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PRELIMINARY REPORT

Parathyroid Hormone–Induced Retraction of MC3T3-E1 Osteoblastic Cells Is Attenuated by the Calpain Inhibitor *N*-Ac-Leu-Leu-Norleucinal

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Parathyroid hormone (PTH) binding to its osteoblastic receptors stimulates cytoplasmic retraction within minutes. We hypothesized that the calpains (*cal*cium-activated p*apain*-like enzymes) contribute to PTH-induced osteoblastic retraction by catalyzing regulatory hydrolysis of cytoskeletal structural proteins or enzymes important in cytokinesis. *N*-Ac-Leu-Leu-norleucinal (ALLN), a reversible calpain inhibitor, was tested for its ability to inhibit PTH-induced retraction in murine MC3T3-E1 osteoblastic cells. ALLN inhibited PTH-induced retraction for 30 minutes in cells cultured on polystyrene cultureware or gelatin-coated glass cover slips, supporting the hypothesis that PTH-induced activation of the calpains contributes to short-term changes in MC3T3-E1 cell shape. Inhibition of PTH-induced retraction occurred on two substrata, suggesting that interactions between the extracellular matrix and cell surface proteins are not the sole determinants of morphology. Intracellular events, such as hydrolysis of focal adherens junction proteins on the cytoplasmic face of the plasma membrane, may contribute to PTH-induced retraction.

OSTEOBLASTIC RETRACTION occurs within minutes of parathyroid hormone (PTH) treatment and may play a key role in regulating PTH-dependent bone resorption by determining the degree of osteoclastic access to the underlying bone.[1] PTH-induced osteoblastic retraction involves cyclic nucleotides and calcium,[2] actin and myosin depolymerization,[3] loss of stress fibers,[3] and microfilament disorganization.[4] Half of the retraction can be ablated by preincubation with E64d, a membrane-permeable inhibitor of the cathepsin and calpain families of cysteine proteases,[5] suggesting that regulatory proteolysis of structural or regulatory proteins plays a major role in mediating PTH-induced retraction of the osteoblast.

The calpains are cytosolic, calcium-activated regulatory proteases initially synthesized as proenzymes.[6] Hormone binding and stimulation of intracellular calcium ion fluxes result in autoproteolytic processing and subcellular relocalization of activated calpains, which are capable of catalyzing limited proteolysis of cytoskeletal structural proteins and enzymes important in cytokinesis, mitosis, and other regulatory events.[6] Endogenous calpain substrates include key focal adherens junction proteins (vinculin, talin, α-actinin), as well as protein kinase A and C isozymes.[6] PTH-stimulated calpains I and II are present in MC3T3-E1 osteoblastic cells,[7] and we hypothesized that they are the specific regulatory proteases that mediate PTH-induced osteoblastic retraction. To test this hypothesis, the effects of PTH on the morphology of murine MC3T3-E1 cells were examined after preincubation with *N*-Ac-Leu-Leu-norleucinal (ALLN), a synthetic membrane-permeable aldehyde tripeptide that competitively inhibits calpain I, calpain II, and cathepsin L in preference to cathepsins B and H.[8]

MATERIALS AND METHODS

Chemicals and Reagents

ALLN was purchased from Calbiochem (San Diego, CA), and rat PTH-(1-34) was obtained from Bachem (Torrance, CA). Dulbecco's modified Eagle's medium (DMEM), antibiotics, low-protein serum replacement-1, 2% calf skin gelatin, newborn calf

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serum, and all biochemicals and buffers were from Sigma Chemical (St Louis, MO).

Cell Culture

Murine MC3T3-E1 osteoblastic cells were routinely cultured in DMEM containing 10% serum, 2 mmol/L glutamine, and antibiotics.[5] For morphologic studies on polystyrene cultureware, cells were freshly plated at a density of 100,000 cells per 60-mm dish and allowed to adhere overnight. On days 2 to 7 in culture, plates were rinsed with phosphate-buffered saline three times and switched to serum-free DMEM supplemented with 1% low-protein serum replacement-1 for 12 hours. Immediately before the morphologic studies, plates were rinsed three times with phosphate-buffered saline, preincubated with ALLN (50 μg/mL) or ethanol vehicle in DMEM for 10 minutes at 37°C, and observed and photographed at timed intervals after addition of 10 nmol/L PTH-(1-34).[5] A similar protocol was used for cells plated on glass cover slips precoated with 2% calf skin gelatin. Cover slips were fixed for 30 minutes in 10% formaldehyde in phosphate-buffered saline prior to photography.

RESULTS

The morphologies of vehicle-pretreated (Fig 1A, 0 minutes) and ALLN-pretreated (Fig 1B, 0 minutes) MC3T3-E1 cells were similar before the addition of PTH. PTH induced rapid, marked retraction of MC3T3-E1 cells plated on polystyrene cultureware (Fig 1A, 30 minutes). Retraction was attenuated by a 10-minute preincubation in ALLN followed by a 30-minute treatment with PTH (Fig 1B, 30 minutes). Brisk PTH-induced retraction was observed in MC3T3-E1 cells after overnight washout of ALLN (data not shown). The morphology of MC3T3-E1 cells plated on gelatin-coated glass cover slips (Fig 2A) was slightly different from that observed on polystyrene plastic cultureware. However, treatment of MC3T3-E1 cells plated on gelatin-coated cover slips with 10 nmol/L PTH resulted in brisk, marked retraction (Fig 2B), and this retraction was strongly inhibited by preincubating the cells in ALLN before PTH addition (Fig 2C), demonstrating that the inhibition of PTH-induced osteoblastic retraction occurs on at least two substrates.

DISCUSSION

Synthetic inhibitors are frequently used as probes to determine the putative biological roles of the calpains. We demonstrated by image analysis that E64d inhibits 50% of the PTH-induced retraction in MC3T3-E1 cells.[5] However, E64d inhibits lysosomal cysteine proteases such as the

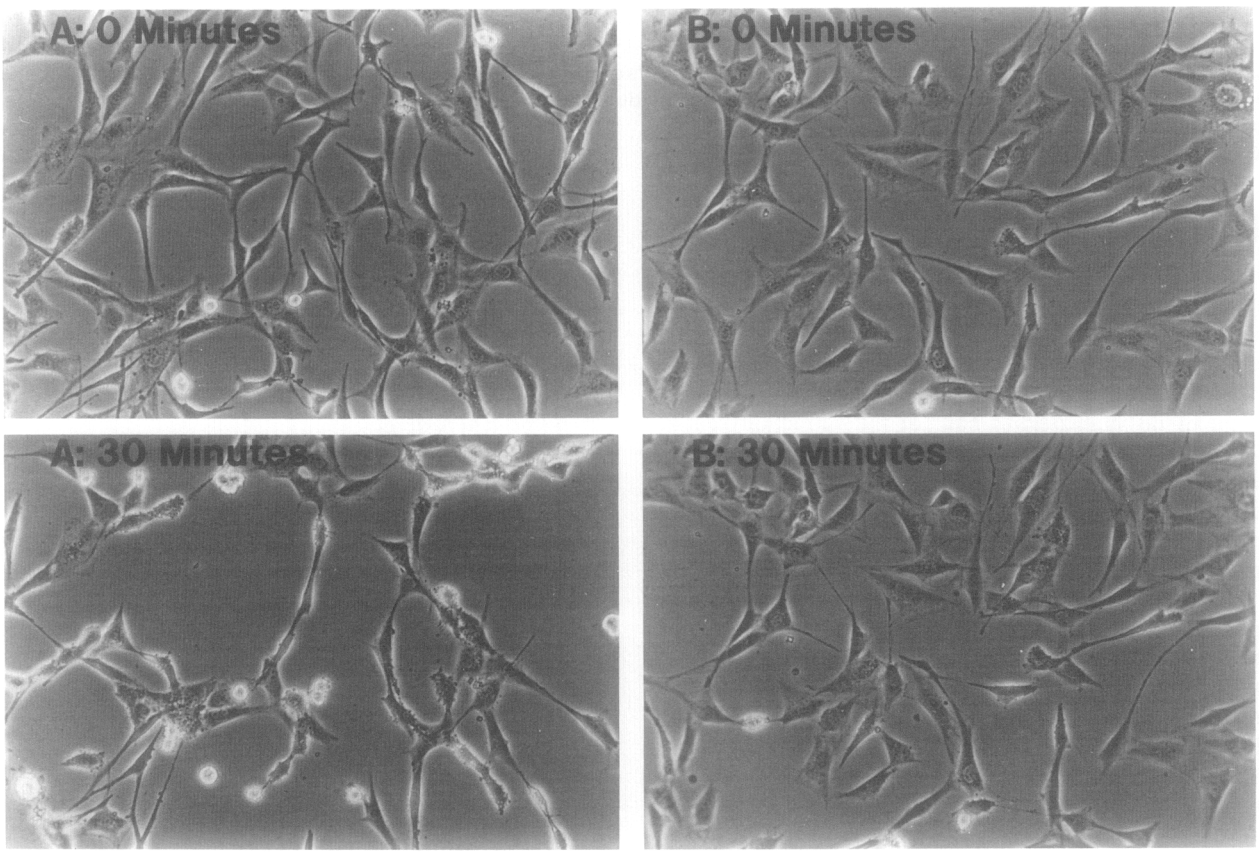

Fig 1. PTH-induced morphologic changes in MC3T3-E1 cells grown on polystyrene cultureware. Log-phase cells were cultured under serum-free conditions for 12 hours, preincubated in ethanol (vehicle) or ALLN (50 μg/mL) in DMEM for 10 minutes at 37°C, and treated with 10 nmol/L PTH for 0 to 30 minutes. (A) Cells pretreated with vehicle for 10 minutes and viewed before addition of PTH (0 minutes), and then treated with PTH for 30 minutes; (B) cells pretreated with ALLN for 10 minutes and viewed before addition of PTH (0 minutes), and then treated with PTH for 30 minutes. (Original magnification ×320.)

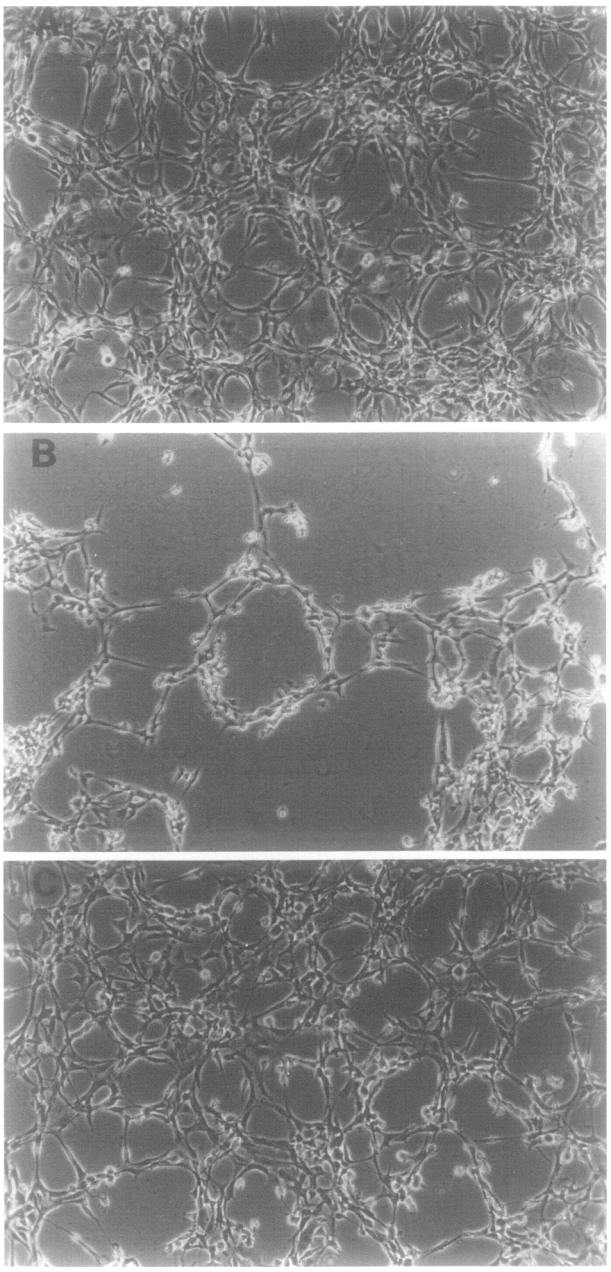

Fig 2. PTH-induced morphologic changes in MC3T3-E1 cells grown on gelatin-coated glass cover slips. Cells were cultured to log phase on gelatin-coated glass cover slips and pretreated with vehicle or 50 μg/mL ALLN for 10 minutes followed by 10 nmol/L PTH for 30 minutes, as outlined in Fig 1. (A) Time-zero control cells; (B) cells pretreated with vehicle, and then treated with PTH for 30 minutes; (C) cells pretreated with ALLN, and then treated with PTH for 30 minutes. (Original magnification ×200.)

cathepsins, in addition to the calpains,[9] and is not sufficiently selective to permit identification of the specific cysteine protease involved in PTH-induced retraction. The tripeptide aldehyde derivative ALLN markedly inhibits three cysteine proteases (calpain I, calpain II, and cathepsin L), but has significantly less activity against cathepsins B and H, papain, trypsin, and chymotrypsin at concentrations of 0.5 to 100 nmol/L.[8] Roles for the calpains have been suggested in memory potentiation,[10] hypoxic myocardial cell injury,[11] hepatic hydroxymethyl glutaryl coenzyme A reductase degradation,[12] and platelet-derived growth factor–induced cell cycle progression,[13] based on ALLN inhibition of these processes in vitro.

The data presented here demonstrate that ALLN markedly attenuates the PTH-induced retraction of osteoblasts over a 30-minute time course. Cathepsins B and D have been detected by immunohistochemistry in osteoclasts and their resorptive surfaces.[14] However, no positive immunostaining has been reported on nonresorbing surfaces or within trabeculae in normal bone,[14] suggesting that little or no cathepsin B or D activity is present in the osteoblast. Procathepsin L and procathepsin L–like proteins that exhibit no enzymatic activity at physiological pH are present in bone homogenates.[15] No cathepsin L activity is observed in whole-bone homogenates, and cathepsin L activity cannot be detected in bone explant culture fluid, even after PTH treatment.[15] Therefore, we conclude that it is the calpains that play a major role in mediating PTH-induced osteoblastic retraction. By analogy to other systems, the calpains (a family of closely related calcium-activated regulatory proteases) may mediate the retractile effects of PTH in the osteoblast by catalyzing proteolysis of key structural or regulatory proteins that are important in maintaining cell shape and cytoskeletal architecture.[6]

Commercial tissue-culture plasticware is manufactured by proprietary techniques that produce surfaces suitable for cell attachment. The surface of glass cover slips is covered with negatively charged silicic acid molecules unsuitable for direct cell attachment. Gelatin-coating provides glass with a surface rich in collagen that is capable of supporting cell attachment. ALLN (a membrane-permeable calpain inhibitor) attenuated PTH-induced osteoblastic retraction on both plasticware and gelatin-coated glass surfaces, suggesting that hormonally regulated intracellular events, such as hydrolysis of focal adherens junction proteins on the cytoplasmic face of the plasma membrane, may contribute to retraction.

REFERENCES

1. Jones SJ, Boyde A: Morphological changes of osteoblasts in vitro. Cell Tissue Res 166:101-107, 1976

2. Lowik CWGM, van Leeuwen JPTM, van der Meer JM, et al: A two-receptor model for the action of PTH on osteoblasts. Cell Calcium 6:311-326, 1985

3. Egan JJ, Gronowicz G, Rodan GA: PTH promotes disassembly of cytoskeletal actin and myosin in cultured osteoblastic cells. J Cell Biochem 45:101-111, 1991

4. Aubin JE, Alders E, Heersche JNM: A primary role for microfilaments, but not microtubules, in hormone-induced cytoplasmic retraction. Exp Cell Res 143:439-450, 1983

5. Tram KK-T, Murray SS, Lee DBN, et al: PTH-induced osteoblast contraction is mediated by cysteine proteases. Kidney Int 43:693-699, 1993

6. Croall DE, DeMartino GN: Calcium-activated neutral protease system: Structure, function and regulation. Physiol Rev 71:813-847, 1991

7. Tram KK-T, Spencer MJ, Murray SS, et al: Identification of CANP activity and regulation by PTH in mouse osteoblastic cells. Biochem Mol Biol Int 29:981-987, 1993

8. Sasaki T, Kishi M, Saito M, et al: Inhibitory effects of di- and tripeptidyl aldehydes on calpains and cathepsins. J Enzyme Inhib 3:195-201, 1990

9. Angliker H, Anagli J, Shaw E: Inactivation of calpain by peptidyl fluormethyl ketones. J Med Chem 35:216-220, 1992

10. Denny JB, Polan-Curtain J, Ghuman A, et al: Calpain inhibitors block long-term potentiation. Brain Res 534:317-320, 1990

11. Iizuka K, Kawaguchi H, Yasuda H: Calpain is activated during hypoxic myocardial cell injury. Biochem Med Metab Biol 46:427-431, 1991

12. Inoue S, Sharma RC, Schimke RT, et al: Cellular detoxification of tripeptidyl aldehydes by an aldo-keto reductase. J Biol Chem 268:5894-5898, 1993

13. March KL, Wilensky RL, Roeske RW, et al: Effects of thiol protease inhibitors on cell cycle and proliferation of vascular smooth muscle cells in culture. Circ Res 72:413-423, 1993

14. Hicks DG, deMesy Jensen KL, Parker JF, et al: Immunohistochemical analysis of lysosomal enzyme activity during abnormal bone remodeling. J Bone Miner Res 8:S392, 1993 (abstr)

15. Delaisse J-M, Ledent P, Vaes G: Collagenolytic cysteine proteinases of bone tissue. Biochem J 279:167-174, 1991