treated HUVECs.

On the contrary, Kim et al. [48] reported an activation of Akt/GSK-3β in SH-SY5Y cells after 6–24 h 25 μM Cd exposure, suggesting that Akt/GSK-3β pathway could play a survival role in Cd-induced cell death. However, we can hypothesize that the discrepancy with our data might indicate differences in the regulation of Akt/GSK-3 β pathway depending on cell type, concentration, and length of Cd treatment. It is worth to mention that the effects of Cd are strongly dose-dependent [20] and opposite effects have also been shown when different doses of Cd are used [49]. Interestingly, our results indicate that low-dose Cd exposure can affect AR activity trough an Akt/GSK-3β-dependent mechanism in endothelial cells in vitro. As the previous studies have also suggested [37], our results further demonstrate that Cd abolished DHT-induced cell proliferation by an AR-dependent mechanism, as depicted in "Result".

In addition, Cd is a potent prooxidative stressor, because it weakens the most important antioxidant scavenger systems including glutathione, thioredoxin, superoxide dismutase, and catalase [15, 50–52] and it inhibits mitochondrial electron transport chain inducing hydrogen peroxide and peroxide ions generation causing a modification of redox-signaling transduction pathways leading to oxidative stress induction [53–55]. In turn, Cd-induced oxidative stress enhances lipid peroxidation causing a lipid and lipoprotein profile derangement, as demonstrated in preclinical animal models [9, 15, 16, 56, 57]. Sakurai et al. [58] have indeed demonstrated that overexpression of TrxR1 enhanced TNF-α- induced DNA-binding activity of NF-kB in endothelial cells, suggesting that TrxR1 might act as a positive regulator of NF-kB and might play a pivotal role in cellular inflammatory responses [58]. Recent studies indicate that Cd exposure alters lipid metabolism in humans and elevated blood Cd concentration is associated with prevalence of dyslipidaemia [59], contributing to initiate or promote the process linked to the development of atheromatic lesions [13]. Since the results presented in our manuscript also indicate alteration of adhesion molecules upon Cd exposure, this could be an additional mechanism linked to the atherogenic effect induced by this endocrine disruptor.

Of interest, in vitro and in vivo studies have indicated a relationship between Cd exposure and inflammation correlated with several vascular diseases [39, 60–62]. Sub-toxic Cd concentrations trigger proinflammatory response by modulating production and release of cytokines and inflammatory molecules [38, 63], key inflammatory components of atheromatic process [39, 64]. Indeed, HUVECs exposure to Cd resulted in an increase of TNF-α, IL-6, and IL-8 mRNA expression levels. This effect was blocked by co-incubation in the presence of ERβ inhibitor, suggesting a direct sex-steroid receptor-dependent mechanism.

Our present data confirm these previous published results, further suggesting that this environmental pollutant could induce the above-mentioned vascular system modification by an endocrine-disrupting mechanism, altering the sex-steroid receptor levels and function.

Furthermore, our data suggest that Cd alters physiological cellular homeostasis of endothelial cells by disrupting

intracellular pathways such as p38 and Thioredoxin reductase-1. For instance, Phuagkhaopong et al. [65] demonstrated that Cd stimulated IL-6 and IL-8 expression and release in human astrocytes by activation of the p38 and NF-kB pathways [65], well-known pathways mediating upregulation of cyclooxygenase-2 (Cox-2) in response to stimulation with TNF-α [66]. In its inactive form NF-kB is sequestered in cytoplasm compartment with IkB complex, but, once activated, IkB phosphorylation, activates NF-KB. It was demonstrated that NF-κB signaling modulates pro-atherogenic program in endothelial cells [67] and its activity is sensitive to inhibition of p38 [68]. In accordance with these previous data, our results showed an increase of IkB phosphorylation in Cd-treated cells, likely suggesting an NF-kB activation pathway involvement as also indicated by the abrogation of Cd-induced IkB phosphorylation by the presence of SB203580.

In addition, SB203580 was also able to blunt Cd-induced TrxR1 protein expression levels. Thioredoxin reductase-1 (TrxR1) is a pivotal intracellular redox sensor and antioxidant enzyme which can be induced by Cd trough the activation of Nrf2 transcription factor and its binding to ARE in the TrxR1 gene promoter [42]. Overexpression of TrxR1 enhanced TNF-α -induced DNA-binding activity of NF-kB in endothelial cells, suggesting that TrxR1 may act as a positive regulator of NF-kB playing a pivotal role in cellular inflammatory response [58]. Thus, it might be hypothesized that Cd overexpression of TrxR1, could be modulated by NF-κB pathway trough p38 MAPK activation.

In conclusion, our study demonstrates for the first time that Cd alters AR and ER expression and function in endothelial cells, likely affecting steroid hormone receptor signaling linked to a proinflammatory state possibly leading to endothelial cell injury and vascular dysfunction. This alteration might be, at least in part, a novel described mechanism of both gender-specific atherogenic damages induced by endocrine disruptors and, thus, to the induction of atherogenic events with increased risk of cardiovascular diseases in individuals exposed to environmental pollutants.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent No informed consent was needed since no human studies are included in the manuscript.

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