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Original Article

Hylonome

The role of calcium channels in osteocyte function

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Abstract

Osteocytic response to stretching, which is potentiated by PTH, is distinct from that of osteoblast to high frequency strain. A MAPK dependent signaling pathway is suggested in the osteoblast response. At least two different types of mechano-transduction pathways are present in bone cells of osteoblastic lineage.

Keywords: Osteocyte, Stretch-Activated Channel, PTH, Mechanotransduction

Introduction

Cells in bone are equipped with mechanisms to sense diverse physical forces and transduce signals so that they adjust themselves to their mechanical environment. Study of osteocytic stretch response mechanisms, which sense even a very low frequency and low rate strain, showed that certain types of mechanical stress are received only by certain stages of osteogenic cells[1-3]. In cell processes of primary young osteocytes, we have reported PTH-potentiated Ca^{2+} influxes, which utilize PKA signaling pathways for the downstream anabolic responses such as production of IGF-I and osteocalcin[4]. The upregulation of mRNA levels of these molecules occurred in a manner similar to that of typical immediate early genes, c-fos or COX-2 [3]. The upregulation was dependent on the presence of extracellular Ca^{2+}, which suggests the involvement of stretch-activated (SA) cation channels. Ca^{2+} influx, visualized in fura-2 preloaded rat young osteocytes, has been mainly localized along the cell processes[4]. Either mRNAs for the subunits of ion channels were sequenced and quantitated or the current measured to provide a molecular basis for these responses. As a result, 1) a Gd^{3+}-sensitive non-selective cation channel, which is generally called SA-cat (molecular identity is unknown)[2], 2) ENaC, a volume sensitive epithelial-like Na^+ channel[5], and 3) Cl^- and K^+ channels were detected. Secondarily driven Ca^{2+} channels such as the voltage dependent L-type channel[6] or Na^+/Ca^{2+} exchanger, which functions as a Ca^{2+} channel when intracellular [Na^+] is elevated[7], have also been detected in the young osteocyte cultures. In these channels examined, most molecular properties are not osteocyte-specific, however. While the expression levels of the ENaC increased along with osteocytic cell differentiation, we were unable to detect any significant increase in the Na channel activity *per se* upon stretching. These results suggest that multiple ion channels may be functioning in collaboration to process the mechanical input into osteocytic cells. L-type channels, for example, can be activated by depolarization, which may be dependent on the primary local flow of Ca^{2+} or Na^+ ions into the cells.

We then searched for mechanotransduction pathways unique to osteoblastic cells and for crosstalk among signaling pathways. Differential responses are likely to be relevant in integrating a variety of mechanical stimuli, which our bone may receive, into the anabolic reactions.

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Methods

ST2 cells

Stromal ST2 cell line of murine bone marrow origin was obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells within the 5th or 6th subculture were plated in 6-well dishes at $1x10^5$ cells/cm^2 (9.4-cm^2/well) 24-48 hr prior to the exposure to ultrasound. Alpha Minimum Essential Medium (α-MEM) was used with supplements, 10 % fetal bovine serum (GIBCO) and the above-mentioned antibiotics mixture (GIBCO) in the presence or absence of 25μg/ml ascorbate-2-phosphate (Wako Pure Chemical Industries Ltd., Osaka, Japan) or 10^{-8}M Dexamethasone (Sigma-Aldrich Inc., St. Louis, MO) for one week.

Preparation of osteoblastic and osteocytic cells from rat long bone

The following experimental protocols were approved by the animal care committee either at Kanagawa Dental College or at Kitasato University School of Medicine. Osteoblastic as well as osteocytic cells were enriched by sequential collagenase digestion as reported earlier[8]. Pieces of 3-day-old rat femur and tibia bone collars were split, stripped carefully of most periosteal soft tissues, and then washed by repeated pipetting to remove bone marrow cells. Following the first brief digestion with 0.75 mg/ml collagenase (Wako), cells released by the second and third digestions (20 min each) at 37 °C were collected and cultured as osteoblasts. After each sequential release of osteoblastic cells, strips of long bone fragments were flushed by repeated pipetting with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan); PBS (-), and cells released were combined with the digests to be inoculated at the density of $5x10^4$ cells/cm^2 in the 6-well plate (Falcon®). Alpha-MEM was used with supplements, 10% fetal bovine serum (GIBCO) and antibiotics mixture (GIBCO). Residual bone strips were further cut into 1 to 2 mm^3-pieces, incubated with collagenase again, washed with PBS (-) and placed in a culture dish. The outgrowth of flat cells and some of the released cells, distinct from fibroblastic cells, were used as sources of osteocytic cells after they reached confluence. Final cell density of these cells is less than one-tenth of the osteoblasts, being approximately 2-5,000/cm^2.

Bone marrow-derived cells

Six-week-old Wistar rats were euthanized, their femora were excised and cleaned, and the epiphyses were removed. Marrow contents were flushed out with α-MEM squirted through a 20G x 11/2" needle, and single cells were isolated in suspension by repeated pipetting. Cells from each femur, $2 x 10^7$ cells, were cultured in a 25-cm^2 flask (Falcon®) in α-MEM containing 10% fetal bovine serum (GIBCO) and antibiotics in a humidified atmosphere of 5% CO2 in air at 37°C. After a 24-hr incubation to allow cells to attach to the bottom of the plate, nonadherent cells were removed by rinsing the plate with PBS (-). Cultures were maintained for another week. After reaching 70% confluence, the cells were detached by treatment with 0.05% trypsin, replated either in 75-cm^2 flasks (Falcon®) or in 6-well plates (9.4-cm^2/well) both at a density of $5.3X10^4$/cm^2. Cells were cultured for two more weeks before experimental use; either with or without Dexamethasone ($1 x 10^{-8}$ M from a 1-M stock solution in ethanol) or 0.25 mM ascorbate 2-phosphate.

Results and discussion

The effect of pulsed ultrasound was studied using SAFHS, sonic accelerated fracture healing system, which has been reviewed as a device that accelerates healing by 38% in human tibial diaphysis and distal radius[9,10]. Although the SAFHS seems to facilitate the overall process of fracture repair, the enhanced osteoblastic activity, but not the induction of cell proliferation, has been implied[11,12]. In our studies, effects of 20-min exposure to 200-μs burst of pressure pulses (sine wave of 1.5 MHz repeated at a frequency of 1.0 kHz), which was repeated every millisecond, were examined in mouse bone marrow-derived ST2 cells, and primary rat bone- and bone marrow-derived cells. By using conventional and semi-quantitative RT-PCR analyses, ST2 cells cultured in the presence of ascorbate or Dex, or the primary cells showed that steady state levels of immediate early genes such as c-fos or COX-2 were upregulated in relatively young osteoblastic cells upon exposure to the ultrasound. IGF-I, osteocalcin and other bone protein messages were also upregulated in the osteoblastic cell population. Very mature osteoblasts and osteocytes derived from newborn rat tibia, on the other hand, were almost insensitive to the pulsed ultrasound compared to the younger osteoblasts.

We have further presented that osteoblastic mechanotransduction pathways in response to the pulsed ultrasound are distinct from that interactive with PTH. We could not detect any extracellular Ca^{2+} influxes induced by the ultrasound. Apparently, Ca^{2+}/PKA pathway, which is potentiated by PTH and plays an essential role in stretched osteocytes, is not involved. Instead, response of osteoblasts to the ultrasound was modulated by inhibitors of p38 MAPK and of upstream effector, PI3K. We believe that is probably why the SAFHS is extremely effective in accelerating bone maturation in distraction osteogenesis. In addition to stimulating tensile stress-sensitive population, such as osteoprogenitor cells[13,14], by stretching callus once a day, exposure to the ultrasound stimulates osteoblastic cells sensitive to the ultrasound. Synergistic upregulation in response to both types of mechanical stimuli is expected.

As summarized in Figure 1, the anabolic effect of the pulsed ultrasound was not influenced at all by PD98059. It demonstrates that the activation of ERK, typically leading cells to proliferation[15,16], is not involved. On the other hand, inhibition of p38 by SB203580 or of PI3K by LY 294002, both specific inhibitors, eradicated the upregulation providing evidence that the p38-dependent bone cell differentiation pathway is accelerated. This particular choice of discretionary route may be consistent with the characteristic features of accelerated fracture healing by the ultrasound: promotion of osteogenic differentiation but not of cell proliferation[9,11]. The responses we observed are a likely reflection of the osteogenic phase in the SAFHS-accelerated fracture repair. Positioned downstream of IGF-IR and IRS-1, PI3K is a key molecule in cell proliferation, apoptosis and differentiation[17,18]. Regulatory pathways have been reviewed in the integrin-stimulated cells[19], and in FAK signaling complex in myocardium mechanotransduction[20]. In endothelial cells, PI3K-dependent NO generation and the phosphorylation of NO synthase have been studied as consequences of fluid shear stress[21,22]. Further characterization

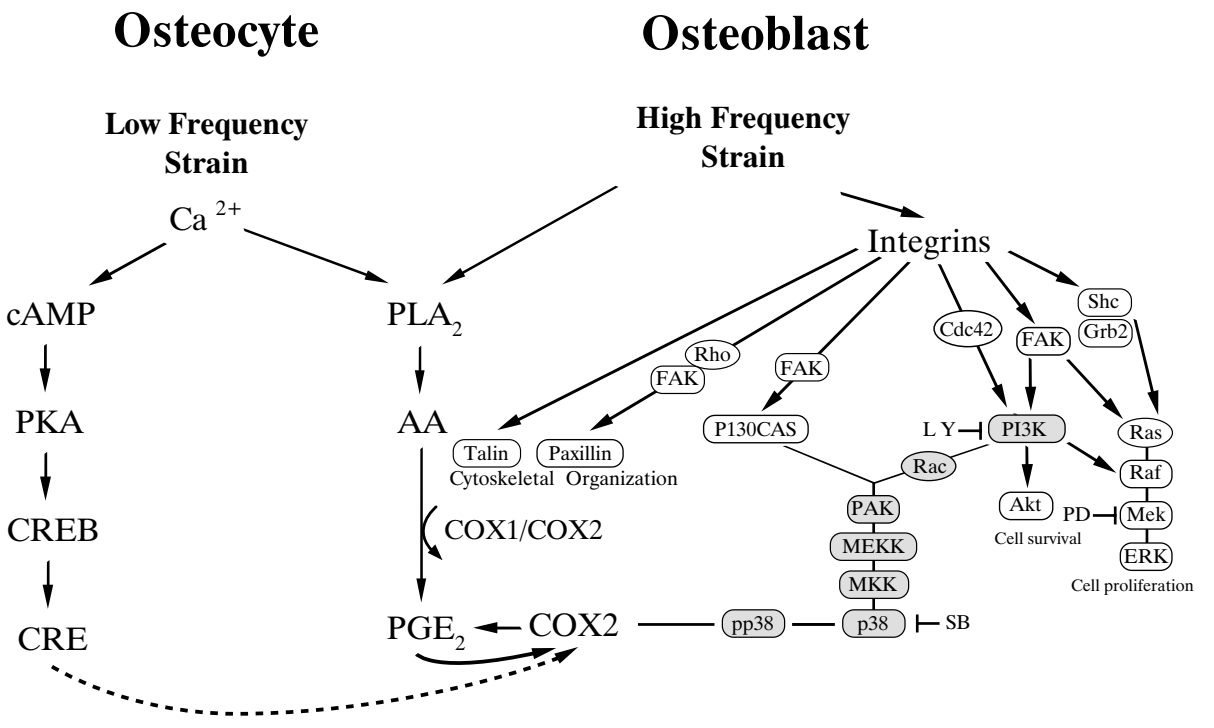

Figure 1. Possible mechanotransduction pathways involved in osteoblast and osteocyte. Within an array of signaling intermediates in osteoblasts exposed to the pulsed ultrasound, PI3K activation cascades started from integrins was modified from Hynes[19] with permission from the publisher. A stream of ellipsoids filled with grey represents an experimentally supported activation cascade.

of PI3K-related upstream/downstream pathways in shear stress-activated bone cells will shed light on the complex nature of mechanical stimulation of bone formation and prevention of bone loss.

Our findings support the notion that low frequency and low rate strain is sensed by the osteocytic cells through Ca^{2+} channels and potentiated by PTH whereas other strain is sensed by other types of cells in the osteogenic lineage through different machinery. Thus, differentiation of osteocytes from osteoblasts, which is accompanied by the loss of proliferation, development of extensive cell processes, and stretch-activated Ca^{2+} influx pathways are essential in generating their distinct mechano-sensing and signal transduction mechanisms.

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