DNA Synthesis Is Not Necessary for Osteoclastic Responses to Parathyroid Hormone in Cultured Fetal Rat Long Bones

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ABSTRACT Osteoclasts, the principal cells mediating bone resorption, are believed to increase their size, number, and resorbing activity in response to parathyroid hormone (PTH) through mechanisms dependent upon the fusion of specific mononuclear precursor cells into either new or existing multinucleated osteoclasts. To address the question of whether these actions of PTH are dependent on the replication of osteoclast precursor cells, we examined the ability of an inhibitor of DNA synthesis, hydroxyurea (HU), to alter bone resorption, osteoclast formation, and DNA synthesis in cultured fetal rat bones treated with PTH. We found that HU significantly reduced [^{3}H]thymidine incorporation into the bones and labeling of osteoclast nuclei by >90%, but did not prevent PTH from stimulating bone resorption, measured as the release of ^{45}Ca, or from increasing the number of osteoclasts in the bones. In bones cultured without PTH, HU decreased the rate of bone resorption, but not the number of osteoclasts per bone.

We conclude that in fetal rat bone cultures, PTH can increase osteoclast number and stimulate bone resorption by affecting existing osteoclasts and osteoclast precursors, and that replication of osteoclast precursor cells is not necessary for PTH to stimulate a resorptive response. In unstimulated cultures it appears that HU inhibits bone resorption by affecting mechanisms that are independent of changes in osteoclast number and that may be influenced by cell replication or other unknown factors.

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INTRODUCTION

Osteoclasts are believed to be the principal cellular mediator of bone resorption (1–4). These cells are multinucleated but have never been shown to multiply by cell division. Instead, they increase their size and number in response to resorptive stimuli through fusion of a mononuclear precursor cell into either new or existing osteoclasts (1–4). While there is good evidence that the osteoclast precursor cell is derived from a hematopoietic stem cell (5–7), little is known about the effect of resorptive stimuli on the replication of this precursor or the relationship of cell replication to bone resorption. Parathyroid hormone (PTH)[1] is an important stimulator of bone resorption both in vivo and in vitro, and increases the number of osteoclasts in bone (8–10). PTH also increases DNA synthesis in bone cells (2, 11, 12, 13). However, definitive evidence demonstrating that the cells that replicate in response to PTH also differentiate into osteoclast precursor cells is lacking. We therefore examined an inhibitor of DNA synthesis, hydroxyurea (HU) (14), for its effects on fetal rat bone organ cultures to determine if PTH required a mitogenic response to mediate its effects on resorption and osteoclast formation.

METHODS

Culture techniques. Our method of fetal rat bone organ culture has been described in detail elsewhere (15, 16). Briefly, 19-d fetal rat forelimb bones labeled in utero with ^{45}Ca were dissected free of surrounding muscle, cartilage, and fibrous tissue, and cultured in 0.5 ml of BGJ medium (Grand Island

[1] *Abbreviations used in this paper:* HU, hydroxyurea; PTH, parathyroid hormone; TCA, trichloroacetic acid.

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Biological Co., Grand Island, NY) supplemented with 5% fetal calf serum (Grand Island Biological Company) that was heat-inactivated at 57°C for 3 h and treated with dextran-coated charcoal to remove endogenous stimulators of resorption (17). In this method, 500 mg of dextran (average molecular weight of 151,000; Sigma Chemical Co., St. Louis, MO) was dissolved in 125 ml of water and mixed with 5 g of activated charcoal (Norit A, Matheson Coleman and Bell, Norwood, OH) at 4°C for 1 h. A dextran-charcoal pellet was made by centrifugation (1,000 *g* for 10 min) and the supernatant discarded. Heat-treated fetal calf serum in a ratio of 1 ml serum per gram of charcoal was added to the pellet and the solution was continually mixed for 12 h at 4°C. This suspension was centrifuged to remove the charcoal from the serum (10,000 *g* for 2 h at 4°C) and the supernatant was then filtered to sterilize the serum and remove any additional charcoal (Nalgene sterilization filter unit, 0.2-μm pore size, Sybron Corp., Rochester, NY).

Bones were precultured for 24 h before being exposed to bovine PTH (1-84), a gift from the National Institutes of Health Hormone Distribution Program. Groups of bones treated with HU (Boehringer Mannheim Biochemicals, Indianapolis, IN) had this agent added to both the preculture and experimental medium. Bone shafts were incubated at 37°C in humidified 95% air/5% CO_2.

HU was dissolved directly into the medium. Stock solutions were dissolved in 0.001 N HCl containing 0.1% bovine serum albumin and then diluted 1/1,000 or greater in the medium.

DNA synthesis. DNA synthesis was assessed as [^{3}H]-thymidine incorporation into the cold acid insoluble fraction of bones using previously described techniques (13, 18, 19, 20, 21). 2 h before the end of an experiment, 1 μCi of [methyl-^{3}H]thymidine (specific activity 20 Ci/mM, New England Nuclear, Boston, MA) was added to the medium of each bone. Experiments were terminated by washing the bones in saline, blotting them on filter paper, and placing them in a counting vial with 200 μl of 5% trichloroacetic acid (TCA) at 4°C. After 1 h, the TCA was removed to another counting vial and the bones were washed with a second aliquot of cold TCA. Both TCA samples were then pooled and the bones were washed with 1 ml of 70% ethanol, air-dried, and dissolved in 400 μl of *N*-chlorosuccinimide tissue solubilizer (Amersham Corp., Arlington Heights, IL) at room temperature for at least 8 h. The medium, TCA extracts, and *N*-chlorosuccinimide digest were counted in ACS scintillation fluid (Amersham Corp.) for ^{3}H and ^{45}Ca.

To minimize the possibility that changes in the endogenous thymidine pool size induced by HU altered the incorporation of [^{3}H]thymidine into the bones, experiments were performed in which a relatively larger concentration of unlabeled thymidine (100 μM) was added to the culture medium (18, 19). In these experiments, endogenous thymidine represented only a small fraction of the pool, and variations in its release induced by HU would have had only minimal effects on [^{3}H]thymidine incorporation.

Nonspecific incorporation of [^{3}H]thymidine into the cold acid insoluble fraction of bones was assessed by measuring the incorporation of [^{3}H]thymidine into the cold acid insoluble fraction of bones devitalized by three cycles of freezing at −80°C and thawing, and by measuring incorporation of [^{3}H]thymidine into the hot acid extracts of bone according to the method of Schneider (22) as modified by Canalis et al. (18, 19). In this procedure, DNA present in the bones after extraction with cold TCA was solubilized by treating the bones with 5% TCA at 90°C for 15 min. ^{3}H counts in the hot acid extracts were designated as the DNA fraction of the total [^{3}H]thymidine incorporated into the bones. ^{3}H counts remaining in the bone after hot acid extraction were designated as the non-DNA fraction of total [^{3}H]thymidine present in the bones after extraction with cold TCA.

Histology. For all histologic studies except autoradiography, bones were fixed in neutral buffered formalin, embedded in glycol-methacrylate, and 3–5 μm undecalcified sections were stained with toluidine blue. For autoradiography, bones were incubated with 5.0 μCi/ml [^{3}H]thymidine for the last 48 h of culture, sectioned, decalcified, dipped in NT2-B photographic emulsion (Eastman Kodak Co., Rochester, NY), and developed for 24–48 h. Slides were counter-stained with hematoxylin and eosin and random ordered. Osteoclasts were identified by their multinucleation, ruffled border, proximity to bone surfaces, and characteristic cytoplasm (9, 10). Labeled nuclei were defined as having five or more overlying silver grains.

Statistical methods. Calcium release and thymidine incorporation were analyzed according to the Student's *t* test. Autoradiography results were compared by chi-square analysis.

RESULTS

After 24 h, 1 mM HU caused a >95% inhibition of [^{3}H]thymidine incorporation into the cold acid insoluble fraction of cultured fetal rat bones (Table I). In bones devitalized by freeze-thawing the decrease in [^{3}H]thymidine incorporation into the cold acid insoluble fraction was similar to that seen in bones treated with HU. After 120 h, incorporation of [^{3}H]thymidine into the cold acid insoluble fraction of HU-treated bones was also decreased by >95%. In bones cultured for 6 h with 100 μM of unlabeled thymidine and then [^{3}H]thymidine, hot acid extraction of control bones solubilized >90% of the [^{3}H] counts remaining after cold acid extraction (Table II), indicating that the majority of [^{3}H]thymidine in the cold acid insoluble fraction was in DNA. When nonspecific incorporation of [^{3}H]thy-

TABLE I
Effects of HU on [^{3}H]Thymidine Incorporation into Fetal Rat Bone Cultures

	^{3}H cpm × 10^{-2}/total ^{45}Ca cpm	
	Cold acid extractable ^{3}H fraction	Cold acid insoluble ^{3}H fraction
Experiment I: 24-h culture		
Live bone control	38.1±2.3	20.2±3.8
Devitalized bone control	35.5±2.7	0.5±0.1
Live bone + HU (1 mM)	40.2±2.3	0.8±0.1*
Devitalized bone + HU (1 mM)	39.1±5.6	0.5±0.1
Experiment II: 120-h culture		
Live bone control	62.4±6.7	65.0±9.0
Live bone HU (1 mM)	58.7±8.2	1.6±0.6*

Values are mean±SE for 2 h of [^{3}H]thymidine labeling and six bones per group.
* Significant effect of HU, $P < 0.01$.

TABLE II

Effect of 100 μM Unlabeled Thymidine on [^{3}H]Thymidine in Fetal Rat Bone Cultures Treated with HU for 6 h

Groups	^{3}H cpm × 10^{-2}/total ^{45}Ca cpm		
	Cold acid extractable ^{3}H fraction	Hot acid extractable ^{3}H fraction	Hot acid insoluble ^{3}H fraction
Live bone control	15.8±2.9	1.84±0.36	0.10±0.02
Devitalized bone control	15.8±2.3	0.08±0.02	0.06±0.02
Live bone HU (1 mM)	15.2±3.2	0.24±0.04*‡	0.07±0.01
Devitalized bone HU	14.1±1.6	0.08±0.02	0.08±0.01

Values are means±SE for 2 h of [^{3}H]thymidine labeling and six bones per group.
* Significant effect of HU, $P < 0.01$.
‡ Significantly greater than devitalized bone, $P < 0.01$.

midine into the hot acid extractable or DNA fraction of devitalized bones was subtracted from the incorporation of [^{3}H]thymidine into the hot acid extracts of the corresponding live bones, 6 h of 1 mM HU treatment decreased [^{3}H]thymidine incorporation by 91%. However, incorporation of [^{3}H]thymidine into the hot acid extracts of live bones was significantly greater than into devitalized bones, indicating that some residual DNA synthesis probably occurred in live bones treated with 1 mM HU.

After 24, 48, and 120 h, release of ^{45}Ca from unstimulated cultures was inhibited by 1 mM HU (Tables III and IV). After 24 h, 100 nM PTH stimulated both ^{45}Ca release and [^{3}H]thymidine incorporation into the bones (Table IV). HU significantly decreased the magnitude of the 24-h resorptive response to 100 nM PTH by >40% and the incorporation of [^{3}H]thymidine into the cold acid insoluble fraction by >90%. However, because HU decreased both unstimulated and PTH-stimulated resorption equally, it did not alter the ratio of PTH-

TABLE III

Effects of HU on the Time Course of ^{45}Ca Release and [^{3}H]Thymidine Incorporation for Bones Stimulated by 1 μM PTH

Group	^{45}Ca Percent release	^{3}H cpm × 10^{-2}/total ^{45}Ca cpm	
		Cold acid extractable ^{3}H fraction	Cold acid insoluble ^{3}H fraction
Experiment I: 24 h			
Control	17±1	50.8±7.5	75.2±8.8
PTH (100 nM)	43±1*	52.5±3.6	122.1±7.6*
HU (1 mM)	10±1‡	61.3±4.9	3.2±0.4‡
HU and PTH	26±2*‡	69.2±5.1	3.6±0.2‡
Experiment II: 48 h			
Control	24±1	31.1±2.5	37.3±3.5
PTH (100 nM)	81±3*	29.2±3.9	32.9±3.1
HU (1 mM)	12±1‡	38.0±3.3	1.2±0.2‡
HU and PTH	75±4*	39.1±4.1	1.0±0.1‡
Experiment III: 120 h			
Control	56±6	27.7±1.0	26.54±3.8
PTH (100 mM)	99±1*	22.5±2.2	12.33±1.1*
HU (1 mM)	17±1	33.1±3.4	0.04±0.01‡
HU and PTH	99±1	21.4±2.9	0.02±0.01‡

Values are mean±SE for six bones per group.
* Significant effect of PTH, $P < 0.01$.
‡ Significant effect of HU, $P < 0.01$.

TABLE IV
Effect of HU on ^{45}Ca Release in Fetal Rat Bone Cultures

Group	^{45}Ca percent release		
	24 h	48 h	120 h
Control	10±1	17±1	36±3
PTH (30 nM)	16±3*	43±5‡	90±5‡
PTH (10 nM)	12±2	24±2	72±6‡
PTH (3 nM)	12±1	20±1	42±4
HU (1 mM)	7±1	12±1§	15±1§
HU and PTH (30 nM)	19±2‡	45±2‡	96±1‡
HU and PTH (10 nM)	12±1‡	26±2‡	92±3‡
HU and PTH (3 nM)	9±1‖	15±1§	50±6‡

Values are mean±SE for 6–12 bones per group.
Bones were cultured with HU for 144 h and PTH for the last 120 h.
Significant effect of PTH: * $P < 0.05$, ‡ $P < 0.01$.
Significant effect of HU: § $P < 0.01$, ‖ $P < 0.05$.

TABLE V
Effects of HU on the Number of Osteoclasts per Bone Section and the Number of Nuclei per Osteoclast in Fetal Rat Bone Cultures

Group	Osteoclasts per bone section	Nuclei per osteoclast
Control	7.5±1.4	4.2±0.6
Control + PTH (100 nM)	15.6±1.7*	4.2±0.2
HU (1 mM)	7.0±0.9	3.4±0.2
HU + PTH	13.7±1.7*	3.2±0.2‡

Values are mean±SE for 4–8 sections per group.
Bones were cultured with inhibitors of DNA synthesis for 72 h and with PTH for the last 48 h.
* Significant effect of PTH, $P < 0.01$.
‡ Significant effect of HU, $P < 0.01$.

treated to untreated resorptive responses (2.6±0.1 without HU vs. 2.7±0.2 with HU). After 48 and 120 h, HU decreased [^{3}H]thymidine incorporation into the cold acid insoluble fraction by >90%, but had no effect on resorptive responses to 100 nM PTH (Table III). After 48 h, 100 nM PTH had no effect on [^{3}H]thymidine incorporation into the bones, but did cause a significant decrease in this parameter after 120 h. After 24, 48, or 120 h, [^{3}H]thymidine in the cold acid extract of bones was not significantly altered by either 100 nM PTH or HU, although there was a trend for 100 nM PTH to decrease this measurement after 120 h.

At concentrations of PTH below 100 nM, HU decreased ^{45}Ca release only at 3 nM and only after 24 and 48 h (Table IV). As with 100 nM PTH, after 24 h, the decrease in the resorptive response with 3 nM PTH was proportional to the decrease seen in unstimulated resorption with HU alone. At all other time points and concentrations of PTH, the resorptive response with HU was equal to or greater than that seen in bone cultured without HU. In fact, because HU decreased unstimulated resorption, we could detect significant resorptive responses at lower PTH concentrations when HU was present in the medium.

At concentrations below 100 nM, there was no effect of PTH on [^{3}H]thymidine uptake into either the cold acid extractable or the cold acid insoluble fractions (data not shown).

After 48 h, 100 nM PTH increased the number of osteoclasts per bone section by twofold, regardless of whether or not HU was present in the cultures (Table V). There was no significant difference between HU-treated groups and control bones in the number of nuclei contained in osteoclasts (Table V), although the trend was for HU to decrease this parameter. In bones treated with HU and PTH, the decrease in the number of nuclei per osteoclasts was significant compared with bones treated with PTH alone. Since HU did not completely inhibit DNA synthesis, it was possible that the increased number of osteoclasts found in bones treated with HU and PTH resulted from a PTH-mediated increase in the replication of a small subpopulation of HU-resistant osteoclast precursors that then became available for incorporation into osteoclasts. To test this hypothesis, we examined autoradiographs of bones cultured with [^{3}H]thymidine for 48 h. In these experiments (Table VI), PTH alone significantly increased the percentage of osteoclast nuclei that contained [^{3}H]thymidine. HU decreased the percentage of osteoclast nuclei that were labeled by 96% in control cultures and by 93% in cultures containing PTH. In HU-treated cultures, PTH induced an increase in the percentage

TABLE VI
Effects of HU on the Incorporation of Recently Replicated Nuclei into Osteoclasts in Fetal Rat Bone Cultures

Group	Number of unlabeled osteoclast nuclei counted	Number of [^{3}H]thymidine labeled osteoclast nuclei counted	Percent of osteoclast nuclei that were labeled
Control	111	16	12.6
PTH (100 nM)	380	139	26.8*
HU (1 mM)	190	1	0.5‡
HU + PTH	305	6	1.9‡

Values obtained from counting nuclei in from 31 to 125 osteoclasts.
Bones were cultured with HU for 72 h and with PTH and [^{3}H]thymidine for the last 48 h.
* Significant effect of PTH, $P < 0.01$.
‡ Significant effect of HU, $P < 0.01$.

of osteoclast nuclei containing label that was greater than in controls. This result may indicate that some replication of osteoclast precursors occurred in bones treated with PTH and HU. However, the actual number of labeled nuclei detected in either HU-treated group was small and not significantly different, implying that the majority of nuclei found in osteoclasts of bones treated with either HU or HU + PTH derived from precursors that had not recently divided.

DISCUSSION

The mechanism by which HU inhibits DNA synthesis in cells has been well studied and is believed to result from an action of this agent on the enzyme ribonucleotide reductase (23–25). In cell cultures this effect is rapid and occurs within 30 min. In our studies, HU inhibited [^{3}H]thymidine incorporation into DNA maximally after 6 h, the earliest time point we examined. HU can also have inhibitory effects on cellular RNA and protein synthesis (23). However, we doubt these latter actions contributed to the decrease in resorption seen in either unstimulated cultures or cultures treated with 100 nM PTH for 24 h because, at other concentrations of PTH, 1 mM HU either had no effect or enhanced ^{45}Ca release, and because HU did not block the increase in the number of osteoclasts present in bones treated with 100 nM PTH. We do not believe that changes in the free thymidine pool caused by HU could be responsible for the decreased [^{3}H]thymidine incorporation seen because HU reduced [^{3}H]thymidine incorporation into the DNA fraction of the cold acid insoluble [^{3}H] counts by 91%, even in the presence of 100 μM unlabeled thymidine. This result, and our histologic data showing HU to cause a marked decrease in [^{3}H]thymidine labeling of osteoclast nuclei, leads us to conclude that in our bone cultures 1 mM HU markedly inhibited DNA synthesis in the precursor cells of the osteoclasts.

The effect of 100 nM PTH on [^{3}H]thymidine incorporation was biphasic, being increased at 24 h and decreased at 120 h. This result suggests that 100 nM PTH produced a synchronous wave of DNA synthesis in the bones. The decreases in PTH-stimulated ^{45}Ca release caused by HU after 24 h with 100 and 3 nM PTH and after 48 h with 3 nM PTH were proportional to the decrease in ^{45}Ca release caused by HU alone and are possibly related to this latter effect. Why other concentrations of PTH and other time points for the two concentrations mentioned above also did not show a proportional decrease when HU was present is unknown, but suggest that an additional resorptive mechanism might be induced by PTH in the presence of HU. Because HU did not prevent PTH from inducing increases in ^{45}Ca release and osteoclast formation, we conclude that in fetal rat bone cultures these actions of PTH do not require the replication of osteoclast precursor cells. Rather, it appears that PTH can activate the available pool of osteoclast precursor cells to fuse into new osteoclasts through a process that is independent of cell replication. Further evidence that PTH activates preexisting osteoclasts to resorb and causes increased fusion of osteoclast precursors into preexisting osteoclasts comes from previous studies of bone cultures which have shown PTH to rapidly activate existing osteoclasts (10) and to form new osteoclasts before the incorporation of [^{3}H]thymidine into osteoclast nuclei (26).

In agreement with our results, studies of the effects of DNA synthesis inhibitors on myoblasts have shown that these cells are also capable of becoming multinucleated when cell replication is inhibited (27). Recently, Kreiger et al. (28) found that x-irradiation did not inhibit PTH from stimulated calcium release from newborn mouse calvarial cultures or from increasing osteoclast number after 48 h of culture. However, in contrast to our results, they did not find x-irradiation to decrease unstimulated levels of bone resorption. The reason for this difference between our results and theirs is unknown, but may result from species differences, differences between the action of HU and x-irradiation on bone, or differences in the technique used to measure bone resorption.

In contrast to previous studies (10), we failed to detect a change in the number of nuclei in osteoclasts after PTH treatment. However, this response is small and has not always been found by others (29). If changes in the number of nuclei per osteoclast do relate to bone resorbing activity, it is possible that the decrease in ^{45}Ca release seen after 24 h of culture in bones treated with 100 nM PTH and HU was related to the decreased number of nuclei present in the osteoclasts of these bones. It is less likely that the inhibition of ^{45}Ca release seen in bones treated with HU alone is related to changes in the number of osteoclasts per bone or nuclei per osteoclast since differences in these latters' parameter were not significant between this group and control. Hence, an unknown mechanism appears responsible for the decrease in ^{45}Ca release seen in unstimulated HU-treated bones, which may or may not be related to the decrease in cell replication caused by HU.

Because PTH stimulates DNA synthesis in bone but does not appear to require this effect to induce new osteoclast formation, it seems less likely that osteoclast precursors respond mitogenically to this hormone. Rather, it seems that the cells that replicate in response to this hormone are instead precursors of osteoblasts or another nonresorbing bone cell. In this regard, PTH has recently been shown to increase cell replication in a clonal rat osteosarcoma cell line having many bio-

chemical characteristics of osteoblasts (30). Alternatively, if PTH affects the replication of osteoclast precursors it is possible that these responses are important only for mediating effects that occur later than can be measured in our relatively short-term cultures.

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