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Biochemical and Biophysical Research Communications 306 (2003) 144–150
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Parathyroid hormone induces the nuclear orphan receptor NOR-1 in osteoblasts

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Received 22 April 2003

Abstract

Parathyroid hormone (PTH) significantly affects osteoblast function by altering gene expression. We have identified neuron-derived orphan receptor-1 (NOR-1) as a PTH-induced primary gene in osteoblastic cells. NOR-1, Nurr1, and Nur77 comprise the NGFI-B nuclear orphan receptor family and Nurr1 and Nur77 are PTH-induced primary osteoblastic genes. Ten nM PTH maximally induced NOR-1 mRNA at 2 h in primary mouse osteoblasts and at 1 h in mouse calvariae. Cycloheximide pretreatment did not inhibit PTH-induced NOR-1 mRNA. PTH activates cAMP-protein kinase A (PKA), protein kinase C (PKC), and calcium signaling. Forskolin (PKA activator) and PMA (PKC activator) mimicked PTH-induced NOR-1 mRNA. Ionomycin (calcium ionophore) and PTH(3–34), which do not activate PKA, failed to induce NOR-1 mRNA. PKA inhibition with H89 blocked PTH- and FSK-induced NOR-1 mRNA. PMA pretreatment to deplete PKC inhibited PMA-induced, but not PTH-induced, NOR-1 mRNA. We conclude that NOR-1 is a PTH-regulated primary osteoblastic gene that is induced mainly through cAMP-PKA signaling.

Keywords: PTH; NOR-1; Osteoblast; Orphan nuclear receptor; NGFI-B

Parathyroid hormone (PTH) significantly affects bone metabolism [1–5], primarily through PTHR1, a G protein-coupled receptor found on osteoblasts that also binds PTH-related peptide (PTHrP). PTHR1 is coupled to cAMP-protein kinase A (PKA), protein kinase C (PKC), and calcium signaling pathways [6]. Receptor-bound PTH triggers these pathways to activate a cascade of gene expression beginning with primary genes, some of which regulate downstream gene transcription. We hypothesize that PTH-induced primary genes are critical determinants of osteoblast function and toward this end we have performed representational difference analysis (RDA) to identify PTH-induced primary genes in osteoblasts [7]. We report here that among the genes identified by RDA was neuron-derived orphan receptor-1 (NOR-1).

NOR-1 is a member of the nerve growth factor-induced gene-B (NGFI-B) family of nuclear orphan receptors that includes Nur77 [8] and Nurr1 [9]; for review see [10]. NGFI-B genes have 90% homology in their DNA binding domain, 20–30% in their N-terminal transactivation domain, and 60–65% in their C-terminal domain. Functionally, NGFI-B proteins are transcription factors involved in neuroendocrine regulation, neuronal differentiation, liver regeneration, and T-cell apoptosis [8,11–16]. NOR-1, Nur77, and Nurr1 form homodimers and heterodimers with each other [17]. Nur77 and Nurr1 also heterodimerize with the 9-*cis*-retinoic acid receptor, RXR, which has been called a ''master regulator'' for its ability to affect multiple nuclear receptors through heterodimeric pairing [18]. This is important to bone biology because steroid nuclear receptors, especially the vitamin D receptor, affect osteoblast function (for review see [19]). Interestingly, NOR-1 does not heterodimerize with RXR [20].

Nurr1 [7] and Nur77 [21] are PTH-induced primary genes in osteoblasts. We report here that the third NGFI-B family member, NOR-1, is also a PTH-regulated primary gene in osteoblasts that is induced mainly through cAMP-PKA signaling.

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doi:10.1016/S0006-291X(03)00931-8

Materials and methods

Reagents and cell culture. Reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified. Bovine PTH was used in all experiments. Calvariae and calvariae-derived osteoblasts (MOB) were extracted from 7-day-old CD-1 mice [7].

RNA extraction and Northern blot analysis. Total RNA was extracted, fractionated in 1% agarose gel containing 3.7% formaldehyde, transferred to Gene Screen Plus hybridization membrane (NEN Life Science Products, Boston, MA), and subjected to Northern blot analysis utilizing a purified NOR-1 or GAPDH PCR fragment as probes. 18S and 28S ribosomal RNA were visualized with ethidium bromide (EtBr) staining. For each experiment, one representative northern blot is shown.

Statistics. Experiments were conducted at least three times. Comparisons between control and treated groups were performed using the Student's t test. p values less than 0.05 were considered statistically significant.

Results

PTH-induced NOR-1 mRNA are time- and dose-dependent in osteoblastic cells

To identify PTH-induced primary genes in osteoblasts, we performed representational difference analysis (RDA; [7]) on RNA from confluent osteoblastic MC3T3-E1 cells pretreated with 10 μg/ml CHX for 1 h followed by vehicle (C) or 10 nM PTH for 1.5 h (Fig. 1A). Sequence analysis of the RDA-generated DNA fragment matched the nuclear orphan receptor neuron-derived orphan receptor-1 (NOR-1).

While MC3T3-E1 cells are a widely used and accepted osteoblast model [22], we conducted the remainder of our studies on primary mouse osteoblasts (MOB cells) because of their greater biological relevance. PTH-induced NOR-1 mRNA was significantly increased from 1 to 4 h of treatment with peak induction at 2 h (Figs. 1B and C). In addition, NOR-1 mRNA levels were significantly induced by 0.001–10 nM PTH for 2 h (Fig. 2A), with 0.02 nM PTH producing half maximum NOR-1 mRNA induction (Fig. 2B).

PTH-induced NOR-1 gene expression in the absence of de novo protein synthesis in osteoblasts

We identified NOR-1 as a PTH-induced primary gene in the MC3T3-E1 cell line. To verify this finding in normal osteoblasts, we pretreated MOB cells with 3 μg/ml CHX for 30 min followed by treatment with 10 nM PTH for 0–4 h. CHX pretreatment did not inhibit, but sustained, PTH-induced NOR-1 mRNA (Fig. 3).

PKA and PKC activators significantly induced NOR-1 mRNA in MOB cells

PTH binds to PTHR1 on the plasma membrane of osteoblasts and activates the cAMP-PKA, PKC, and calcium signaling pathways [6]. To determine which PTHR1-coupled signaling pathway(s) mediates NOR-1 mRNA induction, MOB cells were treated with selected signaling agonists and antagonists. Both 10 μM FSK and 1 μM PMA, which activate the PKA and PKC pathways, respectively, significantly increased NOR-1 mRNA (Fig. 4). Conversely, 1 μM iono, a calcium ionophore, did not increase NOR-1 mRNA.

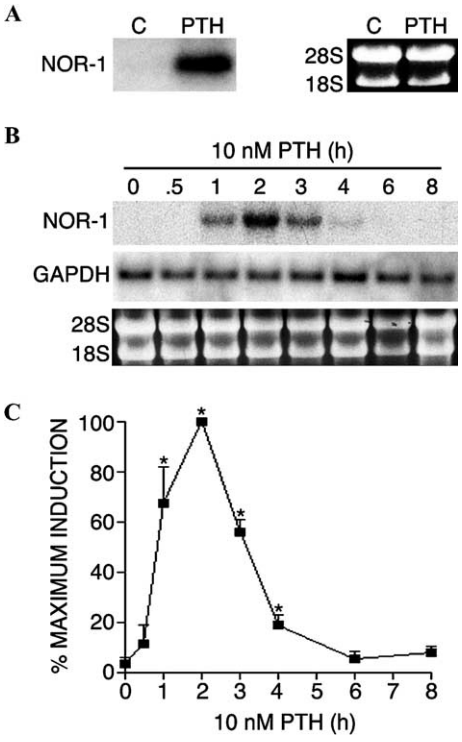

Fig. 1. PTH rapidly and transiently induced NOR-1 mRNA in osteoblastic cells. (A) MC3T3-E1 cells were pretreated with 10 μg/ml CHX for 1 h followed by vehicle (C) or 10 nM PTH for 1.5 h. Northern blot analysis of NOR-1 mRNA (left panel) and ethidium bromide staining of 28S and 18S ribosomal RNA (right panel). (B) MOB cells were treated with 10 nM PTH for 0–8 h. Northern blot analysis of NOR-1 (upper panel) and GAPDH (middle panel) mRNA. EtBr staining of 28S and 18S rRNA (lower panel). (C) Quantitation of NOR-1 mRNA in MOB cells. $*p < 0.05$ compared to vehicle-treated cells (0).

Blocking PKA, but not PKC, signaling inhibited PTH-induced NOR-1 mRNA

Because PKA and PKC pathways induced NOR-1 mRNA, we next tested selective PKA and PKC pathway antagonists for their ability to block NOR-1 mRNA induction. MOB cells pretreated with the PKA antagonist H89 (30 μM) significantly inhibited both 10 nM PTH- and 10 μM FSK-induced NOR-1 mRNA (Fig. 5). MOB cells pretreated overnight with 1 μM PMA to deplete PKC showed significant reduction of 1 μM PMA-, but not 10 nM PTH-, induced NOR-1 mRNA (Fig. 6). Finally, 1–100 nM PTH(3–34), a PTH analog that activates PKC and calcium, but not PKA, signaling, did not induce NOR-1 mRNA (Fig. 7).

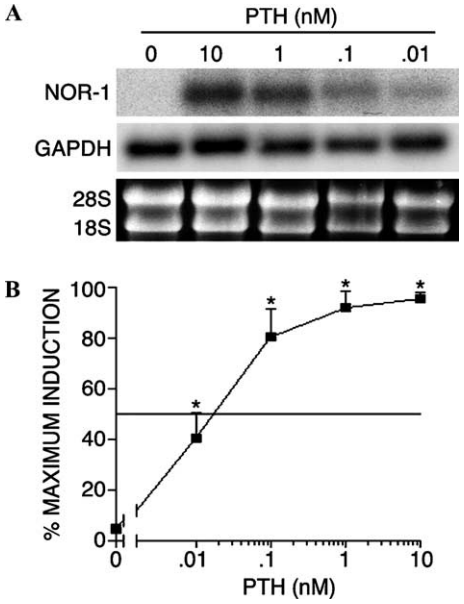

Fig. 2. PTH-induced NOR-1 mRNA in MOB cells is dose-dependent. MOB cells were treated with 0–10 nM PTH for 2 h. (A) Northern blot analysis of NOR-1 (upper panel) and GAPDH (middle panel) mRNA. EtBr staining of 28S and 18S rRNA (lower panel). (B) Quantitation of NOR-1 mRNA. $*p < 0.05$ compared to vehicle-treated cells (0).

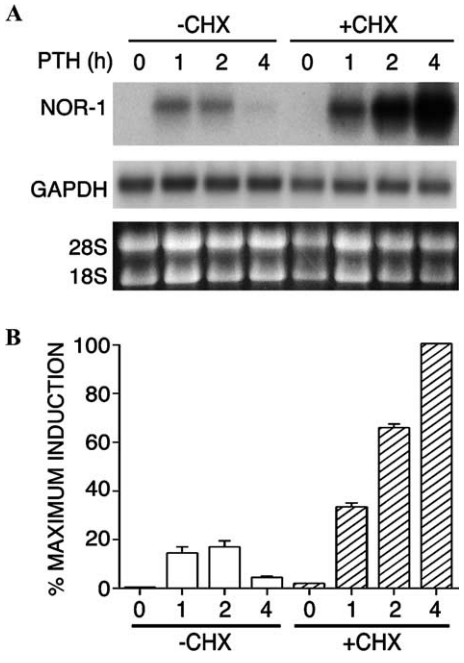

Fig. 3. PTH-induced NOR-1 mRNA in MOB cells does not require new protein synthesis. (A) MOB cells were pretreated with 3 μg/ml CHX for 1 h followed by 10 nM PTH for 1–2 h. Northern blot analysis of NOR-1 (upper panel) and GAPDH (middle panel) mRNA. EtBr staining of 28S and 18S rRNA (lower panel). (B) Quantitation of NOR-1 mRNA. $*p < 0.05$ compared to vehicle-treated cells (0).

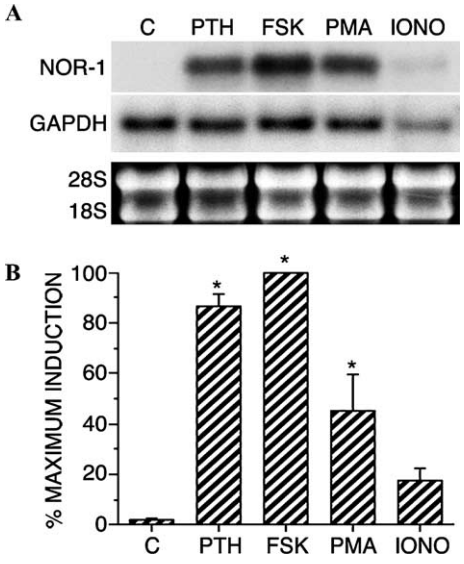

Fig. 4. PKA and PKC agonists induced NOR-1 mRNA. MOB cells were treated with vehicle (C), 10 nM PTH, 10 μM FSK, and 1 μM PMA of 1 μM iono for 2 h. (A) Northern blot analysis or NOR-1 (upper panel) and GAPDH (middle panel) mRNA. EtBr staining of 28S and 18S rRNA (lower panel). (B) Quantitation of NOR-1 mRNA. $*p < 0.05$ compared to control cells (C).

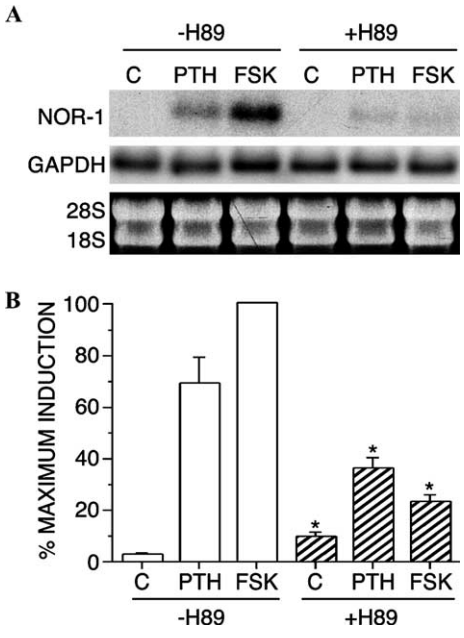

Fig. 5. PKA inhibition blocked PTH- and FSK-induced NOR-1 mRNA. MOB cells were pretreated with vehicle (−H89) or 30 μM H89 (+H89) for 0.5 h followed by vehicle (C), 10 nM PTH or 10 μM FSK for 2 h. (A) Northern blot analysis of NOR-1 (upper panel) and GAPDH (middle panel) mRNA. EtBr staining of 28S and 18S rRNA (lower panel). (B) Quantitation of NOR-1 mRNA. $*p < 0.05$ compared to cell pretreated with vehicle (−H89).

PTH-induced NOR-1 mRNA in neonatal mouse calvariae

To confirm that PTH-induced NOR-1 mRNA in MC3T3-E1 and MOB cells are valid models for osteoblasts in situ, calvariae from 6–8-day-old mice were cultured with 10 nM PTH for 0–6 h (Fig. 8). PTH-induced NOR-1 mRNA levels peaked at 1 h and declined thereafter.

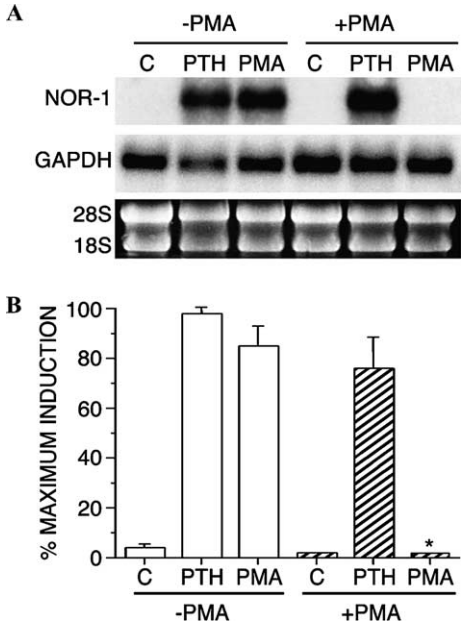

Fig. 6. PKC inhibition reduced PMA-, but not PTH-, induced NOR-1 mRNA. MOB cells were pretreated with vehicle (−PMA) or 10 μM PMA (+PMA) overnight followed by vehicle (C), 10 nM PTH or 1 μM PMA for 2 h. (A) Northern blot analysis of NOR-1 (upper panel) and GAPDH (middle panel) mRNA. EtBr staining of 28S and 18S rRNA (lower panel). (B) Quantitation of NOR-1 mRNA. $*p < 0.05$ compared to cells pretreated with vehicle (−PMA).

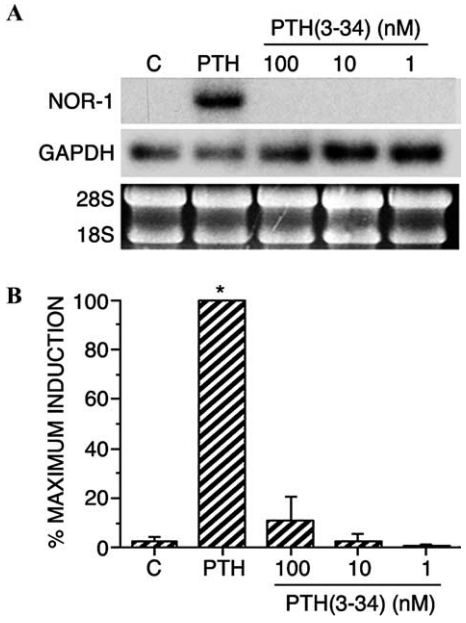

Fig. 7. PTH(3–34) did not induce NOR-1 mRNA. MOB cells were treated with vehicle (C), 10 nM PTH(1–34) or 1–100 nM PTH(3–34) for 2 h. (A) Northern blot analysis of NOR-1 (upper panel) and GAPDH (middle panel) mRNA. EtBr staining of 28S and 18S rRNA (lower panel). (B) Quantitation of NOR-1 mRNA. $*p < 0.05$ compared to control cells (C).

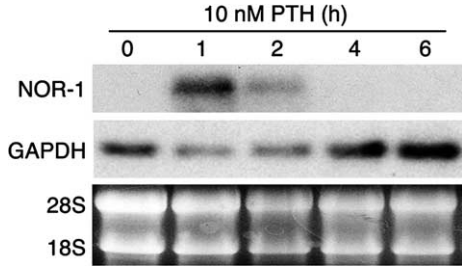

Fig. 8. PTH-induced NOR-1 mRNA in cultured calvariae. Neonatal mouse calvariae were cultured in 10 nM PTH for 0–6 h. Northern blot analysis of NOR-1 (upper panel) and GAPDH (middle panel) mRNA. EtBr staining showing 28S and 18S rRNA (lower panel).

Discussion

We report here that NOR-1, like the two other NGFI-B family members Nurr1 [7] and Nur77 [21], is a PTH-induced primary gene in osteoblasts that is induced primarily through cAMP-PKA signaling. There are slightly different temporal patterns of NOR-1, Nurr1, and Nur77 mRNA induction in MOB cells, with peak PTH-induced NOR-1 (Figs. 1B and C) and Nurr1 [7] mRNA at 2 h, while Nur77 mRNA [21] peaks at 1 h. The temporal patterns of PTH-induced NOR-1, Nurr1, and Nur77 mRNA in neonatal mouse calvariae were identical with peak induction at 1 h (Fig. 8; [7,21]). All three genes were maximally responsive to 10 nM PTH (Fig. 3; [7,21]). However, NOR-1 induction was more sensitive to PTH treatment than Nurr1. Half-maximum induction of NOR-1 required only 0.02 nM PTH (Fig. 3B), while the half-maximum dose for Nurr1 induction was 0.1 nM PTH [7]. In addition, de novo protein synthesis is not required for NOR-1 (Fig. 3), Nurr1 [7] or Nur77 [21] induction by PTH. Taken together these data for NGFI-B genes are consistent with the rapid and transient pattern of other PTH-induced primary genes that are transcription factors, including c-fos and c-jun [23] and ICER [24].

The cAMP-PKA, PKC, and calcium pathways are all coupled to PTHR1, the G protein-coupled receptor that transduces PTH and PTH-related peptide (PTHrP) signaling. Using selective pathway activators and inhibitors, we found that cAMP-PKA and PKC activation significantly induced NOR-1 mRNA (Fig. 4), suggesting that both of these pathways may contribute to PTH-induced NOR-1 mRNA expression. However, only cAMP-PKA inhibition had an effect on PTH-induced NOR-1 mRNA (Figs. 5 and 6). Interestingly, PKC depletion significantly inhibited PMA-induced NOR-1 mRNA, but did not affect PTH-induced NOR-1 mRNA (Fig. 6). While PKC activation can induce NOR-1 mRNA in MOB cells, the PTH response does not rely on PKC signaling. Instead, PTH-induced NOR-1 is mediated primarily by cAMP-PKA signaling.

NOR-1 and Nurr1 are similar in their dependence upon the cAMP-PKA pathway for PTH-mediated

induction (Figs. 4 and 5; [7]). This is anticipated, given that the NOR-1 promoter has three cAMP response elements (CREs; [25]) and the Nurr1 promoter has one consensus CRE [26,27]. However, NOR-1 and Nurr1 differ in their response to PKC activation. PMA treatment to activate PKC significantly induced NOR-1 mRNA in MOB cells (Figs. 4 and 6), while Nurr1 mRNA are unresponsive to PKC activation [7]. Interestingly, in other cell types, NGFI-B family members are variably responsive to cAMP-PKA and PKC activation. Nurr1 is induced by both FSK and TPA in NB-OK-1 human neuroblastoma cells, while NOR-1 and Nur77 are induced only by FSK [28]. In addition, NOR-1, Nurr1, and Nur77 are induced in NTera2 teratocarcinoma-derived neurons by both dibutyryl cAMP and PMA [29]. Cell context appears to be a critical determinant of which pathways mediate NOR-1, Nurr1, and Nur77 induction.

We are intrigued by the identification of NOR-1, Nurr1, and Nur77 as PTH-induced primary genes in osteoblasts. As nuclear orphan receptors, NGFI-B genes act as transcription factors, which suggests a role in regulating PTH-induced secondary genes and subsequent osteoblast function. NGFI-B proteins activate transcription as monomer, homodimers, or NGFI-B heterodimers [17]. Nur77 and Nurr1 also heterodimerize with the retinoid X-receptor, RXR [30]. No known ligand is required for activation of NGFI-B monomers, homodimers, or heterodimers. But, NGFI-B receptor-RXR heterodimers require 9-*cis*-retinoic acid receptor-mediated transcription. Interestingly, RXR is an important mediator of skeletal development and of osteoblast function, especially when heterodimerized with the vitamin D receptor (VDR; [31,32]).

An interesting aspect of VDR-mediated osteoblast regulation is that when its ligand, 1,25-dihydroxyvitamin D_3, and PTH are given together, osteoblast function is significantly altered. There is increased bone resorption [33], decreased expression of PTHR1 [34], and increased expression of VDR, CYP24, and osteocalcin [35–37]. Given that PTH induces all three NGFI-B genes (which, in the case of Nurr1 and Nur77, heterodimerize with RXR) and VDR [38–41], and that VDR-RXR heterodimers affect osteoblast function [31,32], it is plausible that PTH-induced NGFI-B orphan receptors may modulate the PTH-vitamin D effects through competition between NGFI-B proteins and VDR for RXR binding.

While loss of NOR-1 expression leads to aberrant semicircular canal development in the inner ear [42], NOR-1 gene translocation leading to significantly increased NOR-1-mediated transcriptional activation is associated with extraskeletal myxoid chondrosarcomas. Three known translocations place the entire NOR-1 protein downstream of either the Ewing's sarcoma protooncogene (EWS) N-terminal domain [43,44], the EWS-related protein RBP56/TAF2N/TAF68 [45,46] or the basic helix-loop-helix transcription factor TCF12 [47]. These configurations generate NOR-1-mediated transactivation through the NGFI-B response element (NBRE) that, at least for the EWS-NOR1 chimera, is approximately 270 times higher than wild type NOR-1 [48]. The appearance of a chondroblastic phenotype in transformed cells with hyperexpression of NBRE-regulated target genes, along with our findings that PTH regulates all three NGFI-B genes in osteoblasts, suggests that NOR-1, Nurr1, and Nur77 may influence bone cell function.

In summary, we showed that NOR-1 is a PTH-induced primary gene in osteoblasts and that NOR-1 induction is mediated through the cAMP-PKA signaling pathway. As potential mediators of PTH's calcitropic effects on bone, NOR-1 and its NGFI-B family members Nurr1 and Nur77, may be important for crosstalk between PTH and steroid hormone receptor signaling pathways.

Acknowledgment

Supported by NIH Grant R01 DE13316.

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