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Disruption of Pdia3 gene results in bone abnormality and affects 1α,25-dihydroxy-vitamin D_3-induced rapid activation of PKC☆

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ABSTRACT

1,25-Dihydroxy-vitamin D3 [1α,25$(OH)_2D_3$] is a critical regulator of bone development. Protein disulfide isomerase A3 (Pdia3) is a multifunctional protein that has been associated with rapid membrane-initiated signalling by 1α,25$(OH)_2D_3$ in several cell types. To identify the physiological roles of Pdia3 in skeletal development, we generated Pdia3-deficient mice. No homozygous mice were observed at birth, indicating that the targeted disruption of the Pdia3 gene resulted in embryonic lethality. Pdia3 deficiency also resulted in skeletal manifestations as revealed by μCT analysis of femurs from 15-week-old heterozygous mice. The $Pdia3^{+/-}$ mice had increased metaphyseal bone volume and trabeculae compared to $Pdia3^{+/+}$ mice. In contrast, the area and thickness of cortical bone at the femoral mid-diaphysis of $Pdia3^{+/+}$ mice significantly exceeded that of $Pdia3^{+/-}$ mice. *In vitro* studies in osteoblast-like MC3T3-E1 cells showed that silencing of Pdia3 abolished 1α,25$(OH)_2D_3$-induced rapid activation of protein kinase C (PKC) while overexpression of Pdia3 resulted in augmentation of PKC activity by 1α,25$(OH)_2D_3$. Taken together, these data indicated that Pdia3 plays a crucial role in 1α,25$(OH)_2D_3$-regulated bone formation and the Pdia3-PKC signalling pathway might be involved in this process.

1. Introduction

1,25-Dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] is a critical regulator of bone and cartilage development. Two receptors have been described: the classic nuclear steroid hormone vitamin D receptor (VDR) and a membrane-associated receptor, Pdia3, also referred to as 1,25D_3-membrane-associated rapid response steroid binding protein (1,25-MARRS), ERp60 and ERp57 [1].

Pdia3 is a member of the family of protein disulfide isomerases and has been shown to have multiple functions. It functions as a chaperonin, facilitating proper protein folding and reshuffling in the rough endoplasmic reticulum (ER); it participates in assembly of major histocompatibility complex (MHC) class I molecules; and it regulates ER stress and controls cell survival [2]. Furthermore, it has been demonstrated that Pdia3 is required for 1α,25$(OH)_2D_3$-activated protein kinase C-α (PKCα) signalling in a number of cell types [3–5]. However, its function in bone and cartilage physiology remains unknown.

Pdia3 is predominantly located in ER, but it has also been found in the cytosol and nuclei, in plasma membranes, and in extracellular matrix vesicles [6,7]. Our latest results showed that Pdia3 also translocates to lipid rafts/caveolae in both growth zone chondrocytes and osteoblasts with treatment with 1α,25$(OH)_2D_3$, where it forms a complex with caveolin-1 (unpublished data). Furthermore, our studies on caveolin-1 knockout mice indicate that caveolae are required for 1α,25$(OH)_2D_3$-induced rapid membrane responses and play important roles in regulating growth plate physiology and bone development [8,9]. These observations suggest the hypothesis that the presence of Pdia3 in caveolar microdomains is involved in the mechanism of 1α,25$(OH)_2D_3$ action.

In order to better understand the physiological roles of Pdia3 in 1α,25$(OH)_2D_3$-regulated skeletal development and the potential mechanism involved, we generated Pdia3-deficient mice to investigate the effects of disruption this gene on bone phenotype *in vivo*. We also tested the hypothesis that alteration of the expression of Pdia3 affects 1α,25$(OH)_2D_3$-induced rapid PKC activation *in vitro*.

2. Materials and methods

2.1. Animals

The murine embryonic stem cell line (RST613, BayGenomics, USA) with a gene-trap insertion in Intron 1 of the Pdia3 gene

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was used to generate a germ line deletion. Standard blastocyst injection was used to produce chimeric mice. Heterozygous germ line-targeted SV129/J mice were crossed with C57Bl/6 mice. Animals heterozygous for the Pdia3 mutation were viable and fertile. However, homozygous null animals were not observed after birth, indicating that targeted disruption of the Pdia3 gene results in embryonic lethality. Pdia3 heterozygous mice and their wild type littermates were produced by breeding of heterozygous animals. Animals had unlimited access to drinking water and were maintained on standard chow. All animal studies were performed with the approval of the IACUC of Emory University. To confirm that heterozygous mice expressed less Pdia3 mRNA than their wild type littermates, we assessed Pdia3 mRNA levels in their livers by real-time PCR.

2.2. μCT imaging and analysis

High-resolution μCT images of the left femur of 15-week-old Pdia3$^{+/+}$ and Pdia3$^{+/-}$ ($n=6$) mice were obtained using a Scanco VivaCT40 (Scanco Medical, Basserdorf, Switzerland) with a 12 μm voxel resolution. Specific regions of analysis included the distal femoral epiphysis, the distal femoral metaphysis, and the femur mid-diaphysis. Standard Scanco software was used to determine microarchitecture morphology [9]. Parameters derived at the femur epiphysis and metaphysis included tissue volume (mm^3), bone volume (mm^3), and bone volume/tissue volume (BV/TV %), as well as trabecular number (#/mm), thickness (mm), and spacing (mm). At the cortical femur mid-diaphysis, mean cortical area (mm^2) and cortical thickness were determined through the scanned volume of bone. Independent Student's *t*-tests were used to assess the differences between groups.

2.3. In vitro study

To silence Pdia3, mouse osteoblast-like MC3T3-E1 cells were transfected with lentivirus particles with five different sequences for Pdia3 or empty vectors (Sigma, USA). Cells were then selected with media containing 2.0 μg puromycin for two weeks. A stable transfected cell line (Sh-Pdia3) with 80% knock down efficiency was chosen for following experiments. For overexpression, MC3T3-E1 cells were transfected with plasmids coding Pdia3 or empty vectors (Origene, USA). Cells were then selected with media containing 550 μg G418 for two weeks. A stable transfected cell line (Ov-Pdia3) with a 100% over-expression rate was used in the following studies. The efficiency of silencing and overexpression at the mRNA level was determined by real-time PCR; western blots and immunofluorescence staining were used to assess protein levels.

MC3T3-E1 cells were plated at 10,000 cells/cm^2 in 24-well plates with full media (α-MEM supplemented with 10% fetal bovine serum [FBS] and 1% penicillin–streptomycin [P/S]). After 48 h, full media were changed to differentiation media (α-MEM supplemented with 10% FBS, 1% P/S and 1% vitamin C). 12 days after plating, media were replaced with vehicle or 10^{-8} M 1α,25(OH)$_2$D$_3$ in full media. PKC activity was then measured after a 9-min incubation as described previously [9]. Briefly, at harvest cell layers were washed twice with PBS and dissolved in RIPA (20 mM Tres-HCl, 150 mM NaCl, 5 mM disodium EDTA, 1%NP-40). PKC activity was measured using a commercial kit (RPN77, GE Healthcare,

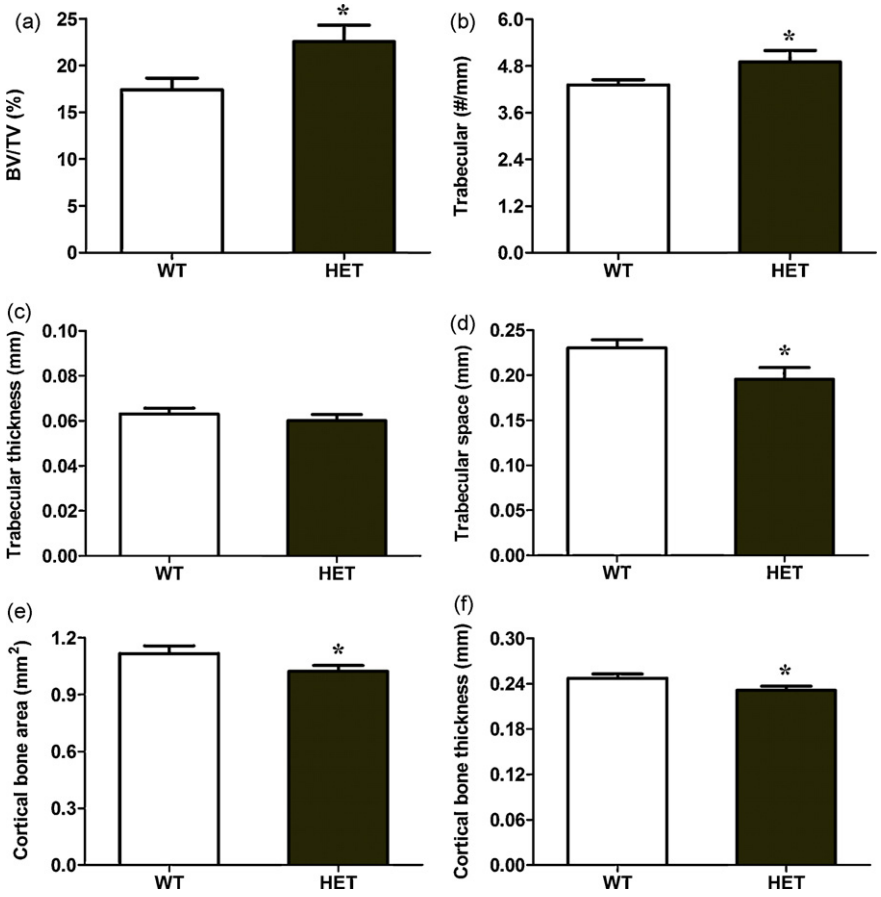

Fig. 1. μCT analysis of left femur of 15-week-old Pdia3 wild type (WT) and Pdia3 heterozygote (HET) mice. Trabecular BV/TV (a), trabecular number (b), thickness (c) and space (d) in the distal femoral metaphysis, cortical bone area (e) and thickness (f) in the femur mid-diaphysis were measured respectively. (6 mice per group, mean ± S.E.M, *$p<0.05$ vs. wild type).

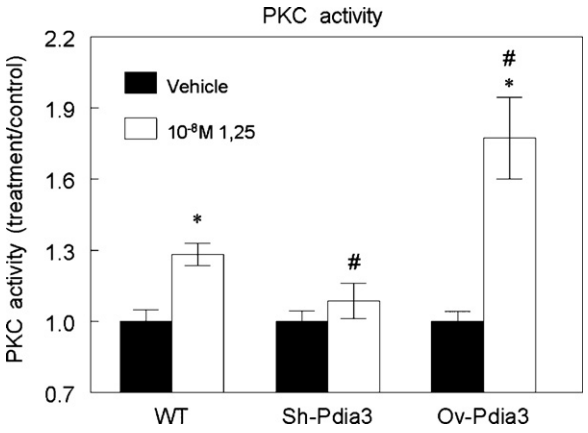

Fig. 2. Alteration of Pdia3 expression affects rapid activation of PKC by 1,25$(OH)_2D_3$ in MC3T3-E1 cells. Post-confluent wild type (WT), Sh-Pdia3 and Ov-Pdia3 MC3T3-E1 cells were treated with vehicle or 10^{-8} M of 1,25$(OH)_2D_3$. PKC activities were measured after for 9 min of incubation and normalized by total protein amount ($n = 6$ per group, mean ± S.E.M, $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. WT).

Piscataway, NJ) according to the manufacturer's instructions and normalized by total protein. One-way ANOVA with Bonferroni's post-test was performed to assess the differences between groups.

3. Results

Real-time PCR analysis of RNA isolated from liver showed that heterozygotes from 5-weeks-old to 30-weeks-old expressed 30-50% less Pdia3 mRNA compared to wild type animals at the same ages (data not shown). The effects of disruption of the Pdia3 gene on bone microarchitecture morphology were evaluated by μCT analysis (Fig. 1). At 15 wk, within the distal femoral metaphysis, $Pdia3^{+/-}$ mice showed significant increases in both BV/TV (+29.7%, $P = 0.027$) and trabecular thickness (+20.6%, $P = 0.046$) compared to $Pdia3^{+/+}$ mice with a corresponding significant decrease in trabecular space (−19.2%, $P = 0.034$). The trabecular thickness remained unchanged. However, within the distal femoral epiphysis, no significant difference in trabecular bone between $Pdia3^{+/+}$ mice and $Pida3^{+/-}$ mice was observed (data not shown). In contrast, at the femur mid-diaphysis, $Pdia3^{+/+}$ mice displayed slightly bigger cortical area (+8%, $P = 0.049$) and thickness (+5%, $P = 0.036$) than $Pdia3^{+/+}$ mice.

The effects of alteration of Pdia3 expression on rapid responses to 1α,25$(OH)_2D_3$ were examined in wild type, Sh-Pdia3 and Ov-Pdia3 MC3T3-E1 cells (Fig. 2). Treatment of wild type MC3T3-E1 cells with 1α,25$(OH)_2D_3$-induced significant increases in PKC activity. However, the rapid activation of PKC by 1α,25$(OH)_2D_3$ was abolished in Sh-Pdia3 cells. Moreover, 1α,25$(OH)_2D_3$ treatment resulted in greater augmentation of PKC activity in Ov-Pdia3 cells than in wild type cells ($P < 0.05$).

4. Discussion

Pdia3 knockout mice were first generated by Garbi et al. [10]. However, no detailed phenotypical changes were reported due to the lethality of null mice. In our study, no homozygous mice were observed at birth, confirming that the targeted ablation of the Pdia3 gene resulted in embryonic lethality. We then tested the assumption that Pdia3 deficiency could lead to skeletal manifestations in heterozygotes by μCT analysis of femurs from 15-week-old heterozygous and wild type mice. Our results showed that disruption of Pdia3 gene resulted in increases in the formation of trabecular bone in the distal femur metaphysis of heterozygotes with minor decreases in cortical bone in the femur mid-diaphysis. Our assessment of Pdia3 mRNA in the liver supports the conclusion that Pdia3 mRNA was reduced in heterozygotes, although we did not specifically measure Pdia3 expression in the femurs. With this caveat in mind, however, to our knowledge, this is the first report on the effects of disruption of Pdia3 gene on the skeletal phenotype.

Interestingly, a similar phenomenon was previously observed in caveolin-1 (Cav-1) knockout mice [9]; at the whole bone level, the deletion of Cav-1 results in increased bone formation. The fact that Pdia3 heterozygous mice shared a similar bone phenotype to caveolin-1 knockout mice supports our hypothesis that the Pdia3–caveolin-1 complex might be involved in the signalling pathway of 1α,25$(OH)_2D_3$-regulated bone development. Although our results demonstrated that Pdia3 disruption resulted in bone abnormality, it is still not clear that the exact mechanism by which Pdia3 exerts its control of the bone phenotype, in view of the fact that Pdia3-deficiency is not limited to bone cells. Thus, other systemic factors controlling skeletal development might also be affected and may have contributed to the bone phenotype. Therefore, it is necessary to generate a skeletal conditional Pdia3 knockout mouse, which will overcome the limitation of embryonic lethality and allow direct insights into how the bone phenotype was achieved.

Here we tested one possibility that Pdia3 regulates bone phenotype via 1α,25$(OH)_2D_3$ membrane-associated signalling pathway. 1α,25$(OH)_2D_3$-induced rapid membrane responses in growth plate chondrocytes are blocked by antibodies against Pdia3 [11], which indicates that Pdia3 might be involved in the membrane signalling pathways mediating 1α,25$(OH)_2D_3$ actions. In this study, the rapid activation of PKC by 1α,25$(OH)_2D_3$ in osteoblasts was completely abolished upon silencing of Pdia3, whereas it was amplified when Pdia3 was over-expressed. These results confirm that Pdia3 is required for 1α,25$(OH)_2D_3$-initiated rapid membrane signalling via PKC. Based on the hypothesis that 1α,25$(OH)_2D_3$ modulates skeletal development via rapid membrane signalling [12], it is possible that down-regulation of Pdia3 expression in $Pdia3^{+/-}$ mice leads to interrupted Pida3-mediated 1α,25$(OH)_2D_3$ signalling pathways and subsequent actions controlling skeletal development. Furthermore, the results from *in vitro* study also suggest that Pdia3 as a key molecule involved in 1α,25$(OH)_2D_3$-membrane signalling pathway could serve as a new drug target for controlling 1α,25$(OH)_2D_3$ action via modulating Pdia3 expression.

In conclusion, our results show that disruption of Pdia3 gene contributed to bone abnormality. 1α,25$(OH)_2D_3$-initiated Pdia3-PKC signalling pathway might be involved in this process. The precise mechanism by which Pdia3 exerts its function in skeletal development needs to be investigated in future.

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