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Estradiol determines the effects of PTH on ERα-dependent transcription in MC3T3-E1 cells

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ABSTRACT

Bone remodeling is a continuous process regulated by several hormones such as estrogens and parathyroid hormone (PTH). Here we investigated the influence of PTH on estrogen receptor alpha (ERα)-dependent transcriptional activity in MC3T3-E1 osteoblasts. Cells that were transfected with an ER-responsive reporter plasmid and treated with PTH showed increased luciferase activity. However, in the presence of 17β-estradiol, we observed that PTH inhibited ERα-mediated transcription. cAMP mimicked the effects by PTH, and the findings were confirmed in COS-1 cells transfected with expression vector encoding the catalytic subunit of cAMP-dependent protein kinase (PKA). Furthermore, PTH exhibited specific effects on the mRNA expression of the decoy receptor osteoprotegerin (OPG) and the receptor activator of NF kappa-B ligand (RANKL) in MC3T3-E1 osteoblasts. In the absence of 17β-estradiol, PTH and cAMP enhanced the OPG/RANKL ratio, whereas, OPG/RANKL was suppressed when estradiol was present. In conclusion, our results indicate that the presence of estradiol determines whether PTH and cAMP stimulates or inhibits ERα-dependent activity and the OPG/RANKL mRNA expression in an osteoblastic cell line.

1. Introduction

Bone metabolism is a finely tuned process where new bone formation mediated by osteoblasts is counterbalanced by bone resorption through osteoclasts. Disturbances in this process may lead to osteoporosis characterized by loss of bone mass and altered microarchitecture, resulting in an increased risk of fractures. PTH influences bone architecture by signaling through G protein-coupled receptors on osteoblasts leading to elevated intracellular levels of inositol-tri-phosphate (IP3) and cyclic adenosine monophosphate (cAMP), increasing the activity of PKA (reviewed in [1,2]). Persistent high levels of PTH, as seen in patients with primary hyperparathyroidism (PHPT), causes cortical bone loss, but tends to preserve trabecular bone, at least based on high-resolution quantitative CR scanning studies [3]. Intermittent treatment with high doses of PTH increases new bone formation, mainly in lumbar spine [4]. The cellular basis for the anabolic effect of PTH is not fully understood.

Estrogens have a bone-protective effect and estrogen deficiency is a major determinant of age-related bone loss [5]. Binding of estrogen to the estrogen receptors (ERα or ERβ) facilitates ER dimerization and interaction of the receptors with estrogen response elements (EREs) in the promoter of the ER target genes. Transcriptional coactivators, such as steroid receptors coactivators (SRCs) can be recruited to ER and stimulate gene transcription, either in an estrogen-dependent or -independent manner [6]. Both ERα and ERβ are important for normal development of the skeleton, though they seem to have different effects on bone growth and maturation [7].

One of the most thoroughly investigated signaling systems between osteoblasts and osteoclasts is the interaction between receptor activator of nuclear factor-kappaB ligand (RANKL) and its decoy receptor osteoprotegerin (OPG). Both RANKL and OPG are produced by osteoblasts. RANKL, a member of the tumor necrosis factor cytokine family, binds to the RANK-receptor on the osteoclast and acts as a key differentiation factor [8,9], while OPG inhibits the binding of RANKL to RANK [10]. RANKL and OPG are regulated by a number of hormones, including estrogen and PTH, having effects on either OPG or RANKL or both [11,12]. As OPG and RANKL have opposite effects on bone the ratio between

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OPG:RANKL might be a more useful measure to reflect the balance in bone metabolism than the evaluation of each of these two hormones separately (reviewed in [13]). Other cytokines, such as TNFα, IL6 and IL4 are also involved in bone metabolism and regulate osteoclastogenesis and osteoblast function [14].

The aim of this study was to investigate the influence of PTH on ERα-dependent transcription in bone cells. The PTH-mediated regulation of the ERα activity through cAMP/PKA signaling, as well as the effect of PTH on the OPG/RANKL-system in the presence or absence of 17β-estradiol was explored by using the mouse-derived osteoblast cell line MC3T3-E1.

2. Material and methods

2.1. Plasmids

The luciferase reporter plasmid ERE-TATA-luc and the expression plasmid pSG5-hERα were generously supplied by Dr. E. Treuter (Stockholm, Sweden). The pSG5-HA-GRIP1 expression plasmid encoding Steroid Receptor Coactivator 2 (SRC-2) was kindly provided by Dr. M.R. Stallcup (Los Angeles, CA, USA). The pCMV5-Cα expression plasmid encoding the catalytic subunit of PKA was provided by Dr. G.S. McKnight (Seattle, WA, USA).

2.2. Cell cultures

COS-1 African monkey kidney cells and the osteoblast cell line MC3T3-E1 subclone 4 (obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA)) were cultivated under standard conditions (37 °C, 5% CO_2) in Dulbecco's modified eagles medium (DMEM) (Invitrogen, Carlsbad, CA, USA) and α-minimum essential medium (α-MEM) (Invitrogen), respectively, supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin solution. Differentiation of MC3T3-E1 cells was performed for 14 days in α-MEM medium supplemented with 25 μg/ml ascorbic acid (Sigma–Aldrich) and 3 mM β-glycerophosphate (Sigma–Aldrich, St. Louis, MO, USA). MC3T3-E1 cells were not used after cell passage 25.

2.3. Transfection experiments

Two days before transfection cells were seeded in 24-well plates in phenol red-free DMEM or α-MEM supplemented with 10% (COS-1) or 2% (v/v) (MC3T3-E1) charcoal stripped FBS. Cells were transfected using SuperFect (QIAGEN, Valencia, CA, USA). Twenty-four hours after transfection MC3T3-E1 cells were stimulated with PTH (0.1 μM) (Sigma–Aldrich, St. Louis, MO, USA) or n6-benzoyl-cAMP (n6-bnz-cAMP) (150 μM) (Biolog Life Science Institute, Bremen, Germany) in combination with forskolin (10 μM) and 3-Isobutyl-1-methylxanthine (IBMX) (50 μM) to activate PKA. COS-1 cells were treated with 8-para-chlorophenylthio-cAMP (8-CPT-cAMP) (300 μM) (Biolog Life Science Institute) also in combination with forskolin (10 μM) and IBMX (50 μM). The cells were also treated with estradiol (E2) as indicated in the figure legends. Forty-eight hours after transfection cells were harvested and lysed in a buffer containing 25 mM Tris Acetate-EDTA (pH 7.8), 2 mM dithiothreitol, 1 mM EDTA, 10% (v/v) glycerol, and 1% (v/v) Triton X-100. ERα-dependent transcriptional activity was measured by luciferase activity, using the luciferase assay kit (BIOThema AM, Handen, Sweden).

2.4. qPCR analysis

Total RNA was isolated from MC3T3-E1 cells using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Concentrations of mRNA were estimated using Nanodrop (Saveen Werner, Malmö, Sweden). The Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany) was used for cDNA-synthesis followed by quantitative real time PCR (qPCR) analysis using the LightCycler480 SYBR Green I Master kit and the LightCycler480 rapid thermal cycler system (Roche Applied Science). Primers used were: *36B4*, 5′-aagcgcgtcctggcattgtct-3′ (forward) and 5′-ccgcaggggcagcagtggt-3′ (reverse); *IL6*, 5′-agttgccttcttgggactga-3′ (forward) and 5′-tccacgatttcccagagaac-3′ (reverse); *OPG*, 5′-ctgcctgggaagaagatcag-3′ (forward) and 5′-ttgtgaagctgtgcaggaac-3′ (reverse); *RANKL*, 5′caatgaatgccttgtgttgg-3′ (forward) and 5′-tttgttgggcatttgtctca-3′ (reverse). *BGLAP*, 5′-ttggtgcacactagcagac-3′ (forward) and 5′-accttattgccctcctgctt-3′ (reverse). Target gene mRNA expression levels were quantified relative to the housekeeping gene 36B4.

2.5. Statistical analysis

Variables are reported as mean ± standard deviation (SD). All experiments were performed in triplicate transfections and results from three representative experiments were used. Statistical analyses were performed using SPSS Statistics 19 for MAC (IMB Corporation, New York, NY, USA). Independent samples *t*-test was used for calculating statistical significance between groups. All tests were two-sided and $p < 0.05$ was considered significant.

3. Results

3.1. PTH stimulates ligand-independent activity of ERα

The exact role of PTH in regulation of ER-dependent processes in bone is still enigmatic. In order to investigate how PTH signaling modulates ERα-dependent transcriptional activity, undifferentiated MC3T3-E1 osteoblast cells were transfected with the ER-responsive luciferase reporter plasmid, ERE-TATA-luc and treated with PTH. Interestingly, we observed that PTH stimulated ERα-dependent transcriptional activity 2.5-fold in absence of the ligand 17β-estradiol (E2) ($p < 0.001$) (Fig. 1A). PTH is known to activate the cAMP/PKA signaling pathway [2]. Thus, MC3T3-E1 cells transfected with ERE-TATA-luc were also treated with the cAMP-analog, n6-bnz-cAMP, and the cAMP-elevating agents forskolin and IBMX (cAMP). Subsequent elevation of the luciferase activity (2.6-fold, $p = 0.005$) indicates that the increased ERα activity was mimicked by cAMP, suggesting that PTH stimulated ERα activity through the cAMP/PKA signaling pathway. Next, we examined the effects by PTH and cAMP/PKA on ERα-dependent transcriptional activity in MC3T3-E1 cell upon treatment with 17β-estradiol (E2). In contrast to the results in absence of ligand, PTH and cAMP both inhibited ER-dependent transcription after exposure to E2 (0.50-fold, $p = 0.018$, and 0.77-fold, $p < 0.001$, respectively) (Fig. 1B).

The ligand-dependent steroid receptor coactivators (SRCs) are crucial regulators of ER-dependent transcription. Since we have previously reported that cAMP/PKA regulates SRC-2 [15], we wanted to examine the potential role of this coactivator in MC3T3-E1 osteoblasts that were treated with PTH and cAMP. As expected, co-transfection of cells with a plasmid expressing SRC-2 (pSG5-HA-GRIP1) resulted in a marked increase in ERE-TATA-luc activity, both in the presence and absence of E2 (Fig. 1C and D compared to A and B). Similar to the results above, cells overexpressing SRC-2 demonstrated a stimulatory effect of PTH (3.3-fold, $p = 0.014$) and cAMP (2.6-fold, $p = 0.001$) in absence of E2 (Fig. 1C), whereas the presence of E2 resulted in a pronounced PTH- and cAMP-mediated inhibition of the ERα activity (0.67-fold, $p = 0.014$ and 0.34-fold, $p = 0.003$, for PTH and cAMP respectively) (Fig. 1D). The results imply that the effect of PTH on the ER-responsive luciferase reporter plasmid is not mediated through SRC-2. Together, these results

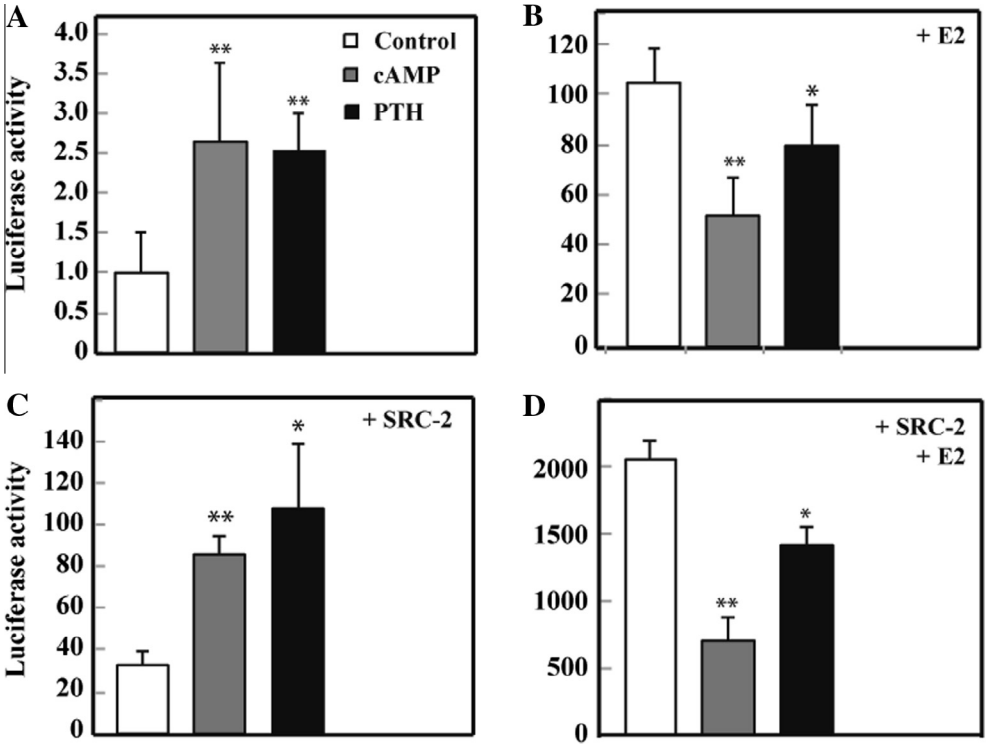

Fig. 1. PTH and cAMP regulates ERα activation in MC3T3-E1 osteoblasts. (A) and (B) MC3T3-E1 osteoblasts were transfected with the estrogen-responsive luciferase reporter plasmid, ERE-TATA-luc (0.6 μg). Twenty-four hours after transfection, cells were treated with PTH (0.1 μM) or n6-benzoyl-cAMP (150 μM), IBMX (50 μM) and forskolin (10 μM) (cAMP) without (A) or in combination with 17β-estradiol (0.1 μM) (B). (C) and (D) Cells were transfected with an expression plasmid encoding SRC-2 (pSG5-HA-GRIP1) in addition to the ERE-TATA-luc reporter plasmid and treated with PTH and cAMP, as described above in the absence (C) or in presence of 17β-estradiol (D). Forty-eight hours after transfection the cells were lysed and subjected to luciferase assays. The figures show the mean ± SD of triplicate transfections of three representative experiments. $^{*}p$-value < 0.05 and $^{**}p$-value < 0.005 vs. control cells.

indicate that the presence of E2 determines whether PTH stimulates or inhibits ERα-dependent transcriptional activation.

In order to confirm the results observed in MC3T3-E1 cells in another cell system, COS-1 cells were co-transfected with the ERE-TATA-luc reporter construct and an expression plasmid encoding ERα. The COS-1 cells were treated with 8-CPT-cAMP, forskolin and IBMX (cAMP), and similar to the MC3T3-E1 cells, cAMP stimulated ERα activity 8.56-fold in the absence of E2 ($p = 0.003$) (Fig. 2A). We also employed an expression plasmid encoding the catalytic subunit of PKA (PKA-Cα) to ensure that the cAMP-effect was mediated by PKA and not through the exchange proteins activated by cAMP (Epac), which enhanced the activity 6.5-fold ($p = 0.005$) (Fig. 2A). Of note, a 0.62-fold decrease in luciferase activity was observed in cells with overexpression of SRC-2 together with PKA-Cα ($p = 0.005$). Though, cAMP increased the luciferase activity 1.9-fold ($p = 0.002$) (Fig. 2C). In accordance with a previous report from our group [6], this may be caused by increased degradation of SRC-2 due to prolonged PKA-activation induced by PKA-Cα overexpression. After treatment of the COS-1 cells with E2, we observed that the ERα-dependent activity was inhibited by cAMP and PKA-Cα both in the absence of overexpressed SRC-2 (0.64-fold decrease, $p = 0.007$ when treated with cAMP, and 0.76-fold decrease, $p = 0.021$ when co-transfected with PKA-Cα), and in the presence of SRC-2 (0.75-fold, $p = 0.041$, and 0.41-fold, $p = 0.013$, when treated with PTH or co-transfected with PKA-Cα, respectively) (Fig. 2B and D).

3.2. Estradiol determines the effect of PTH on OPG/RANKL ratio in MC3T3-E1 osteoblasts

To further explore the regulatory role of PTH and cAMP on transcription of endogenous ER target genes in osteoblasts we analyzed alterations in the mRNA expression of osteoprotegerin (OPG) and the receptor activator of NF kappa-B ligand (RANKL) that plays a central roles in the intercellular signaling between osteoblasts and osteoclast. As shown in Fig. 3, undifferentiated MC3T3-E1 osteoblasts revealed elevation of the OPG/RANKL mRNA ratio when exposed to PTH (2.1-fold, $p = 0.046$) or cAMP (2.3-fold, $p = 0.048$) for 24 h. E2 treatment alone also increased the OPG/RANKL ratio ($p < 0.001$). In contrast, stimulation with PTH or cAMP in the presence of E2 resulted in a decreased OPG/RANKL ratio compared to treatment with E2 alone (0.79-fold, $p = 0.025$, and 0.36-fold, $p = 0.001$, respectively) (Fig. 3A), showing that PTH and cAMP lead to an elevation of the OPG/RANKL-ratio in an E2-independent manner.

As differentiated MC3T3-E1 osteoblasts express higher mRNA levels of bone-related proteins [16], we examined the expression of OPG/RANKL mRNA in differentiated osteoblast cells [17,18]. Differentiation of the cell line was confirmed by measuring an increased level of bone gamma-carboxyglutamic acid-containing protein (*BGLAP*) (osteocalcin) expression after 7 and 14 days of differentiation compared to undifferentiated MCT3T-E1 cells (Supplementary Fig. S1A). MC3T3-E1 cells differentiated for 14 days were treated with E2 for 1, 2, 4, and 24 h followed by analysis of OPG/RANKL mRNA expression. As shown in Supplementary Fig. S1B, the OPG/RANKL mRNA ratio increased, reaching a top at 4 h of E2 stimulation. Although, no significant effect by cAMP was observed, treatment of the cells with PTH for 4 h clearly increased the OPG/RANKL mRNA ratio (2.1-fold, $p = 0.003$). In contrast, both PTH (0.26-fold, $p < 0.001$) and cAMP (0.25-fold, $p = 0.001$) inhibited the E2-mediated elevation of OPG/RANKL ratio (Fig. 3B). Taken together, these results demonstrate that E2 also determines the effect of PTH on OPG/RANKL ratio in differentiated MC3T3-E1 osteoblasts.

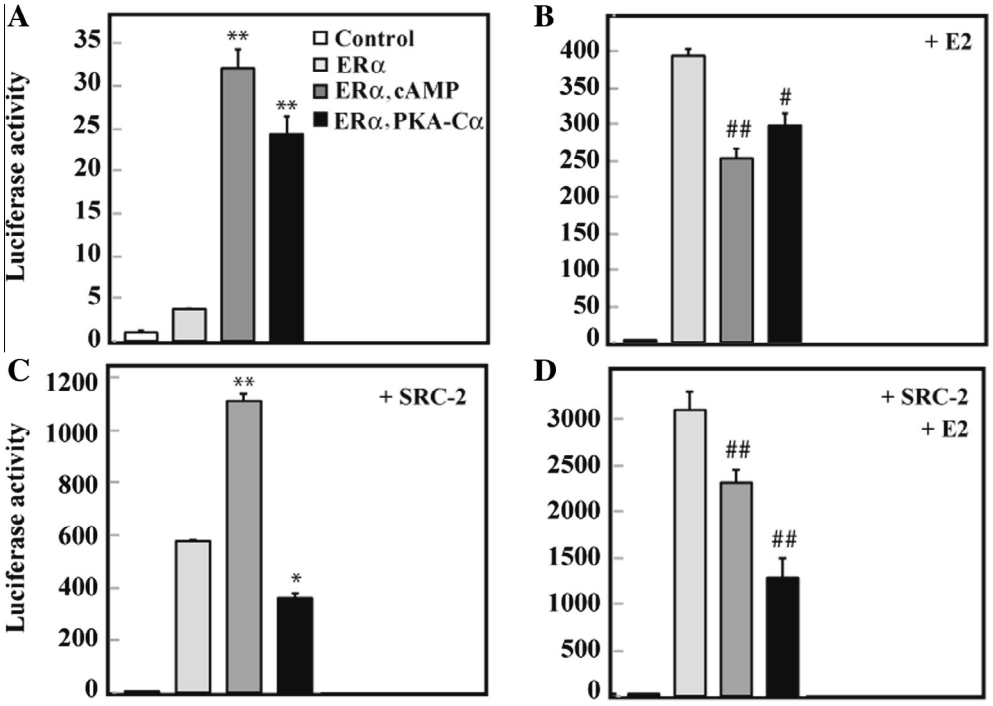

Fig. 2. cAMP/PKA signaling regulates ERα activation in COS-1 cells. (A) and (B) COS-1 cells were transfected with the estrogen-responsive luciferase reporter plasmid, ERE-TATA-luc (0.6 μg) along with an expression plasmid encoding ERα (0.05 μg) and PKA-Cα (0.1 μg). Twenty-four hours after transfection the cells were treated with 8-CPT-cAMP (300 μM), IBMX (50 μM) and forskolin (10 μM) (cAMP) in absence (A) or in presence of 17β-estradiol (10 nM) (B). (C) and (D) Cells were cotransfected with the expression plasmid encoding SRC-2 (pSG5-HA-GRIP1) and treated with cAMP as described above, in absence (C), or in presence of 17β-estradiol (D). $^{*}p$-value < 0.05 and $^{**}p$-value < 0.005 vs. cells transfected with an expression plasmid encoding ERα or ERα and SRC-2 without treatment, $^{\#}p$-value < 0.05 and $^{\#\#}p$-value < 0.005 vs. cells transfected with an expression plasmid encoding ERα or, ERα and SRC-2 treated only with E2.

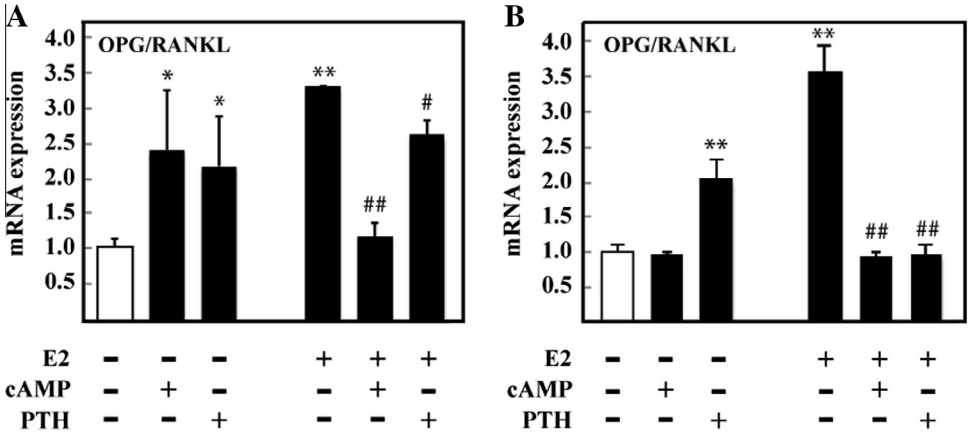

Fig. 3. PTH and cAMP stimulation in MC3T3-E1 cells regulate gene expression of OPG/RANKL. (A) Undifferentiated MC3T3-E1 cells were treated with PTH (0.1 μM) or n6-benzoyl-cAMP (150 μM), IBMX (50 μM) and forskolin (10 μM) (cAMP) for 24 h in combination with or without 17β-estradiol (0.1 μM). The expression of OPG/RANKL mRNA was measured using qPCR. The results present mean values ± SE obtained from three independent qPCRs. $^{*}p$-value < 0.05 and $^{**}p$-value < 0.005 vs. cells without treatment, $^{\#}p$-value < 0.05 and $^{\#\#}p$-value < 0.005 vs. cells treated only with E2. (B) Expression of OPG/RANKL mRNA in MC3T3-E1 cells differentiated for 14 days and treated with PTH (0.1 μM) or n6-benzoyl-cAMP (150 μM), IBMX (50 μM) and forskolin (10 μM) for 4 h in combination with or without 17β-estradiol (0.1 μM). The expression of OPG/RANKL mRNA was measured using qPCR. The results present mean values ± SE obtained from three independent qPCRs. $^{**}p$-value < 0.005 vs. cells without treatment, $^{\#\#}p$-value < 0.005 vs. cells treated only with E2.

3.3. PTH stimulates IL6 expression in MC3T3-E1 osteoblasts independently of E2

It has been reported that both E2 and PTH regulate interleukin 6 (IL6) in osteoblasts [17,18]. Thus, we wanted to examine the influence of PTH on the endogenous expression of IL6 in differentiated MC3T3-E1 cells in presence and absence of E2. As shown in Fig. 4, IL6 mRNA expression was significantly reduced in cells treated with E2 (0.51-fold, $p < 0.001$). Furthermore, we observed that cells exposed to PTH or cAMP for 4 h showed a similar increase in the expression of IL6 mRNA levels both in the absence of E2 as well as when E2 was present (all $p < 0.001$). Thus, in contrast to the regulation of OPG/RANKL reported above, it appears that PTH and cAMP stimulate IL6 expression independently of E2.

4. Discussion

In the present study we observed that PTH stimulated ligand-independent transcriptional activation of ERα, while in the presence

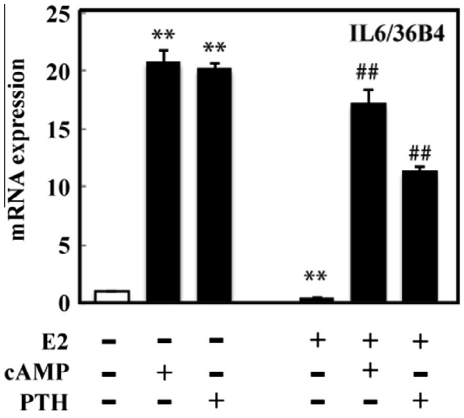

Fig. 4. Expression of IL6 in MC3T3-E1 cells is modulated by PTH in a specific manner. Expression of IL6 mRNA in MC3T3-E1 cells differentiated for 14 days and treated with PTH (0.1 μM) or n6-benzoyl-cAMP (150 μM), IBMX (50 μM) and forskolin (10 μM) for 4 h in combination with or without 17β-estradiol (0.1 μM). The expression of IL6 mRNA relative to the housekeeping gene 36B4 was measured using qPCR. The results present mean values ± SE obtained from three independent qPCRs. $^{**}p$-value < 0.005 vs. cells without treatment, $^{\#\#}p$-value < 0.005 vs. cells treated only with E2.

of 17β-estradiol PTH suppressed ERα activity. This was observed in both MC3T3-E1 osteoblasts and COS-1 cells. Our results suggest that PTH, acting through the cAMP/PKA-signaling pathway, induces activation of ERα in a ligand-independent manner. Previously, it has been reported that E2 modulates PTH-induced expression of IL6 in osteosarcoma cells [19] and fibronectin in osteoblast-like (Saos-2) cells [1]. However, to our knowledge, an effect of PTH-mediated signaling on ERα activity has not been demonstrated before (Supplementary Fig. S2). In transfected COS-1 cells, PKA is known to stimulate phosphorylation and activation of ERα in a ligand-independent manner [20]. PKA activation protects ERα from estrogen-induced degradation and mediates stabilization of ERα protein in GH_3 cells [21], and in breast cancer cell lines PKA has been shown to suppress E2-dependent activation of ERα through changes in ERα phosphorylation and reduced binding of E2 to ERα [22]. Although the interactions between E2-dependent and PKA-stimulated pathways are well characterized in different other cells, the physiological implications of these effects should be examined in more detail. Concerning the bone biology, it would be of particular interest to study whether PTH-mediated regulation of ERα in osteoblasts is modulated by menopausal status.

Here we also demonstrated that PTH enhanced the ratio between OPG and RANKL mRNA expression in the absence of E2, while PTH decreased the OPG/RANKL-ratio after exposure to E2. Previous reports have shown that E2 decreases RANKL levels, resulting in an increased ratio of OPG/RANKL [23], while PTH increased the RANKL mRNA-expression in osteoblasts [12]. The observed increase in the OPG/RANKL-ratio by E2 is in accordance with the bone-protective effect of estrogen [24], whereas exposure to PTH in the presence of E2 decreased the OPG/RANKL ratio, compared to exposure to E2 alone. PTH has dual effects on bone metabolism [25]. Continuous administration of PTH leads to bone resorption, whereas intermittent administration of PTH used in treatment of osteoporosis induces bone formation. Using MCF-7 cells, we have previously reported that the ER-coactivator SRC-2 was stimulated after short-term elevation of cAMP, whereas prolonged activation of the cAMP/PKA pathway reduced the recruitment of SRC-2 to an ER-responsive gene promoter [6]. Thus, it is tempting to speculate that the cross-talk between PTH/cAMP and ERα in osteoblasts could be partly mediated by SRC-2. However, in the present study overexpression of SRC-2 did not change the effects of PTH on the ERα-dependent transcriptional activity in MC3T3-E1 cells.

Several cytokines regulating bone metabolism, such as IL1, IL6, TNFα and macrophage colony stimulating factor (M-CSF), are influenced by estrogen [26,27]. We observed a decrease in mRNA levels of IL6 in MC3T3-E1 osteoblasts after treatment with E2 and an increase in IL6 mRNA levels after PTH-treatment. Previously, others have described similar effects of E2 and PTH on the release of IL6 [17,18,28]. The increase of IL6 upon stimulation by PTH and cAMP was independent of the presence of E2, indicating that other mechanisms may be involved in the regulation of IL6 as compared to OPG/RANKL expression.

In conclusion, we observed that in osteoblasts PTH modulates ERα activity in a dual manner depending on the presence or absence of E2. Both PTH and E2 increased the OPG/RANKL-ratio, but the presence of E2 resulted in inhibitory effects by PTH. Further studies should be performed to assess the biological implications of the interaction between PTH and ERα in more details.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.109.

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