Carboxyl-Terminal Parathyroid Hormone Receptor Regulates Osteocyte Cytoskeleton Through Mechanisms Dependent Upon Calcium Influx

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Abstract: Parathyroid hormone (PTH) exerts classical actions on bone and mineral metabolism by activating PTH/PTHrP receptors (PTH1Rs) on target cells, including osteoblasts and osteocytes in bone. Such bone cells also express an additional receptor for PTH distinct from PTH1Rs, that recognize determinants within the carboxyl (C)-terminal portion of PTH (1-84), the C-terminal PTH receptor ("CPTHR"). CPTHRs previously were found to regulate intercellular communication, cell survival and to increase cytosolic calcium in bone cells in a manner dependent upon voltage-sensitive calcium channels. As intracellular free calcium is known to regulate cytoskeletal function, we sought to determine if CPTHR activation altered cytoskeletal structure in OC-59 osteocytic cells, which lack PTH1Rs but express abundant CPTHRs.

Treatment of OC-59 cells with 100 nM hPTH (53-84) for 10 minutes induced marked condensations of the cytoskeletal components actin and vinculin, as visualized by immunofluorescence in permeabilized cells. This effect was not observed in cells treated with vehicle alone or with the CPTHR ligand for only 2 minutes. These changes also were not seen in cells exposed for 10 minutes to the inactive CPTH analog, [Ala^{55-57}]PTH (53-84), which does not bind to CPTHRs and does not induce a calcium signal in OC-59 cells. Cytoskeletal condensation induced by hPTH (53-84) was blocked by pretreatment with gadolinium chloride, which is known to inhibit CPTHR-dependent calcium responses in these cells. Taken together, these results suggest that calcium influx induced by CPTHR activation may play an important role in regulating the cytoskeleton in osteocytes.

INTRODUCTION

Parathyroid hormone (PTH) is the major physiologic regulator of blood calcium and phosphate, and it exerts potent effects upon cells in bone, cartilage and kidney [1]. The type 1 PTH/PTHrP receptor (PTH1R) recognizes the highly conserved amino (N)-terminal domain of PTH (and the homologous N terminus of PTHrP) and thus is fully activated by both PTH (1-34) and the intact hormone, PTH (1-84) [2]. Carboxyl (C)-fragments of intact PTH (1-84), such as PTH (39-84) or PTH (53-84), do not bind or activate the PTH1R [3]. A possible physiologic role for this region of the hormone is suggested by observations that the amino acid sequence of the C-terminal domain is highly homologous across species [4]. Furthermore, direct physical evidence of a putative receptor, i.e. the carboxyl-terminal PTH receptor (CPTHR) with binding specificity for C-terminal PTH sequences, was obtained by cross-linking of ^{125}I- [Tyr^{34}] hPTH (19-84) (which does not bind to the PTH1R) to 40-kDa and 90-kDa proteins in the ROS 17/2.8 rat osteosarcoma cell line [5,6]. Previous binding and cross-linking studies showed that these peptides could efficiently bind CPTH receptors in other bone cells including osteocytes [7] and osteoclasts [8]. We previously reported the isolation and characterization of clonal osteocytic cells, expressing high levels of CPTHRs, derived from fetuses in which most exons encoding the PTH1R had been ablated by gene targeting in order to eliminate potentially confounding effects of co-expressed PTH1Rs [9]. These clonal osteocytic (OC) cell lines expressed 1,900,000 to 3,400,000 CPTHR binding sites per cell, a level 6- to 10-fold higher than observed on osteoblastic cells obtained from the same fetal calvarial bones and at least 5-fold higher than on ROS 17/2.8 cells [9]. Thus, these osteocytic cells, lacking PTH1Rs but expressing CPTHRs, provide a useful model to study CPTHR signaling. We previously reported that the CPTH fragment hPTH (53-84) rapidly and transiently increases cytosolic free calcium ($[Ca^{2+}]i$), as assessed by single-cell ratiometric microfluorimetry in one such clonal osteocytic cell line (OC-59 cells) [10].

It is known that calcium influx is involved in many cellular functions, including regulation of the cytoskeleton. The cytoskeleton is composed of a network of fibrous elements, consisting primarily of microtubules, actin microfilaments, and intermediate filaments, found in the cytoplasm of eukaryotic cells [11]. The cytoskeleton provides structural support for the cell and permits directed movement of organelles, chromosomes, and the cell itself [11]. Actin microfilaments formed by polymerization of monomeric globular (G) actin, play an important role in muscle contraction, cytokinesis, cell movement, and other cellular functions and structures [12].

In bone, fluid flowing through microchannels surrounding osteocytes may be a primary stimulus for functional adaptation of bone. Osteoblasts, and osteocytes in particular, respond to fluid flow *in vitro* with enhanced nitric oxide (NO) and prostaglandin E_2 (PGE_2) release; both of these signaling molecules mediate mechanically-induced bone forma-

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tion [13]. Because the cytoskeleton is involved in signal transduction, it is hypothesized that the NO and PGE_2 induced by pulsatile fluid flow in both osteoblastic and osteocytic cells involves the actin and microtubule cytoskeleton. It was found that the fluid flow-induced PGE_2 response in osteocytes was inhibited by cytoskeletal disruption, whereas in osteoblasts it was enhanced. These opposite responses to PGE_2 are likely related to differences in cytoskeletal composition (osteocyte structure was more dependent on actin), but may occur *via* cytoskeletal modulation of shear/stretch-sensitive ion channels (such as calcium channels) that are known to be dominant in osteocyte (but not osteoblast) responses to mechanical loading [13]. It is known that phosphorylation of cytoskeletal proteins in response to actin condensation plays a critical role in several signaling functions such as apoptosis. Maintenance of bone structural integrity depends in part on the rate of apoptosis of bone-forming osteoblasts. Tripplet *et al*., recently demonstrated that disruption of focal adhesion tyrosine phosphorylation by disruption of alpha-actinin-integrin interactions at focal adhesions renders osteoblasts susceptible to apoptosis [14].

We have reported previously that CPTHRs in osteocytic cells trigger a number of functional responses, including apoptosis, and that CPTHRs elicit rapid increases in $[Ca^{2+}]i$ in these cells [14]. In this study, we examined the effect of CPTHR signaling, and of associated changes in $[Ca^{2+}]i$, on cytoskeletal elements of osteocytic cells that highly express CPTHRs.

MATERIALS AND METHODS

Cell Culture

Clonal OC-59 osteocytic cells were previously isolated by enzymatic digestion from calvarial bones of 18.5 day-old tsA58 (+)/PTH1R (-/-) fetuses, as described [9]. Cells were cultured at 33°C in a humidified atmosphere (95% air/5% CO_2) using growth medium [α-MEM containing 10% FBS (lot no. 1011961 Life Technologies, Inc. Gaithersburg, MD) and 1% penicillin-streptomycin (PS)].

Human PTH Peptides

Human PTH (53-84) and mutant [Ala $^{55-57}$] hPTH (53-84) were synthesized, analyzed and quantified in the Massachusetts General Hospital Peptide and Oligonucleotide Core Laboratory (Boston, MA).

Immunoflourescent Staining and Image Analysis

Cells were plated in 4-well chamber slides at 10,000 cells per well (10,000 cells/1.7 cm^2). On day 3 of culture, cells were treated with hormone or vehicle (0.01% TFA) for various periods of time. Cells were fixed with 4% paraformaldehyde in PBS for 5 min, permeabilized in PBS containing 0.1% Triton X-100 and then blocked with 2% bovine serum albumin (BSA) in PBS for 60 min at room temperature. The subsequent incubations were carried out with 2% BSA in PBS, and all washes were carried out in PBS containing 0.1% Triton X-100 at room temperature. Cells were incubated with anti-vinculin antibody (1:400 dilution; Vector Lab, CA, USA) for 1 h at 37 °C and then with FITC conjugated secondary antibody (Vector Lab, CA, USA; 1:200 dilution) for 1 h at 37 °C in the dark. For F-actin staining, cells were incubated with rhodamine-phalloidin (Vector Lab, CA, USA; 1:100 dilution) at 37 °C for 10 min and then rinsed. After extensive washing, the slides were mounted with Vectashield mounting medium (Vector Lab, CA, USA) to reduce photobleaching. Control experiments, performed in parallel with the omission of the primary antibodies, did not show the activity. Actin and vinculin fluorescence labeling was examined using a Nikon laser microscope. A 488 nm argon laser was used in combination with a 499/505-530 nm excitation/emission filter set for fluorescein examination. For rhodamine, the 543 nm helium neon laser was used with a 543 nm excitation filter and a 560 nm emission filter. All illustrations were assembled and processed digitally using Adobe Photoshop 7. *Image J* software (NIH, DC, USA) was used to analyze the images of vinculin condensations. Pictures were obtained from different fields and images were analyzed using *Image J* software. The size of the condensations was computed using the area measurement option of the program. This determination was made at 6 different locations per well, 4 wells per group.

RESULTS

Effect of CPTH Fragments on Actin and Vinculin Condensations

To examine the effect of CPTHR activation on osteocyte cytoskeleton, OC-59 cells were cultured for 3 days at 33 °C and treated with 100 nM CPTH (53-84) for 2 and 10 minutes at 37°C. At the end of the incubation period, medium was removed and cells were stained to visualize actin filaments as described in the methods section. Control cultures were treated with PBS. CPTH treatment for 10 minutes induced actin condensations compared to cultures treated for 2 minutes and non-treated cultures (Fig. **1**).

In mammalian cells, vinculin is a membrane-cytoskeletal protein in focal adhesion plaques that is involved in linkage of integrin adhesion molecules to the actin cytoskeleton [15]. To further study the effect of CPTHR activation on the cytoskeleton, we examined the effect of hPTH (53-84) on vinculin condensations in osteocytes. Osteocyte cultures were treated with 100 nM hPTH (53-84) for 2 and 10 minutes. Cells were then stained for vinculin, and vinculin condensation images were analyzed using NIH Image *J* software, as described in Methods. As shown in Fig. (**2**), hPTH (53-84) treatment for 10 minutes induced significant vinculin condensations compared to cultures treated for two minutes and non-treated cultures. To assess the specificity of CPTH fragments on osteocyte actin and vinculin condensations, we used a mutant CPTH fragment that binds very weakly to CPTHRs and has no effect on osteocyte calcium influx, as previously shown [10] As expected, mutant [Ala $^{55-57}$]hPTH (53-84) failed to induce vinculin condensations in OC-59 cells (Fig. **2**). Actin and vinculin condensations were accompanied with smaller cells and more prominent cell protrusions (Figs. **1**, **2**).

The Role of Calcium in CPTH Induced Cytoskeletal Changes

Since calcium influx, such as that induced by hPTH (53-84), is involved in several cellular functions including regulation of cytoskeletal structure [16], we further examined if calcium influx is involved in actin and vinculin condensations induced by CPTHR activation. To this end, we pre-

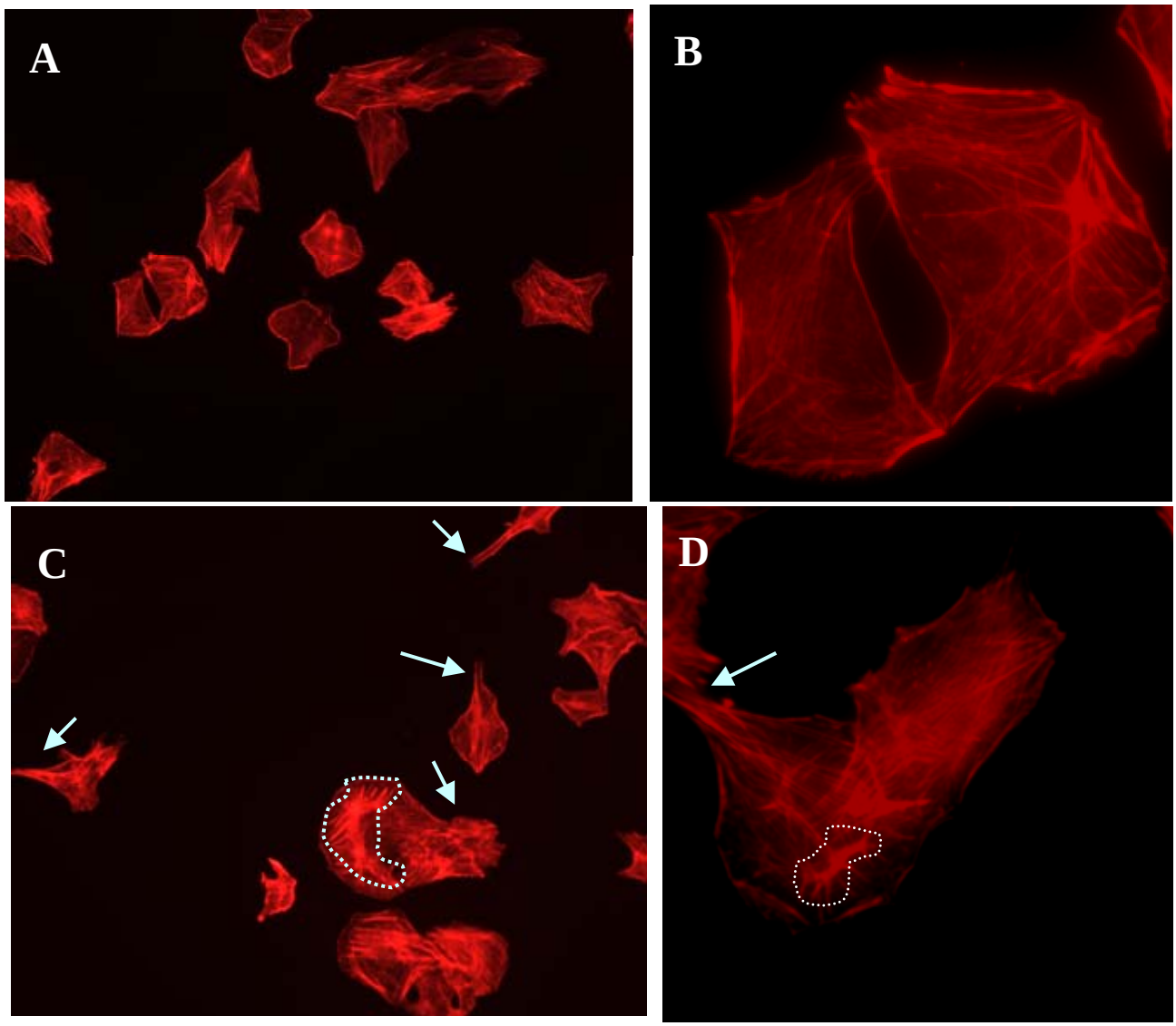

Fig. (1). Effect of hPTH (53-84) on actin condensations in osteocytes. (**A**) Osteocytes treated with vehicle at 20 x magnification. (**B**) Osteocytes treated with vehicle at 40 x magnification. (**C**) Osteocytes treated with 100 nM hPTH (53-84) for 10 minutes at 20 x magnification. (**D**) Osteocytes treated with 100 nM hPTH (53-84) for 10 minutes at 40 x magnification. Dashed lines indicate sample for actin condensations. Arrows indicate cellular protrusions.

treated osteocytic cultures with gadolinium chloride (10 μM) for two minutes. Gadolinium chloride is commonly used to block influx of extracellular calcium by competing with calcium ions. We previously used gadolinium to examine the source of calcium signals in response to hPTH treatment in osteocytic cells [10].

Gadolinium chloride pretreatment (10 μM) abolished the effect of hPTH (53-84) on vinculin condensations in OC-59 cells (Fig. **3**). Gadolinium chloride alone had no effect on vinculin condensation (data not shown).

DISCUSSION

Bone tissue adapts to its functional environment such that its morphology is "optimized" for the mechanical demand, and osteocytes are thought to be the principle mechanosensors (mechanotransducers) of bone [16]. Osteocytes may respond rapidly to strain through glutamate receptor-like mechanisms, through calcium influxes, through gap junctions, and less rapidly through the production of small molecules and factors [17]. Strain may take the form of substrate stretching and/or fluid flow [16]. There is growing evidence for modulation of these mechanotransduction steps by endocrine factors, particularly parathyroid hormone and estrogen [16]. In this study, we demonstrated that CPTHR activation induces rapid changes in the osteocyte cytoskeleton, as manifested by condensation of both actin and vinculin. Based upon that, it is plausible to postulate that these cytoskeletal changes may modify osteocyte responses to mechanical strains.

It has long been recognized that mechanical loading is fundamental for bone health. Mechanical stimulation of the skeleton by exercise is an important anabolic signal in bone, which leads to increased osteoblastic proliferation and matrix deposition, whereas the absence of mechanical stimula-

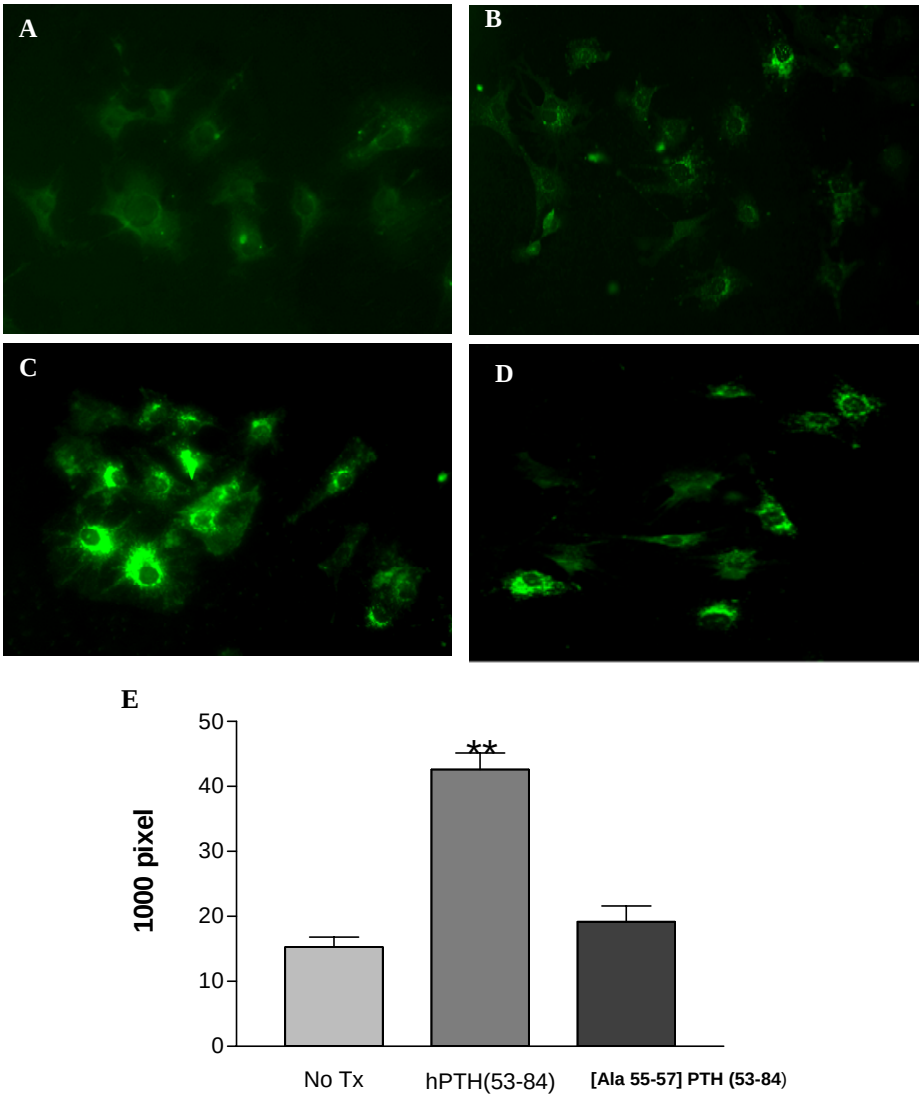

Fig. (2). Effect of hPTH (53-84) on vinculin condensations in osteocytes. (**A**) Osteocytes treated with vehicle. (**B**) Osteocytes treated with 100 nM hPTH (53-84) for 2 minutes. (**C**) Osteocytes treated with hPTH 100 nM for 10 minutes. (**D**) Osteocytes treated with Mutant [Ala^{55-57}] PTH (53-84) for 10 minutes. (**E**) Image analysis for vinculin condensations at various treatment conditions. Pictures stained for vinculin condensations were taken from different fields using a *Nikon* inverted microscope. Images were then analyzed using *ImageJ* (NIH) image analysis software. The size of the condensations was computed using the area measurement feature of this program. In (**E**) statistical analysis of the data was achieved by one-way ANOVA, followed by Bonferroni post-test. (**) represents $P < 0.01$. Data shown are accumulated results from 3 independent experiments with four replicates in each experiment. Data presented in (**E**) are the mean ± SD.

tion, as occurs with immobilization, disuse and exposure to low gravity, causes bone loss [11, 14]. The cellular and molecular mechanisms involved in mechanotransduction have remained largely unknown. Nonetheless, the organized distribution of osteocytes throughout the bone, and their unique ability to form a functional cellular network, make them ideally suited to sense mechanical stimuli and to relay this information to the surrounding cells. How exactly the osteocyte can sense the mechanical forces applied to the bone and then transform this mechanical stimulus into a biological function remains incompletely understood. The identity of the mechanoreceptor (s) is still unknown but it has been hypothesized that ion channels, integrins, connexins and the lipid membrane including the cytoskeleton might be involved in mechanosensation. The distal intracellular signaling systems activated by mechanoreceptors include those emanating from G-proteins (e.g., intracellular calcium shifts), MAPKs, and nitric oxide [18]. In this study, we demonstrated that CPTHR activation induces cytoskeletal changes in osteocytes, suggesting a role for CPTHRs in modifying and /or initiating intracellular signaling pathway (s) that might modulate osteocyte mechanotransduction functions. We previously demonstrated that CPTHRs induce calcium influx *via* VDCC [10]. We now provide evidence that changes in cytoskeletal structure induced by CPTHR activation may involve calcium influx, as they were prevented by gadolinium chloride, which blocks CPTHR-induced increases in cytosolic calcium in these cells [11].

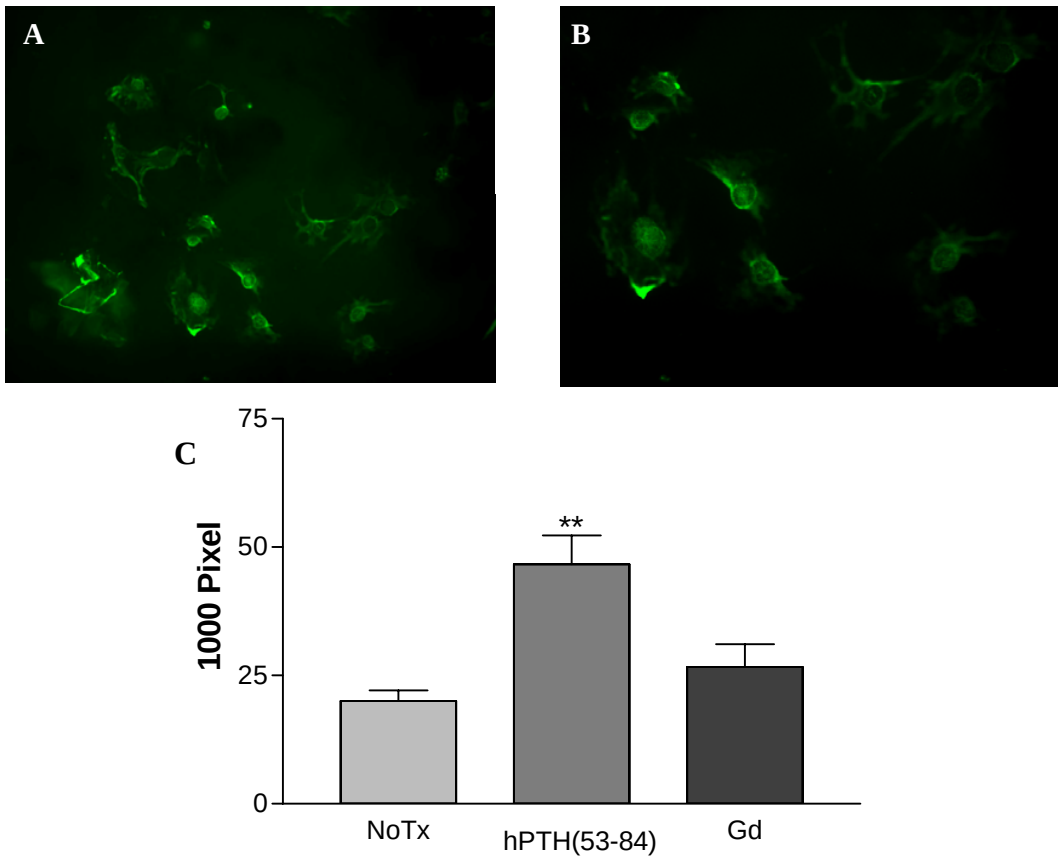

Fig. (3). Effect of gadolium chloride on hPTH induced vinculin condensations in osteocytes. (**A**) Osteocytes treated with vehicle. (**B**) Osteocytes pretreated with gadolinium chloride (10 µM) for 2 minutes before 10 minutes hPTH (53-84) treatment (100 nM). (**C**) Image analysis for vinculin condensations at various conditions. Pictures stained for vinculin condensations were taken from different fields using a *Nikon* inverted microscope. Images were then analyzed using *ImageJ* (NIH) image analysis software. The size of the condensations was computed using the area measurement feature of this program. In (**C**) statistical analysis of the data was achieved by one-way ANOVA, followed by Bonferroni post-test. (**) represents $P < 0.01$. Data shown in figure C are accumulated results from 3 independent experiments with four replicates in each experiment. Data presented in (**C**) are the mean ± SD.

Cell adhesion is mediated by the integrin subunit beta 1, but other integrins or non-integrin adhesion receptors are also involved [19]. Osteocytes make contact with the extracellular matrix *via* small attachment points which colocalize with vinculin. This connection between the bone matrix and the cytoskeleton may be important for osteocytic sensing of mechanical strain, as it supplies a pathway for transduction of extracellular (mechanical) signals into intracellular messages [17]. Based on these observations, CPTHR effects on the osteocyte cytoskeleton might also affect the manner in which osteocytes interact with extracellular matrix.

In conclusion, this study suggests that calcium signals induced by CPTHRs may play an important role in regulation of osteocyte cytoskeletal assembly and structure. Further studies are needed to explore the subsequent effects on other signaling events and the potential role of CPTHRs in modulating the osteocyte response to mechanical strain and osteocyte interaction with extracellular matrix.

REFERENCES

[1] Murray TM, Rao LG, Divieti P, Bringhurst FR. Parathyroid hormone secretion and action: evidence for discrete receptors for the carboxyl-terminal region and related biological actions of carboxyl-terminal ligands. Endocr Rev 2005; 26: 78-113.

[2] Abou-Samra AB, Jüppner H, Force T, *et al*. Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. Proc Natl Acad Sci 1992; 89: 2732-6.

[3] Bringhurst FR, Stern AM, Yotts M, Mizrahi N, Segre GV, Potts JT Jr. Peripheral metabolism of PTH: fate of biologically active amino terminus *in vivo*. Am J Physiol 1988; 255: E886-93.

[4] Khosla S, Demay M, Pines M, Hurwitz S, Potts Jr JT, Kronenberg HM. Nucleotide sequence of cloned cDNAs encoding chicken preproparathyroid hormone. J Bone Miner Res 1988; 3: 689-98.

[5] Inomata N, Akiyama M, Kubota N, Jüppner H. Characterization of a novel parathyroid hormone (PTH) receptor with specificity for the carboxyl-terminal region of PTH- (19-84). Endocrinology 1995; 136: 4732-40.

[6] Murray TM, Rao LG, Muzaffar SA. Dexamethasone-treated ROS 17/2.8 rat osteosarcoma cells are responsive to human carboxylterminal parathyroid hormone peptide hPTH (53-84): stimulation of alkaline phosphatase. Calcif Tissue Int 1991; 49: 120-3.

[7] Divieti P, Inomata N, Chapin K, Singh R, Juppner H, Bringhurst FR. Receptors for the carboxyl-terminal region of PTH (1-84) are highly expressed in osteocytic cells. Endocrinology 2001; 142: 916-25.

[8] Divieti P, Lotz O, Geller A, Juppner H, Bringhurst FR. Inhibition of osteoclast formation by human PTH (7-84) involves direct actions on hematopoietic cells. J Bone Miner Res 2002; 17: S167.

[9] Divieti P, Lanske B, Kronenberg HM, Bringhurst FR. Conditionally immortalized murine osteoblasts lacking the type 1 PTH/PTHrP receptor. J Bone Miner Res 1998; 13: 1835-45.

[10] Selim AA, Mahon M, Juppner H, Bringhurst FR, Divieti P. Role of calcium channels in carboxyl-terminal parathyroid hormone receptor signaling. Am J Physiol Cell Physiol 2006; 291: C114-21.
[11] Wang JH, Thampatty BP. An introductory review of cell mechanobiology. Biomech Model Mechanobiol 2006; 5: 1-16.
[12] Goldmann WH. Mechanical aspects of cell shape regulation and signaling. Cell Biol Int 2002; 26: 313-7.
[13] McGarry JG, Klein-Nulend J, Prendergast PJ. The effect of cytoskeletal disruption on pulsatile fluid flow-induced nitric oxide and prostaglandin E2 release in osteocytes and osteoblasts. Biochem Biophys Res Commun 2005; 330: 341-8.
[14] Triplett JW, Pavalko FM. Disruption of alpha-actinin-integrin interactions at focal adhesions renders osteoblasts susceptible to apoptosis. Am J Physiol Cell Physiol 2006; 291: C909-21.
[15] Arena N, Venti G, Becchetti E, *et al.* Characterization of the cytoskeleton in human normal and otosclerotic osteoblast-like cells. Acta Anat (Basel) 1991; 141: 311-5.
[16] Bringhurst FR. PTH receptors and apoptosis in osteocytes. J Musculoskelet Neuronal Interact 2002; 2: 245-51.
[17] Xu W, Baribault H, Adamson ED. vinculin knockout results in heart and brain defects during embryonic development. Development 1998; 125: 327-37.
[18] Bloomfield SA. Cellular and molecular mechanisms for the bone response to mechanical loading. Int J Sport Nutr Exerc Metab 2001; l: S128-36.
[19] Astrof NS, Salas A, Shimaoka M, Chen J, Springer TA. Importance of force linkage in mechanochemistry of adhesion receptors. Biochemistry 2006; 45: 15020-8.

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