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Pleiotropic roles of Ca^{+2} /calmodulin-dependent pathways in regulating cadmium-induced toxicity in human osteoblast-like cell lines

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Abstract

The heavy metal cadmium is a widespread environmental contaminant that has gained public attention due to the global increase in cadmium-containing electronic waste. Human exposure to cadmium is linked to the pathogenesis of osteoporosis. We previously reported cadmium induces apoptosis and decreases alkaline phosphatase mRNA expression via extracellular signal-regulated protein kinase (ERK) activation in Saos-2 bone-forming osteoblasts. This study examines the mechanisms of cadmium-induced osteotoxicity by investigating roles of Ca^{+2} /calmodulin-dependent protein kinase (CAMK) pathways. Saos-2 or MG-63 cells were treated for 24 or 48 h with 5 μM CdCl_2 alone or in combination with calmodulin-dependent phosphodiesterase (PDE) inhibitor CGS-9343 β ; calmodulin-dependent kinase kinase (CAMKK) inhibitor STO-609; or calmodulin-dependent kinase II (CAMKII) inhibitor KN-93. CGS-9343 β protected against cadmium-induced toxicity and attenuated ERK activation; STO-609 enhanced toxicity and exacerbated ERK activation, whereas KN-93 had no detectable effect on cadmium-induced toxicity. Furthermore, CGS-9343 β co-treatment attenuated cadmium-induced apoptosis; but CGS-9343 β did not recover cadmium-induced decrease in ALP activity. The major findings suggest the calmodulin-dependent PDE pathway facilitates cadmium-induced ERK activation leading to apoptosis, whereas the CAMKK pathway plays a protective role against cadmium-induced osteotoxicity via ERK signaling. This research distinguishes itself by identifying pleiotropic roles for CAMK pathways in mediating cadmium's toxicity in osteoblasts.

Keywords

Cadmium; Calmodulin; Osteoblast; Apoptosis

1. Introduction

Cadmium is a widespread environmental contaminant that poses a threat to human health (Zheng et al., 2008; Jarup and Akesson, 2009). Recent reports of cadmium in electronic waste (e.g., cell phones and computers) and inexpensive jewelry have increased public awareness regarding this toxic metal (Weidenhamer et al., 2011; Song and Li, 2015; Xu et

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al., 2015). Electronic waste is one of the fastest growing types of waste globally and due to the ongoing demand for cadmium in electronics, this metal continues to leach into the environment. Once in the environment, cadmium accumulates in exposed organisms because of its long biological half-life of 10–30 years (Jarup and Akesson, 2009).

Humans are exposed to cadmium primarily through ingesting contaminated food and water, smoking cigarettes, or occupational use (Jarup and Akesson 2009; Satarug et al., 2010). Recent reports of cadmium in children and adult jewelry highlight other possible routes of human exposure (Weidenhamer et al., 2011; Guney et al., 2014). Once in the body, cadmium can accumulate in bone of exposed humans and animals (Satarug et al., 2010; Trzcinka-Ochocka et al., 2010; Akesson et al., 2014). A noteworthy case of cadmium-induced bone disease occurred in Japan in the 1940s which led to an outbreak of Itai-Itai disease in women living in a cadmium-polluted region (Tsuchiya 1976). Over past decades, epidemiological research indicates that even chronic, low-level environmental exposure to cadmium leads to reduced bone mineral density, increased risk of bone fractures and osteoporosis in humans (Akesson et al., 2014) and these findings are confirmed in animal experiments (Regunathan et al., 2003; Brzoska et al., 2010; Brzoska, 2012). Collectively, these studies demonstrate that bone is a critical target of cadmium-induced toxicity. With increased life expectancy, the number of osteoporosis cases is expected to rise, making the need to understand the impact of cadmium on this disease germane.

There are two proposed mechanisms for how cadmium targets the skeleton. First, cadmium is able to indirectly disrupt bone function by damaging the kidneys leading to a secondary effect on bone (Kjellstrom 1992). Alternatively, cadmium can act directly on bone by stimulating bone resorption by osteoclasts or inhibiting bone formation by osteoblasts (Regunathan et al., 2003; Coonse et al., 2007; Chen et al., 2009; Smith et al., 2009; Arbon et al., 2012). In either case, disturbance in the normal pattern of bone remodeling can contribute to the pathogenesis of osteoporosis. We previously reported that cadmium induces apoptosis in human osteoblasts via extracellular signal-regulated protein kinase (ERK) activation, increasing oxidative stress, and activating caspase-3 (Conse et al., 2007; Smith et al., 2009; Arbon et al., 2012). Since apoptosis is an integral component of bone remodeling, disruption of the apoptotic signaling cascades in osteoblasts may contribute to net bone loss, leading to a bone disease state (Mollazadeh et al., 2015).

Regulation of osteoblast proliferation, differentiation, and apoptosis involves complex intracellular communication, which includes Ca^{+2} signaling (Zayzafoon 2006). A principal mediator of osteoblast Ca^{+2} intracellular signaling is the Ca^{+2} binding protein calmodulin (CaM). Upon binding to Ca^{+2} ions, CaM undergoes a conformational change allowing it to activate downstream effector proteins. Three such downstream effector proteins activated by the Ca^{+2} /CaM complex are calmodulin-dependent phosphodiesterase (calmodulin-dependent PDE), calmodulin-dependent kinase kinase (CAMKK) and calmodulin-dependent kinase II (CAMKII). Reports indicate calmodulin-dependent PDE, CAMKK, and CAMKII isoforms are expressed in osteoblasts (Wakabayashi et al., 2002; Zayzafoon et al., 2005; Cary et al., 2013). Several lines of *in vitro* evidence indicate Cd^{+2} , which has a similar ionic radius to Ca^{+2} , can also bind CaM influencing these downstream effector proteins (Chaos et al., 1984; Miles et al., 1989; Shoran and Barren, 2009). Specifically, a recent *in vitro* study using

osteoblasts derived from fetal rat calvarias, demonstrates that 1 to 5 μM cadmium treatment significantly increases intracellular Ca^{+2} leading to CaM activation and ultimately apoptotic death (Liu et al., 2014). Other studies specifically implicate the CAMKII pathway as being activated by cadmium exposure resulting in apoptosis in cultured mesangial and neuronal cells (Liu and Templeton, 2007; Chen et al., 2011). However the roles of the other two pathways, calmodulin-dependent PDE and CAMKK, in cadmium toxicity are under-investigated. Taken together, these studies provide evidence in support of the current research to further elucidate the pleiotropic roles of CAMK pathways in cadmium-induced osteotoxicity.

The activation of CAMK pathways can initiate a network of downstream intracellular cascades, including amidogen activated kinase (MAPK) pathways. Several studies identify the ERK signaling pathway, a member of the MAPK family, as a downstream target of CAMK signaling in multiple cell types, including osteoblasts (Nag et al., 2007; Cio et al., 2009; Chen et al., 2011; Banerjee et al., 2014). Traditionally, ERK is generally considered a cell proliferation pathway with an ability to protect cells against apoptosis (Martin et al., 2006; Thevenod and Lee, 2013). However, studies illustrate a dual role of ERK with reports of sustained ERK activation leading apoptotic signaling (Martin and Prognoses, 2010; Yuan et al., 2015). In human Saos-2 and rat osteoblasts, *in vitro* studies report cadmium exposure leads to prolonged ERK activation resulting in apoptotic death (Arbon et al., 2012; Shako et al., 2015), whereas inhibition of ERK can lead to cadmium-induced apoptosis in human MG-63 cells (Hun et al., 2015).

This research builds upon our previous reports (Coonse et al., 2007; Arbon et al., 2012) and others (Liu et al., 2014) by examining the pleiotropic roles of CAMK pathways in cadmium-induced osteotoxicity using Saos-2 and MG-63 human-derived osteoblast-like cells exposed to cadmium only or in combination with well-characterized CAMK pathway-specific inhibitors (Norman et al., 1987; Semi et al., 1991; Tourist et al., 2002). Ultimately, this research aims to elucidate the underlying mechanisms in which exposure to cadmium contributes to the pathogenesis of bone diseases.

2. Materials and methods

2.1. Cell culture

The human osteosarcoma cell lines Saos-2 and MG-63 were purchased from American Type Culture Collection (ATCC, Manassas, VA). Saos-2 cells were cultured in McCoy's 5A medium and MG-63 cells in Eagles MEM medium, each supplemented with 10% FBS (Atlanta Biological, Lawrenceville, GA) and 2 mM L-glutamine, 100 IU/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma-Aldrich, St. Louis, MO). Cells were cultured at 37 °C in air containing 5% CO_2 . For routine maintenance, medium was changed every 3–4 days and cells were subcultured weekly.

2.2. Cell treatment

Cells were plated at different densities depending on the assay. After 24 h, treatment was initiated with 0.1–10 μM CdCl_2 (Sigma-Aldrich, St. Louis, MO), 5 μM calmodulin-

dependent PDE inhibitor CGS-9343 β (Santa Cruz Biotechnology, CA, USA), 5 μ M or 10 μ M CAMKK inhibitor STO-609 (Tocris, Bristol, UK), 2.5 μ M CAMKII inhibitor KN-93 (Tocris, Bristol, UK), or a co-treatment of CdCl₂ and inhibitor for 24 or 48 h (ATCC, Manassas, VA). For the cytotoxicity studies controls received OPTI-MEM serum-free medium only and for inhibitor studies the controls received OPTI-MEM serum-free medium containing 0.01% or less DMSO. A cytotoxicity profile using the MTT assay determined that 5 μ M CGS-9343 β , 5 μ M or 10 μ M STO-609, and 2.5 μ M KN-93 were not cytotoxic and these concentrations were used for co-treatment experiments (data not shown). The CdCl₂ concentrations used are within the concentration range and exposure time reported in the literature (Pulido and Parrish, 2003; Liu and Templeton, 2007; Chen et al., 2011) and specifically for osteoblast cultures (Martineau et al., 2010; Arbon et al., 2012; Liu et al., 2014, 2016).

2.3. Cell viability assay

Cells were plated at a density of 3×10^4 cells/well for Saos-2 or 1×10^4 cells/well for MG-63 in a 96- well plate. After 48 h treatment, cells were washed with phosphate buffered saline and incubated at 37°C with 10 μ M/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide; ATCC, Manassas, VA) for 4 h. The conversion of tetrazolium salt MTT to a colored formazan by mitochondrial dehydrogenase was used to assess cell viability. After the supernatant was removed, 100 μ l of DMSO were added to each well and absorbance was read at 570 nm.

2.4. Western blot analysis

Cells were plated at 3×10^5 cells/well in a 6-well culture plate. After 24 h treatment, cells were lysed and protein concentration was determined using a Bradford assay (Bradford, 1976). Equivalent amounts of protein (20 μ g) were electrophoresed on a 12% SDS gel and transferred onto a PVDF membrane (Bio-Rad, CA, USA). Membranes were blocked in TTBS containing 5% nonfat dry milk for 1 h then incubated overnight at 4°C with primary antibodies (Santa Cruz Biotechnology, CA, USA) for phosphorylated ERK (pERK) or total ERK followed by 2 h incubation at room temperature with HRP antibody (Santa Cruz Biotechnology, CA, USA). Immunoreactive proteins were detected by exposing the membranes to Immuno-Star HRP chemiluminescent (Bio-Rad, CA, USA), visualized using Quantity One 1-D Analysis software, and quantified using Image J software.

2.5. Apoptosis assay

The APOPercentage dye (Biocolor, Carrickfergus, UK) was used to detect apoptosis. The dye is transported into an apoptotic cell during the translocation of phosphatidylserine from the inner leaflet to the outer leaflet of the cell membrane. Cells were plated at a density of 1×10^5 cells/well for Saos-2 or 8×10^4 cells/well for MG-63 in a 24-well plate. After 48 h treatment, cells were washed with OPTI-MEM serum-free medium, incubated for 10 minutes with the APOPercentage dye (1:300 dilution), washed with OPTI-MEM serum-free medium and visualized using a Nikon epifluorescence Eclipse E400 microscope. Digital images were captured using ImagePro software by media Cybergenetics (Silver Spring, MD). Approximately 100 cells were counted and scored as apoptotic negative or positive stained cells.

2.6. Alkaline phosphatase activity assay

Saos-2 cells were plated at a density of 4×10^5 cells/well in a 6-well plate. After 48 h treatment, cells were lysed using 1% Triton X-100 (Sigma–Aldrich, St. Louis, MO) followed by two freeze (-80°C)/ thaw (37°C) cycles and centrifugation at $12,000 \times g$ for 5 min. Total protein was determined with a Bradford assay (Bradford, 1976) and equivalent protein amounts were assayed from each sample. Assay conditions were optimized for substrate concentration, incubation and freeze/thaw time and cell density. Absorbance was read at 405 nm after 20 minute exposure to p-nitrophenyl phosphate substrate (Sigma–Aldrich, St. Louis, MO).

2.7. Statistical analysis

Results are expressed as mean $\pm$ SEM of at least three independent experiments. Data were analyzed using a one-way analysis of variance followed by a Student-Newman-Keuls for multiple comparisons or by a Student's t-test for comparison between two groups. A p-value of <0.05 was considered significant. * denotes significant difference compared to control. † denotes a significant difference compared to CdCl_2 treatment.

3. Results

3.1. Cadmium exposure decreases cell viability

A dose-dependent decrease in viability, measured using an MTT assay, was detected after a 48 h exposure to CdCl_2 in Saos-2 and MG-63 cells. The EC_{50} value was $5\ \mu\text{M}$ and $7\ \mu\text{M}$ for Saos-2 and MG-63 cells, respectively (Fig. 1A and 1B). A marked cytotoxic effect was observed with $10\ \mu\text{M}$ CdCl_2 treatment in both cell lines with less than 20% viable cells remaining at 48 h. Based on the results of this study and our previous work (Arbon et al., 2012), subsequent experiments were conducted using $5\ \mu\text{M}$ CdCl_2 .

3.2. Calmodulin-dependent PDE signaling facilitates whereas CAMKK signaling inhibits cadmium-induced osteotoxicity

In order to assess the involvement of three CAMK pathways in cadmium-induced osteotoxicity, cells were exposed to $5\ \mu\text{M}$ CdCl_2 or in combination with $5\ \mu\text{M}$ of the calmodulin-dependent PDE inhibitor CGS, $10\ \mu\text{M}$ the CAMKK inhibitor STO, or $2.5\ \mu\text{M}$ of the CAMKII inhibitor KN-93 for 48 h. The results of the CAMK pathway inhibitor studies were comparable in the two osteoblast cell lines (Fig. 2). Co-treatment with calmodulin-dependent PDE inhibitor CGS resulted in partial recovery from cadmium-induced toxicity (Fig. 2A and 2B), while co-treatment with CAMKK inhibitor STO exacerbated cadmium-induced toxicity (Fig. 2C and 2D). There was no significant detectable effect on viability when cells were co-treated with CdCl_2 and the CAMKII inhibitor KN-93 compared to CdCl_2 alone (Fig. 2E and 2F). These results demonstrate pleiotropic roles of CAMK pathways in cadmium-induced osteotoxicity. The calmodulin-dependent PDE pathway promotes cadmium toxicity whereas the CAMKK pathway protects against toxicity, since the opposite occurs in the presence of the pathway-specific inhibitors. Unlike in mesangial and neuronal cells (Liu and Templeton, 2007; Chen et al., 2011), this study did not detect involvement of the CAMKII pathway in cadmium's effect on osteoblast viability.

3.3. Calmodulin-dependent PDE signaling facilitates whereas CAMKK signaling inhibits cadmium-induced ERK activation

We previously reported that cadmium induces apoptosis in Saos-2 cells via ERK activation (Arbon et al., 2012). Possible upstream regulators of ERK signaling are the CAMK pathways (Nag et al., 2007; Ciao et al., 2009; Chen et al., 2011; Banerjee et al., 2014). Therefore, we next investigated whether the two CAMK inhibitors that altered cell viability in response to cadmium, calmodulin-dependent PDE inhibitor CGS and CAMKK inhibitor STO, function as upstream regulators of ERK. Treatment with 5 μ M CdCl₂ significantly increased pERK activation at 24 h in both cell lines (Fig. 3). Co-treatment with the calmodulin-dependent PDE inhibitor CGS resulted in recovery of cadmium-induced ERK activation to control level (Fig. 3A and 3B). In contrast, co-treatment with STO exacerbated cadmium-induced ERK activation (Fig. 3C and 3D). These results are consistent with the cell viability studies (Fig. 2) and reveal upstream pleiotropic roles for the CAMK pathways in cadmium-induced osteotoxicity. Cadmium-induced ERK activation is facilitated by calmodulin-dependent PDE signaling whereas cadmium-induced ERK activation is inhibited by CAMKK signaling.

3.4. Cadmium-induced apoptosis is mediated by the calmodulin-dependent PDE signaling pathway

Studies demonstrate a pro-apoptotic role for ERK in several cell types (Martin and Prognoses, 2010; Yuan et al., 2015). In Saos-2 osteoblasts, we previously reported that cadmium exposure leads to ERK activation and apoptotic death using several apoptotic markers including Annexin V staining, DNA fragmentation, *APOPercentage* dye staining, and caspase-3 activity (Coonse et al., 2007; Arbon et al., 2012). Since cell viability studies (Fig. 2) implicate the calmodulin-dependent PDE pathway in facilitating cadmium-induced osteotoxicity, we investigated whether the toxicity involves apoptotic death using the calmodulin-dependent PDE inhibitor CGS. Treatment with 5 μ M CdCl₂ significantly increased the percent of apoptotic cells compared to untreated controls in both cell lines (Fig. 4C), which was also evident when observing stained apoptotic (pink) cells (Fig. 4A and B). Co-treatment with the calmodulin-dependent PDE inhibitor CGS resulted in recovery from cadmium-induced apoptosis to a control level (Fig. 4C). These results demonstrate that cadmium-induced apoptosis is, at least in part, mediated by calmodulin-dependent PDE signaling since inhibition of the pathway results in protection.

3.5. Inhibition of the calmodulin-dependent PDE signaling pathway does not lead to recovery of cadmium-induced decrease in ALP activity

We previously reported that cadmium exposure leads to a decrease in the mRNA expression of the key osteoblast gene alkaline phosphatase (ALP) via ERK activation in Saos-2 cells (Arbon et al., 2012). Therefore, in this study we investigated whether the calmodulin-dependent PDE pathway mediates cadmium's effect on Saos-2 ALP by assessing enzymatic activity. Consistent with our previous findings, 5 μ M CdCl₂ exposure resulted in a significant decrease in ALP activity in Saos-2 cells (Fig. 5). However in contrast to cadmium-induced apoptosis (Fig. 4), we did not detect a recovery effect on ALP activity when cells were co-treated with CdCl₂ and the calmodulin-dependent PDE inhibitor CGS

(Fig. 5). In summary, our major findings are that the calmodulin-dependent PDE pathway facilitates cadmium-induced ERK activation leading to apoptosis. However this pathway does not appear to facilitate cadmium-induced decrease in ALP activity. In contrast, the CAMKK pathway plays a role in protecting against cadmium-induced osteotoxicity via ERK signaling. Taken together, these studies demonstrate pleiotropic roles for the CAMK pathways in mediating cadmium's toxicity in osteoblasts (Fig. 6).

4. Discussion

Cadmium has long been known to cause adverse effects on human health, including increased susceptibility to developing metabolic bone diseases such as osteoporosis (Satarug et al., 2010). Compromised bone health may result from cadmium disrupting normal calcium-signaling in bone-forming osteoblasts. Research indicates cadmium exposure can lead to a transient rise in intracellular Ca^{+2} ions in several cell types, including osteoblasts (Xie et al., 2010; Chen et al., 2011; Liu et al., 2014). Furthermore, osteoblasts can uptake Cd^{+2} ions via membrane-bound transporters, such as transient receptor potential channels (Levesque et al., 2008). Consequently it is possible that a combination of increased intracellular Cd^{+2} and Ca^{+2} ions can bind calmodulin (CaM) leading to activation or inhibition of Ca^{+2} /calmodulin dependent kinase (CAMK) pathways. This study examines pleiotropic roles of three CAMK pathways, calmodulin-dependent phosphodiesterase (calmodulin-dependent PDE), calmodulin-dependent kinase kinase (CAMKK) and calmodulin-dependent kinase II (CAMKII), in cadmium-induced osteotoxicity using Saos-2 and MG-63 human-derived osteoblast-like cells exposed to cadmium only or in combination with different well-characterized CAMK pathway-specific inhibitors (Norman et al., 1987; Semi et al., 1991; Tourist et al., 2002). The two tumor-derived osteoblast cell lines used in this study, MG-63 and Saos-2, are well characterized and exhibit similar, though not identical, phenotypes to normal human osteoblasts (Pautke et al., 2004; Czekanska et al., 2012). MG-63 and Saos-2 cell lines are routinely used to study apoptosis and utilize many of the pathways known to exist in normal osteoblasts such as caspases, MAPKs, GSK-3 β /NF- κ B, Wnt/ β -catenin pathways, and Bcl-2 family members (Arbon et al., 2012; Yin et al., 2013; Shangguan et al., 2014; Hun et al., 2015; Papa et al., 2015; Wang et al., 2015). Hence for mechanistic studies, these cell lines are a suitable model to study apoptosis pathways that also exist in osteoblasts *in vivo* (Bellido and Plotkin, 2011).

Treatment with the three CAMK pathway inhibitors results in inhibitor-specific responses to cadmium exposure. We demonstrate that exposure to cadmium significantly reduces the viability of Saos-2 and MG-63 osteoblasts. The addition of the calmodulin-dependent PDE pathway inhibitor CGS results in recovery whereas the addition of the CAMKK inhibitor STO exacerbates cadmium-induced decrease in cell viability. Co-treatment with the CAMKII inhibitor KN-93 results in no detectable effect on cell viability. We conclude that calmodulin-dependent PDE signaling facilitates cadmium-induced osteotoxicity whereas CAMKK signaling inhibits, since the reverse occurs in the presence of the respective pathway-specific inhibitor. Our results are consistent with other studies that report the presence of calmodulin-dependent PDE inhibitors CGS or W-7 recover loss of cell viability induced by exposure to 2–20 μM CdCl_2 (Powlin et al., 1997; Liu et al., 2014). Regarding the CAMKK pathway, this current study distinguishes itself by identifying a protective role for

this under investigated signaling pathway in cadmium-induced osteotoxicity. The third pathway studied, CAMKII, does not appear to play a critical role in cadmium toxicity in bone; however this may be dependent upon cell type and/or *in vitro* culture conditions. For example, cadmium exposure leads to activation of the CAMKII signaling pathway resulting in apoptosis in cultured mesangial and neuronal cells (Liu and Templeton 2007; Cio et al., 2009; Chen et al., 2011). These non-osseous cell studies elicited a response using 10 μ M KN-93, which was a cytotoxic concentration in our experiments (data not shown). Collectively these studies support pleiotropic roles of the CAMK pathways in mediating cadmium toxicity, which varies among cell types. We next investigated whether the two CAMK inhibitors that altered cell viability in response to cadmium, calmodulin-dependent PDE inhibitor CGS and CAMKK inhibitor STO, function as upstream regulators of ERK.

The activation of CAMK pathways can initiate a network of downstream intracellular cascades, including the ERK pathway, which is a member of the mitogen activated kinase (MAPK) family (Nag et al., 2007; Cio et al., 2009; Chen et al., 2011; Banerjee et al., 2014). We previously reported that cadmium exposure leads to prolonged ERK activation resulting in apoptotic death in Saos-2 osteoblasts (Arbon et al., 2012). This study extends our previous findings by demonstrating cadmium-induced ERK activation leads to apoptosis in another human osteoblast cell line, MG-63. In contrast, another study using MG-63 cells reports ERK inhibition leads to cadmium-induced apoptosis (Hun et al., 2015). We speculate that these opposite roles of ERK signaling in MG-63 cells in response to cadmium exposure may be related to the cadmium concentration used, which was substantially higher in the Hun et al., 2015 study at 30–60 μ M compared to 5 μ M in the current study.

Although reports indicate cadmium leads to ERK activation via CAMKII in non-osseous cells (Cio et al., 2009; Chen et al., 2011), this study is unique in that it focuses on two other CAMK pathways. Consistent with the cell viability experiments, this study demonstrates that cadmium-induced ERK activation is inhibited by CAMKK signaling, since the opposite occurs in the presence of the inhibitor STO. Additional research will be required to determine if CAMKK/ERK are integral mediators of growth and proliferation in osteoblasts. In support of this idea, CAMKK activates ERK leading to outgrowth of neurons (Schmitt et al., 2004) and cell cycle progression in MCF-7 breast cancer cells (Schmitt et al., 2010). In contrast to CAMKK, cadmium-induced ERK activation is facilitated by calmodulin-dependent PDE signaling in osteoblasts, which is a unique and unreported finding that warrants further study.

Evidence justifies our further investigation into the calmodulin-dependent PDE pathway as a facilitator of cadmium-induced osteotoxicity. It is known that Cd^{+2} ions can directly bind CaM leading to suppression or activation of the calmodulin-dependent PDE pathway that is dependent on intracellular Cd^{+2} and Ca^{+2} concentrations. In cell-free *in vitro* studies, at high concentrations (>50 μ M) cadmium binds CaM and inhibits PDE activity. At low concentrations (<50 μ M), similar to what is used in this study, cadmium binds CaM and activates PDE activity (Suzuki et al., 1985; Flik et al., 1987). Thus, we next examined whether the calmodulin-dependent PDE pathway mediates cadmium's effect the specific osteoblast endpoints of apoptosis and alkaline phosphatase (ALP) activity.

We previously reported that cadmium-induced apoptosis occurs via ERK activation in Saos-2 osteoblasts (Arbon et al., 2012). This study extends our prior work by demonstrating that cadmium-induced apoptosis is also, at least in part, facilitated by the calmodulin-dependent PDE signaling pathway. This work corroborates a study using primary rat osteoblasts whereby treatment with 10 μ M of the PDE inhibitor W-7 rescues cells from apoptosis induced by 2 μ M CdCl₂ exposure (Liu et al., 2014). Thus, the calmodulin-dependent PDE pathway appears to be part of the complex network of signaling pathways involved in cadmium-induced apoptosis in osteoblasts.

Another endpoint assessed in this study was ALP activity; a key marker of osteoblast differentiation and mineralization. We previously reported that cadmium exposure leads to a decrease in ALP mRNA expression via ERK activation in Saos-2 cells (Arbon et al., 2012). Consistent with our previous findings, this study shows that cadmium exposure significantly decreases ALP activity in Saos-2 cells. In contrast to our results on cadmium-induced apoptosis, we do not detect a recovery effect on ALP activity when cells are co-treated with cadmium and the calmodulin-dependent PDE inhibitor CGS. These results suggest that osteoblast ALP is regulated independent of calmodulin-dependent PDE signaling. A study using mouse MC3T3-E1 osteoblasts reports that the calmodulin-dependent PDE 1 inhibitor vinpocetine is unable to rescue cells from BMP-4 induced ALP activity (Wakabayashi et al., 2002). Similar to vinpocetine, CGS is a calmodulin-dependent PDE 1 inhibitor. Interestingly, the same study shows other PDE isoform inhibitors (PDE 2, 3 and 4) enhance BMP-4 induced ALP activity. Taken together, it is possible that other PDE isoforms are involved in cadmium's effect on osteoblast ALP activity.

Another potential role of the calmodulin-dependent PDE pathway in osteoblast function is in cell cycle regulation. For example, the calmodulin-dependent PDE pathway is reported to mediate cadmium's effect on inhibiting the cell cycle progression in human trophoblast cells (Powlin et al., 1997). It would also be interesting to examine cyclic nucleotide metabolism in osteoblasts exposed to cadmium. PDE isoforms are known to degrade intracellular cGMP and cAMP (Bischoff 2004), with calmodulin-dependent PDE 1 preferentially degrading cAMP (Norman et al., 1987). Studies using non-osseous cells report contradictory results. Rat RC2 Leydig cells cultured in 24.2 μ M CdCl₂ exhibit a decrease in intracellular cAMP after 24 h exposure, suggesting PDE may be activated (Zhang et al., 2011). In contrast, intracellular cAMP decreases in response to 25 μ M CdCl₂ in U937 human myeloid leukemia cells, although this was after only a 2 h exposure (Vilaboa et al., 1995). Thus, the complexity of calmodulin-dependent PDE signaling in bone will require further investigation.

In summary, this research distinguishes itself by identifying pleiotropic roles for the CAMK pathways in mediating cadmium's toxicity in human osteoblasts. The major findings suggests the calmodulin-dependent PDE pathway facilitates cadmium-induced ERK activation leading to apoptosis, whereas the CAMKK pathway plays a protective role against cadmium-induced osteotoxicity via ERK signaling. Collectively, our research adds to the current understanding of how cadmium interferes with osteoblast function and how it may contribute to the pathogenesis of bone disease.

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HIGHLIGHTS

- Identification of pleiotropic roles for CAMK pathways in osteoblast cadmium toxicity.
- Calmodulin-dependent PDE pathway facilitates cadmium-induced ERK leading to apoptosis.
- CAMKK plays a protective role against cadmium-induced toxicity via ERK signaling

Fig. 1. Effect of CdCl₂ on viability on Saos-2 (A) and MG-63 (B) cells. Cells were treated with 0.1–10 μM CdCl₂ in OPTI-MEM serum-free medium for 48 h. Controls received OPTI-MEM serum-free medium only. Cell viability was determined using the MTT assay. Results are expressed as percent cell viability. Each bar represents the mean ± SEM of at least 3 independent experiments. * denotes significant from control p < 0.05.

Fig. 2. Effect of CAMK inhibitors on cadmium-induced osteotoxicity. Saos-2 (A, C, E) or MG-63 (B, D, F) cells were treated alone with 5 μ M CdCl₂, 5 μ M CGS, 10 μ M STO, 2.5 μ M KN-93 or with CdCl₂ and inhibitor in co-treatment for 48 h. Controls received OPTI-MEM serum-free medium containing 0.01% or less DMSO. Cell viability was determined using the MTT assay. Results are expressed as percent cell viability. Each bar represents the mean $\pm$ SEM of at least 3 independent experiments. * denotes significant from control $p < 0.05$. † denotes significant from CdCl₂ only $p < 0.05$.

Fig. 3. Effect of the calmodulin-dependent PDE inhibitor CGS and CAMKK inhibitor STO on ERK activation. Relative density of pERK/ERK measured by densitometry at 24 h in Saos-2 (A, C) or MG-63 (B, D) cells treated alone with 5 μ M CdCl₂, 5 μ M CGS, 5 μ M STO, 2.5 μ M KN-93 or with CdCl₂ and inhibitor in co-treatment. Controls received OPTI-MEM serum-free medium containing 0.01% or less DMSO. Representative Western blots of pERK and ERK for Saos-2 (E, G) and MG-63 (F, H). Each bar represents the mean $\pm$ SEM of at least 3 independent experiments. * denotes significant from control $p < 0.05$. † denotes significant from CdCl₂ only $p < 0.05$.

Fig. 4.

Effect of the calmodulin-dependent PDE inhibitor CGS on cadmium-induced apoptosis. The *APOPercentage* dye was used to detect apoptosis. Representative images of pink (apoptotic) Saos-2 (A) or MG-63 (B) cells treated with 5 μ M CdCl₂, 5 μ M CGS, or CdCl₂ and inhibitor co-treatment at 48 h. Controls received OPTI-MEM serum-free medium containing 0.01% or less DMSO. Approximately 100 cells were counted and scored as apoptotic negative or positive stained cells (C) and expressed as a percentage of apoptotic cells in at least 3 independent experiments. * denotes significant from control $p < 0.05$. † denotes significant from CdCl₂ only $p < 0.05$.

Fig. 5. Effect of the calmodulin-dependent PDE inhibitor CGS on ALP activity in Saos-2 cells. Cells were treated with 5 μ M CdCl₂, 5 μ M CGS, or CdCl₂ and inhibitor co-treatment for 48 h. Controls received Opti-MEM serum-free medium containing 0.01% or less DMSO. Results are expressed as absorbance. Each bar represents the mean $\pm$ SEM of at least 3 independent experiments. * denotes significant from control $p < 0.05$.

Fig. 6. Proposed model of pleiotropic roles of Ca^{+2} /calmodulin-dependent (CAMK) pathways regulating cadmium toxicity in human osteoblast-like cells. The calmodulin-dependent PDE pathway facilitates cadmium-induced ERK activation leading to apoptosis, while having no detectable effect cadmium-induced ALP activity. In contrast, the CAMKK pathway plays a protective role against cadmium-induced osteotoxicity via ERK signaling. This study did not detect involvement of the CAMKII pathway in cadmium-induced osteotoxicity.