1,25-Dihydroxyvitamin D₃ Increases Citrate Secretion from Osteosarcoma Cells*

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Rat osteosarcoma cells respond to 1,25-dihydroxyvitamin D₃ with a 6- to 10-fold increase in the secretion of citric acid. The time required to attain a half-maximal response is 12 h, a time course which is consistent with the postulated steroidal hormone action of this vitamin D metabolite. The citrate response is achieved by physiological concentrations of 1,25-dihydroxyvitamin D₃, with half of the maximal response at a vitamin concentration of 0.03 ng/ml. Both the time course and the dose dependence of the citrate response closely parallel the previously reported stimulation of bone Gla protein synthesis by 1,25-dihydroxyvitamin D₃ in these cells.

Citrate and bone Gla protein bind avidly to bone mineral and are numerically the most abundant organoic acid and protein in bone. The parallel secretion of both in 1,25-dihydroxyvitamin D₃-treated osteoblastic cells suggests that they may act in tandem to mediate an action of this vitamin D metabolite on the mineral phase of bone.

The discovery of large quantities of citric acid in bone and its relative decrease in rickets raised the question of the relationship between citric acid and calcification (1). Subsequent studies established that both vitamin D and parathyroid hormone increase bone and serum citrate (2-10). The regulation of citrate secretion suggested by these observations led to the speculation that citrate may, by virtue of its interactions with calcium (11) and hydroxyapatite (12), play a role in the mobilization of calcium from bone in response to vitamin D and parathyroid hormone (1, 2, 13).

Citrate Analyses-An initial purification of media citrate was achieved by BaSO₄ adsorption. In a typical experiment 2 ml of media with the same level of 1,25(OH)₂D₃ and the cells were cultured for another 12 h prior to the removal of media for citrate analysis. Cell numbers were determined with a Coulter counter after trypsin treatment. BGP levels in culture media were determined by triplicate radioimmunoassay as described (25).

EXPERIMENTAL PROCEDURES

Materials—Rat osteosarcoma cell lines ROS 17/2, 2/3, 25/1, and 25/4 were generously provided by Dr. Gideon Rodan. 1,25-Dihydroxyvitamin D₃ was a gift from Hoffman-LaRoche, and vitamin D₃ was obtained from Sigma. NADH, citrate lyase, and malate dehydrogenase were purchased from Boehringer Mannheim, and 1,5-³C-labeled citrate (52 Ci/mmol) was a product of New England Nuclear. Intact bovine PTH (1-84) was obtained from the National Hormone and Pituitary Program of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, and synthetic bovine PTH (1-34) was purchased from Beckman Bioproducts, Operations.

Osteosarcoma Cell Culture—The procedures used to maintain osteosarcoma cells in culture have been described (24). For measurement of the effect of 1,25(OH)₂D₃ on citrate secretion, cells were grown to confluence in 60-mm culture plates with Coon’s F12 medium supplemented with 10% fetal calf serum. The media was then changed to 2% fetal calf serum and the cells were cultured for another 24 h. At this time the experiment was initiated by changing to 5 ml of serum-free Coon’s F12 medium containing either the desired concentration of 1,25(OH)₂D₃ or ethanol vehicle alone. To assess the time course of the citrate and BGP response to 1,25(OH)₂D₃, the entire cell media was removed from two plates treated with 1 ng/ml of 1,25(OH)₂D₃ and two plates treated with vehicle at each time point. To determine the dose dependence of the citrate response to 1,25(OH)₂D₃, cells were first pretreated with the desired concentration of 1,25(OH)₂D₃ for 24 h. The media was then exchanged for new media with the same level of 1,25(OH)₂D₃ and the cells were cultured for an additional 12 h prior to the removal of media for citrate analysis. Cell numbers were determined with a Coulter counter after trypsin treatment. BGP levels in culture media were determined by triplicate radioimmunoassay as described (25).

Citrates Analyses—An initial purification of media citrate was achieved by BaSO₄ adsorption. In a typical experiment 2 ml of media were combined with 400 mg of BaSO₄, mixed at 25 °C for 5 min, and then centrifuged. The pellet was washed twice with 2 ml of distilled H₂O and then resuspended in 1 ml of 0.1% H₃PO₄ to desorb bound citrate and again centrifuged. In a typical analysis, 100 μl of desorbed citrate solution were applied to two Bio-Rad Aminex HPX-87H organic acid columns (300 × 7.8 mm) joined in series and maintained at 65 °C. Eluent was 0.1% H₃PO₄ delivered at 0.7 ml/min by a Waters high pressure liquid chromatography system. Eluent was monitored at 220 nm, and peak areas were integrated by hand from the recorder trace. When greater sensitivity of citrate analysis was required, citric acid was desorbed from BaSO₄ with 0.2 M HCl and concentrated by drying. The dry desorbed citric acid was then dissolved in 100 μl of 0.1% H₃PO₄ and applied to the organic acid columns. Recovery of citrate was determined by adding 10 μl of ³C-labeled citric acid (0.1 mCi/ml) to media.
samples from control and 1,25(OH)_{2}D_{3}-treated cultures. In both cases, about 52% of the citrate label added to media was recovered after high pressure liquid chromatography. All data presented here have been corrected to give the actual amount of citrate in the original medium.

To verify the identity of the putative citrate component, effluent corresponding to a retention time of 16.8 min was pooled, dried, and analyzed by liquid partition chromatography as described (27). The putative citrate component was also converted to its trimethylsilyl derivative and analyzed by gas chromatography-mass spectroscopy on a Finnigan 4021. An OV17 column (Supelco) was used for gas chromatography, and the mass spectrum was determined by electron impact ionization at 70 eV.

Citrates lyase treatment (26) was used to establish peak identity by selective destruction of citrate in media samples. In a typical experiment 1 ml of media was mixed with 50 μl of 1 M Tris, pH 8.2, 5 μl of 0.04 M ZnSO₄, 10 μl of 0.1 M NADH, and 5 μl of malate dehydrogenase (5 mg/ml in 3.2 M (NH₄)₂SO₄). Forty μl of citrate lyase (37.5 mg/ml in 0.15 M Tris, HCl, pH 8.2) was then added, and the reaction was incubated for 1.5 h at 25 °C before BaSO₄ adsorption and analysis. Control samples were treated in the same manner except 40 μl of 0.15 M Tris, pH 8.2, was substituted for citrate lyase. In some media samples from 1,25(OH)_{2}D_{3}-treated cells, citrate levels were high enough to permit direct determination by the citrate lyase method (26). In these samples, the average level of citrate determined by citrate lyase was in good agreement with the average level determined by the high pressure liquid chromatography method.

**RESULTS**

Media levels of citric acid were quantified by high pressure liquid chromatography on an organic acid column after adsorption on BaSO₄. As shown in Fig. 1, media from cells treated with 1,25(OH)_{2}D_{3} showed increased levels of a component with a retention time of 16.8 min, a time identical to that of citric acid. Treatment of media with citrate lyase prior to BaSO₄ adsorption selectively abolished this putative citrate component (Fig. 1). To further confirm identification, the presumptive citrate fraction from the high pressure liquid chromatography effluent was isolated and subjected to several further tests. Liquid partition chromatography of this component over silica (27) gave a single peak with a retention time identical to citrate. After trimethylsilylation, gas-liquid chromatography of presumptive media citrate yielded a single component in the expected position of citrate which, on mass spectral analysis, proved to be identical to the trimethylsilyl derivative of citric acid.

One additional component resolved by high pressure liquid chromatography of media was also consistently stimulated by 1,25(OH)_{2}D_{3} treatment, a component which eluted with a retention time of 14.9 min (Fig. 1). This component coelutes with transaconitate. If it is transaconitate, the level of transaconitate computed from the molar extinction coefficient is 0.5% of the citrate level in media from 1,25(OH)_{2}D_{3}-treated cells.

Fig. 2 compares the time course of the increase in citrate and BGP levels in culture media following the addition of 1 ng/ml of 1,25(OH)_{2}D_{3}. The time required to attain a half-maximal response to 1,25(OH)_{2}D_{3} is 12 h for citrate compared to 9.5 h for BGP. This time course for the BGP response is essentially identical to that reported earlier (18). Both the accumulation of citrate and of BGP in cell media cease after 24 h (Fig. 2), whether or not cells are treated with 1,25(OH)_{2}D_{3}. This effect has been noted previously for the accumulation of BGP in media (24). If the media is exchanged for fresh media at 24 h, BGP and citrate again accumulate in media and the rate of increase for each substance remains 6- to 10-fold elevated in the cells treated continuously with 1,25(OH)_{2}D_{3}.

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**Fig. 1.** Representative recorder tracings for the quantitative analysis of citrate by high pressure liquid chromatography. 24-h culture media from control cells (left) and cells treated with 1 ng/ml of 1,25(OH)_{2}D_{3} (center); 24-h culture media from cells treated with 1 ng/ml of 1,25(OH)_{2}D_{3} after subsequent digestion with citrate lyase (right) as described under "Experimental Procedures." Culture media was adsorbed onto BaSO₄, eluted with 0.1% H₃PO₄, and applied to two Bio-Rad Aminex columns (300 x 7.8 mm) joined in series and equilibrated with 0.1% H₃PO₄. Flow rate, 0.7 ml/min; temperature, 65 °C.
The dependence of citrate secretion on the concentration of 1,25(OH)₂D₃ was determined after 24-h pretreatment with the vitamin at the test concentration to ensure that the response was maximal. As can be seen in Fig. 3, a 1,25(OH)₂D₃ concentration of 0.03 ng/ml is required for half-maximal stimulation of citrate secretion. This dose dependence is essentially identical to that previously seen for the stimulation of BGP secretion by these cells (18).

The hormonal specificity of the citrate response was evaluated using vitamin D₃ and parathyroid hormone. Vitamin D₃ gave the same magnitude of citrate secretion response as did 1,25(OH)₂D₃, but the concentration required for half-maximal stimulation, 1 μg/ml, was over 10³ greater than the 1,25(OH)₂D₃ concentration needed for this level of response. This difference in dose dependence parallels the difference in the affinity of the cytosolic 1,25(OH)₂D₃ receptor for these molecules. Intact bovine PTH and synthetic bovine PTH (1-34) both failed to stimulate citrate accumulation in media after 24 or 48 h of treatment at hormone concentrations of 4 × 10⁻⁹ and 10⁻⁷ M.

Several clonal osteosarcoma cell lines were tested for a citrate secretion response to 1,25(OH)₂D₃ in order to better define the bone cell phenotype responsible for this effect. As may be seen in Table I, the only cell lines which increase citrate secretion in response to 1,25(OH)₂D₃ are ROS 2/3 and 17/2. These cell lines have a more osteoblastic phenotype than the 25/1 and 25/4 cell lines (28) and are the only cell lines tested which also synthesize BGP (24). The ability to increase citrate secretion in response to 1,25(OH)₂D₃, therefore, appears to be another marker for the osteoblastic phenotype.

**DISCUSSION**

The present study demonstrates that physiological 1,25(OH)₂D₃ concentrations dramatically and specifically elevate media levels of citric acid in osteosarcoma cells. The kinetics of the citrate response is essentially identical to the BGP response of these cells (Fig. 1) and suggests that both the BGP and citrate effects may reflect the activation of specific genes by 1,25(OH)₂D₃ in the manner postulated for the cytosolic calcium-binding protein of intestine (29). To our knowledge, the present results are the first demonstration that 1,25(OH)₂D₃ stimulates the secretion of citrate from bone cells in culture.

In order to determine citrate at the levels present in cell media, it was necessary to develop an assay more sensitive than the citrate lyase procedure. The present assay employs an initial BaSO₄ adsorption step to extract citrate from most proteins and organic acids. Citric acid is then desorbed with acid and analyzed by high pressure liquid chromatography. The sensitivity limit of this assay is determined primarily by the photometer used to monitor the effluent. In our system, with a 0.02 absorbance unit full scale recorder, we could quantitatively measure 1 nmol of citrate in 5 ml of media. This is over 100 times less than the 110 nmol in 5 ml of media limit of the citrate lyase assay. Among the other advantages of this method are that labeled citrate can be used as an internal standard to evaluate recovery and that the presumptive citrate component can be recovered and subjected to mass spectral analysis for structural verification.

Previous studies with bone in organ culture and with isolated osteoblastic cells in primary culture have demonstrated that 1,25(OH)₂D₃ and PTH dramatically decrease the release of ¹⁴C₀₂ from 1,5-¹⁴C-labeled citrate (17, 30, 31). These results have been interpreted as evidence for decreased intracellular decarboxylation of citrate in the tricarboxylic acid cycle of hormone-treated bone cells, a decrease which is presumed to
increase intracellular citrate levels and so drive an increase in the passive diffusion of citrate from cells. There are several arguments against this interpretation of the $^{14}$C02 release studies. In order to explain the 10- to 15-fold increase in citrate secretion observed in our investigations, it would be necessary to postulate that intracellular citrate levels could be increased to this extent without distorting cellular metabolism in the pathways now known to be allosterically regulated by citrate. In addition, decreased citrate decarboxylation in the tricarboxylic acid cycle implies that the aerobic metabolic rate in hormone-treated cells is correspondingly reduced, an effect which has not been observed (15, 31). It seems to us more likely that the decreased release of $^{14}$CO2 from labeled citrate is due to the dilution of citrate label by secreted citrate prior to its entry into the cell. While we presently favor the alternative hypothesis that 1,25(OH)$_2$D$_3$ directly increases the rate of citrate transport from bone cells, it is clear that further studies will be needed to determine the relationship between hormone effects on $^{14}$C02 release from labeled citrate and citrate secretion.

The observation that 1,25(OH)$_2$D$_3$ stimulates a parallel secretion of citrate and BGP from osteoblastic bone cells suggests that these molecules may act in tandem in bone. Since both substances bind strongly to hydroxyapatite (11, 20, 21) and probably account for most of the molecules bound to the surface of bone mineral, the most likely site of their action is bone mineral. Although the mechanisms by which BGP and citrate affect bone mineral are not yet understood, recent studies have demonstrated that warfarin, a vitamin K antagonist, produces an excessive mineralization disorder characterized by complete closure of the growth plate in rats. It has been postulated that this disorder is caused by an inability to inhibit spontaneous hydroxyapatite growth (22) due to an absence of the vitamin K-dependent amino acid Gla in BGP. It is possible that citrate may also play a role in the inhibition of hydroxyapatite growth, perhaps by regulating the solubility of bone mineral (1, 12, 15). If the function of BGP and citrate is indeed the inhibition of bone mineralization, the increased serum 1,25(OH)$_2$D$_3$ levels in Ca$^{2+}$-deficient animals may act to restore serum Ca$^{2+}$ levels partly by stimulating BGP and citrate production and thereby reducing the extent to which newly formed bone is mineralized.
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