Fatty acid oxidation in bone tissue and bone cells in culture

Characterization and hormonal influences

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Fatty acid oxidation and its hormonal modulation were investigated in cultured rat calvaria and in cultivated cell populations. The latter were obtained from calvaria of newborn rats by sequential time-dependent digestion with collagenase, yielding eight cell populations: the early ones containing mainly fibroblasts, the middle ones being osteoblast-like, and late ones osteoblast–osteocyte-like. In calvaria, fatty acid oxidation was increased by adding 0.1 mM- and 1.0 mM-palmitate to the medium, containing 10% (v/v) fetal-calf serum. No effect was found after parathyrin addition *in vitro* or when injected *in vivo*. All cell populations obtained by sequential digestion were found to oxidize palmitate, whereby the osteoblast-like cells showed a lower oxidation rate than the other populations. Both parathyrin and calcitonin had no effect on fatty acid oxidation. 1,25-Dihydroxycholecalciferol at 1–100 nM and 24,25-dihydroxycholecalciferol at 100 nM increased oxidation primarily in the population enriched with osteoblast-like cells. Insulin at 1.6 μ M diminished it in the cell populations enriched with osteoblast-like cells and in the late bone-cell fraction. However, glucagon had no effect. The energy provided by fatty acid oxidation in this system is approx. 40–80% of glucose metabolism, suggesting that this event may be of importance in the energy metabolism of bone.

INTRODUCTION

Borle *et al.* (1960a,b) showed that glucose appears to be an important nutrient for bone tissue. Since these early investigations an impressive body of literature has appeared, centering on studies of glucose utilization by bone and its regulation by insulin (Schmid *et al.*, 1982, 1983) as well as by the calciotropic hormones parathyrin (Cohn & Forscher, 1962; Hahn *et al.*, 1971, 1985; Hekkelman *et al.*, 1974; Neuman *et al.*, 1978; Ituarte *et al.*, 1985) and 1,25(OH)₂D₃ (Brommage & Neuman, 1979). Although knowledge on this subject has advanced considerably, very little is known on fatty acid metabolism by this tissue. Previously we have shown that bisphosphonates, compounds known to cause severe alteration in skeletal tissue (Fleisch, 1983), are inhibitory to the glycolytic pathway of bone cells (Fast *et al.*, 1978) and chondrocytes (Guenther *et al.*, 1979), without appreciably affecting replication of either cell type (Guenther *et al.*, 1979, 1984). Since glucose metabolism constitutes an important pathway in bone tissue, experiments were designed to examine the possibility that energy derived from fatty acid oxidation may offset the bisphosphonate-mediated loss of energy generated by the glycolytic pathway. Data obtained by that study showed that some bisphosphonates induced, concomitantly with the decrease in glycolysis, a rise in fatty acid oxidation in a mixed bone-cell population (Felix & Fleisch, 1981a; Felix *et al.*, 1986).

The present study was undertaken to investigate the role that fatty acids may play in the overall energy metabolism of bone tissue. For this, rat calvaria and, since any effect on a specific cell type might be undetected

in the whole tissue, rat calvaria cells separated into different bone-cell populations by sequential digestion, were studied. These individual cell populations were also used to investigate which hormones might influence this fatty acid oxidation.

METHODS

Materials

Collagenase (*Clostridium histolyticum*, type I; 175 μ mol/min per mg), trypsin (bovine pancreas, type III; 10200 μ mol/min per mg), hyaluronidase (bovine testes, type I; 415 μ mol/min per mg), β -hydroxybutyrate dehydrogenase (*Rhodospseudomonas*, type II; 6.2 μ mol/min per mg), bovine albumin (Cohn fraction V), bovine albumin (essentially fatty-acid-free), insulin (pig pancreas, 25.5 units/mg) and glucagon (mixture of bovine and pig pancreas) were obtained from Sigma, St. Louis, MO, U.S.A. Lactate dehydrogenase (rabbit muscle; 550 μ mol/min per mg) and a kit for enzymic citrate determination were purchased from Boehringer, Mannheim, Germany, and parathyrin (synthetic bovine 1–34) was from Beckman, Palo Alto, CA, U.S.A. Salmon calcitonin (synthetic) was obtained from Sandoz; 1,25(OH)₂D₃ and 24,25(OH)₂D₃ were gifts from Hoffmann–La Roche, Basel, Switzerland. Minimum essential medium (MEM), MEM amino acids (50 $\times$ conc.) and MEM vitamins (100 $\times$ conc.) were obtained from Gibco, Paisley, Scotland, U.K. Foetal-calf serum was bought from Nabi, Miami, FL, U.S.A. Penicillin and streptomycin were purchased from Seromed, Munich, Germany, and fungizone was from Squibb, Princeton, NJ, U.S.A.

Abbreviations used: 1,25(OH)₂D₃, 1,25-dihydroxycholecalciferol; 24,25(OH)₂D₃, 24,25-dihydroxycholecalciferol; MEM, minimal essential medium.

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[U-¹⁴C]Palmitate (800 Ci/mol) and [U-¹⁴C]glucose (329 Ci/mol) were obtained from New England Nuclear, Boston, MA, U.S.A. and 5-(biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole ('butyl-PBD') was from Ciba-Geigy, Basel, Switzerland. All other reagents were of analytical grade and were obtained from Merck, Darmstadt, Germany.

Tissue culture

Calvaria halves were excised from 4-day-old Wistar rats, cut in halves and immediately placed in Tyrode solution until all explants were dissected. They were then incubated in 11 ml glass ampoules (17 mm inner diameter) containing 0.5 or 1 ml of Earle's salt solution, 1 mM-[U-¹⁴C]palmitate (0.2 µCi/ml) and 0.45 mM (3%, w/v) fatty-acid-free bovine serum albumin. A stock solution of 4 mM-palmitate and 1.8 mM-albumin was prepared by the method of Lorch & Gey (1966).

Cell preparation and cultivation

Different bone cell populations were obtained by sequential digestion of calvaria from 1-day-old Wistar rats by a slight modification of the method of Luben *et al.* (1976), as described by Guenther *et al.* (1984). In addition, we have continued the digestion for 100 min, yielding three more cell populations. Briefly, 60 calvaria were slowly stirred at room temperature in 10 ml of a solution containing 0.35 mg of hyaluronidase/ml, 0.6 mg of collagenase/ml, 0.35 mg of trypsin/ml, 96 mM-NaCl, 1.9 mM-KCl, 0.33 mM-NaH₂PO₄ and 80 mM-EDTA. Cells released during seven consecutive digestion periods each of 20 min and one of 60 min were collected by centrifugation for 7 min at 400 *g* and designated as populations I–VIII. Cells from each population were cultured by plating 285 000 cells, suspended in 10 ml of medium in 75 cm² plastic flasks (Corning Glass Works, Corning, NY, U.S.A.). The culture medium, MEM with Earle's salts, was supplemented with 10% (v/v) foetal-calf serum, 2 mM-glutamine, 60 µg of penicillin/ml, 100 µg of streptomycin/ml and 0.25 µg of fungizone/ml, and, from day 2 of culture onwards with 0.28 mM-ascorbic acid. After cells had reached confluence, usually after 10 days, they were brought into suspension by treating them for 1½ h with 10 ml of a solution containing 10 mg of collagenase, 5 mg of trypsin, 116 mM-NaCl, 5.4 mM-KCl, 5.6 mM-glucose, 26.2 mM-NaHCO₃ and 50 µg of Phenol Red. The cells were subsequently centrifuged at 400 *g* for 7 min, and the resulting cell pellet was resuspended at a density of 100 000 cells/ml of medium. Then 0.5 ml portions of this suspension were placed into glass ampoules (inner diameter 17 mm) and cultured for 7 days, at which time the cells were confluent.

Cells were counted with a Coulter counter (Fast *et al.*, 1978).

Addition of hormones

The various hormones were dissolved in the following vehicle solutions: 0.1 mM-insulin and -glucagon in 5 mM-HCl containing 0.3 mM-albumin; 3.6 µM-parathyryl in a solution containing 150 mM-NaCl, 21 mM-glutathione, 10 mM-HCl and 0.15 mM-albumin; 5.5 µM-calcitonin in 33 mM-acetic acid/24 mM-sodium acetate/128 mM-NaCl. A portion (11 µl) of 1 mM-1,25(OH)₂D₃ or -24,25(OH)₂D₃ (dissolved in ethanol) was added to 1 ml of foetal-calf serum, which was then added to the

medium, giving a final concentration of ethanol of 0.01% for 1,25(OH)₂D₃ and 0.1% for 24,25(OH)₂D₃. Control experiments without the hormones were carried out with the respective vehicles.

Fatty acid oxidation

Bone tissue. Before the start of the incubation, the ampoules were flushed with O₂/CO₂ (19:1) for 1 min, closed with a rubber sleeve and then sealed with Balco 270 (Bally, Schönenwerd, Switzerland). After an incubation period of 5 h at 37 °C, 0.2 ml of ethanolamine was injected through the rubber sleeve into a suspended plastic centre well containing a folded filter paper to absorb ¹⁴CO₂ formed. The reaction was stopped by adding 0.35 ml of 0.33 M-HClO₄ into the culture medium. The ampoules were then shaken for 1 h at room temperature, after which the ethanolamine and the filter were transferred to scintillation vials and the radioactivity was determined. The calvaria halves were recovered and their DNA contents, expressed as deoxyribose, determined (Shinoda *et al.*, 1983).

To measure water-soluble radioactivity, a further 50 µl of 9.61 M-HClO₄ was added to the ampoules to precipitate the proteins in the medium. The supernatant was neutralized with 2.5 M-KHCO₃, and 0.5 ml of the neutralized solution were mixed with 0.5 ml of 3 M-acetate buffer. The non-water-soluble radioactivity was twice extracted with light petroleum (b.p. 40–80 °C). The total fatty acid oxidation is expressed as the sum of ¹⁴CO₂ and of water-soluble radioactivity. Its rate was found to be constant during the time of incubation.

Bone cells. The medium was removed on day 7 and replaced with MEM, containing 10% foetal-calf serum and [U-¹⁴C]palmitate (0.5 µCi/ml). Non-radioactive palmitate was added from a stock solution containing 2 mM-palmitate and 0.4 mM fatty-acid-free bovine serum albumin. The ampoule was flushed for 1 min with air/CO₂ (19:1), and then closed with a rubber sleeve and sealed. After a 16 h incubation, ¹⁴CO₂ production and the content of the water-soluble radioactivity in the medium were determined as described above for calvaria halves.

For calculation of the specific radioactivity of the palmitate, the fatty acid originally present in the serum [0.11 ± 0.02 mM (3); mean ± S.E.M. (*n*)] was regarded as palmitate.

Characterization of water-soluble radioactivity

H.p.l.c. was carried out with an ion-exchange column (Animex HPX-87; Bio-Rad, Richmond, CA, U.S.A.) with 17 mM-H₂SO₄ as eluent at a flow rate of 0.6 ml/min; 270 µl fractions were collected. To precipitate the proteins, 125 µl of 9.61 M-HClO₄ was added to 1 ml of medium. After centrifugation, the supernatant was neutralized with 2.5 M-KHCO₃. The extract was concentrated 2.5–4.5-fold by freeze-drying, and then 100 µl was injected into the column, or the extract was used for treatment with enzymes.

Citrate was converted into malate and acetate with a commercial kit from Boehringer. A 0.2 ml portion of buffer containing 0.51 M-glycylglycine, pH 7.8, 60 µM-Zn²⁺ and 1.5 mM-NADH was added to 1 ml of 5-fold-concentrated extract and incubated with malate dehydrogenase (3.8 µmol/min per ml) and lactate dehydrogenase (7.9 µmol/min per ml) at room temperature.

Then 550 μ l of this incubation mixture was further incubated with citrate lyase (0.29 μ mol/min per ml) for 30 min. Extract treated with all the enzymes, or extract treated only with malate dehydrogenase and lactate dehydrogenase, were injected into the chromatography column.

To convert any possible β -hydroxybutyrate into acetoacetate, 20 μ l of 0.5 M-glycine was added to 1 ml of extract, the pH adjusted to 10.0 and 0.1 ml of 47 mM-NAD⁺ was added. Half of the mixture was incubated with β -hydroxybutyrate dehydrogenase (0.05 μ mol/min per ml) for 30 min.

To test the existence of acetoacetate, 1 ml of extract was mixed with 20 μ l of 0.5 M-sodium phosphate buffer, adjusted to pH 6.9; 20 μ l of 17 mM-NADH was added. Then β -hydroxybutyrate dehydrogenase (0.05 μ mol/min per ml) was added to 500 μ l of the supernatant to transform acetoacetate into β -hydroxybutyrate. Extracts treated or not treated with β -hydroxybutyrate dehydrogenase were injected into the chromatography column.

Glucose oxidation

[U-¹⁴C]Glucose was used, and ¹⁴CO₂ was determined as described for palmitate oxidation.

Lactate determination

After protein precipitation and neutralization, the lactate in the medium was determined by using lactate dehydrogenase (Engel & Jones, 1978).

Determination of alkaline phosphatase

Alkaline phosphatase activity was determined as previously described (Felix & Fleisch, 1979).

Parathyrin response of bone-cell populations

The response of bone cells to a challenge of 24 nm-parathyrin during 10 min at 37 °C was carried out as previously reported (Felix & Fleisch, 1981b). Cyclic AMP production was measured as described by Brown *et al.* (1971).

Statistics

The values are expressed as means $\pm$ S.E.M. for calvaria halves or glass ampoules containing the cultured cells. Differences of means (in consideration of variable variances) were tested by the *t*-test of Welch. The mean ratios (treated/control) were compared by *t*-test with the hypothetical mean 1. When pairs of calvaria halves were compared, pair analysis was made. For these tests see Sachs (1973).

RESULTS

Whole calvaria

Influence of O₂. Although the oxidation of palmitate was not influenced by varying the O₂ partial pressure, this was not the case for lactate production, which decreased when O₂ was raised beyond 20% (Table 1). Therefore, 95% O₂ was used to supply optimal O₂.

Influence of palmitate concentration; ATP production. The increase in palmitate concentration in the medium resulted in an increase of the oxidation rate (Table 2). This rate was not significantly influenced by changing the molar ratio palmitate/albumin from 5 to 2.2. On the

Table 1. Effect of O₂ partial pressure on palmitate oxidation and lactate production in calvaria *in vitro*

Calvaria halves from 4-day-old rats were incubated for 5 h under three different partial O₂ pressures. The gas mixtures used were O₂/CO₂/N₂ (20:5:75, 58:5:37 and 95:5:0). The medium contained 1 mM-[U-¹⁴C]palmitate, 0.45 mM-albumin and 10% foetal-calf serum. The results are means $\pm$ S.E.M., for the numbers of calvaria halves given in parentheses. ***P* < 0.01, ****P* < 0.001, significantly different from the values obtained with 20% O₂.

O ₂ partial pressure (%)	Palmitate oxidation (μ mol/5 h per mol of deoxyribose)	Lactate production (mol/5 h per mol of deoxyribose)
20	601 $\pm$ 35 (7)	74.1 $\pm$ 8.5 (7)
58	620 $\pm$ 28 (4)	43.0 $\pm$ 0.8 (4)**
95	642 $\pm$ 23 (7)	50.9 $\pm$ 2.9 (7)***

other hand, the production of lactate was not affected by changes of either the palmitate concentration or the palmitate/albumin ratio.

The amount of energy supplied by fatty acid oxidation was found to be quite remarkable. At concentrations of 1 mM-palmitate and 5.5 mM-glucose, 0.85 $\pm$ 0.06(19) μ mol of this fatty acid/mol of deoxyribose were oxidized in 5 h [mean $\pm$ S.E.M. (*n*)], and 72 $\pm$ 5(20) μ mol of lactate/mol of deoxyribose were formed in 5 h. When [U-¹⁴C]-glucose was used under the same conditions, 1.6 $\pm$ 0.1 (4) μ mol of glucose/mol of deoxyribose were oxidized to CO₂ in 5 h. Calculation of the ATP produced gives 110 μ mol for fatty acid, 72 μ mol for lactate and 57 μ mol for glucose oxidation.

Water-soluble radioactivity. Radioactive products formed by the oxidation of [U-¹⁴C]palmitate were shown to be not only ¹⁴CO₂, but also water-soluble radioactivity (about 0.7–1.7 times that of ¹⁴CO₂). By h.p.l.c., this radioactivity was resolved into two peaks at positions where citrate and lactate were found to be eluted. The presence of citrate was confirmed after treating the alleged citrate peak with citrate lyase and malate dehydrogenase. This peak disappeared, and new products, malate and acetate, were identified. No peaks were found in the position where acetoacetate or hydroxybutyrate would be eluted from the column. Treatment with hydroxybutyrate dehydrogenase to convert either hydroxybutyrate or acetoacetate did not change the elution profile obtained with the column.

Effect of parathyrin. For assessment of the effect *in vitro*, the hormone was added either for 5 h, during which [¹⁴C]palmitate oxidation was measured, or during 19 h preincubation and a 5 h measurement period. Parathyrin was also administered to the animal *in vivo* by subcutaneous injection, 12 h before it was killed (Table 3). In none of these conditions did parathyrin influence fatty acid oxidation. However, lactate production was increased when the hormone was added during the 5 h measurement period, indicating that the hormone was active under test conditions employed. In the 19 h preincubation experiment, palmitate oxidation was shown to decline, whereas lactate production markedly increased in the controls, but without being influenced by

Table 2. Influence of palmitate concentration on palmitate oxidation and lactate production in calvaria *in vivo*

Calvaria halves from 4-day-old rats were incubated in media with different [U-¹⁴C]palmitate concentrations and palmitate/albumin ratios. The medium contained 10 % foetal-calf serum. The O₂ partial pressure was 95 %. The results are means $\pm$ S.E.M. for three calvaria halves. Duplicates are shown if only two calvaria were tested.

Palmitate added (mM)	Palmitate/albumin (mol/mol)	Palmitate oxidation (μ mol/6 h per mol of deoxyribose)	Lactate production (mol/6 h per mol of deoxyribose)
Tracer	5	38, 45	51, 36
0.1	5	179 $\pm$ 22 (3)	49, 48
1.0	5	697 $\pm$ 48 (3)	47, 42
1.0	2.2	836 $\pm$ 71 (3)	41, 50

Table 3. Effect of parathyrin administered *in vitro* and/or *in vivo* on palmitate oxidation and lactate production in calvaria *in vitro*

Calvaria halves from 4-day-old rats were incubated in medium with 1 mM-[U-¹⁴C]palmitate and 0.45 mM-albumin but without foetal-calf serum. (a) Parathyrin (74 nM) was added for 5 h, the time during which palmitate oxidation was measured. (b) The calvaria were preincubated for 19 h with 74 nM-parathyrin and then treated as in (a). (c) Parathyrin was administered *in vivo* by subcutaneous injection at a dose of 0.15 nmol/g body weight 12 h before the rats were killed. The calvaria were then incubated as in (a). The results are the means $\pm$ S.E.M., for the numbers of calvaria halves given in parentheses. **P* < 0.05, ***P* < 0.01, significantly different from the control with vehicle alone.

		Palmitate oxidation (μ mol/5 h per mol of deoxyribose)	Lactate production (mol/5 h per mol of deoxyribose)
(a) Incubation			
Vehicle		857 $\pm$ 66 (20)	72 $\pm$ 5 (20)
Parathyrin		846 $\pm$ 69 (21)	100 $\pm$ 7 (21)**
(b) Preincubation Incubation			
Vehicle	Vehicle	131 $\pm$ 25 (24)	179 $\pm$ 9 (24)
Parathyrin	Vehicle	123 $\pm$ 21 (9)	195 $\pm$ 16 (10)
Parathyrin	Parathyrin	179 $\pm$ 30 (19)	178 $\pm$ 8 (19)
Vehicle	Parathyrin	154 $\pm$ 24 (15)	189 $\pm$ 11 (16)
(c) Injection Incubation			
Vehicle	Vehicle	972 $\pm$ 78 (15)	62 $\pm$ 4 (15)
Parathyrin	Vehicle	922 $\pm$ 62 (12)	60 $\pm$ 4 (12)
Parathyrin	Parathyrin	1011 $\pm$ 73 (13)	83 $\pm$ 5 (13)*

Table 4. Characterization of calvaria-cell populations

Individual cell populations were grown to confluence in secondary culture for 7–8 days. Cyclic AMP response to 24 nM-parathyrin and alkaline phosphatase activity were determined as described in the Methods section. Results are given as means $\pm$ S.E.M. for five individual cultures dishes.

Cell population	Alkaline phosphatase (nmol/min per 10 ⁶ cells)	Cyclic AMP production (pmol/10 ⁶ cells)		
		Control	Treated	Treated/control
I	11 $\pm$ 2	25 $\pm$ 5	50 $\pm$ 11	2.0 $\pm$ 0.6
II	11 $\pm$ 1	30 $\pm$ 10	68 $\pm$ 13	2.3 $\pm$ 0.9
III	20 $\pm$ 2	45 $\pm$ 13	300 $\pm$ 41	6.7 $\pm$ 2.1
IV	42 $\pm$ 3	20 $\pm$ 7	990 $\pm$ 111	49.5 $\pm$ 18.2
V	69 $\pm$ 2	49 $\pm$ 17	710 $\pm$ 53	14.5 $\pm$ 5.1
VI	48 $\pm$ 3	35 $\pm$ 14	298 $\pm$ 68	8.5 $\pm$ 3.9
VII	26 $\pm$ 1	43 $\pm$ 10	350 $\pm$ 63	8.1 $\pm$ 2.4
VIII	16 $\pm$ 1		Not determined	

Table 5. Palmitate oxidation and lactate production by different calvaria cell populations *in vitro*

Cells were subcultured and were grown to confluence in glass ampoules. After 16 h of incubation in medium containing [^{14}C]palmitate and 10% foetal-calf serum, palmitate oxidation and lactate production were measured. The results are the means $\pm$ S.E.M., for the numbers of ampoules shown in parentheses. (a) Balanced means of ten experiments in which all populations were tested. (b) Balanced means of five experiments in which populations I, V and VIII were measured in each experiment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from population I.

Cell population	(a)		(b)	
	Palmitate oxidation (pmol/16 h per 10^6 cells)	Lactate production ($\mu\text{mol}/16$ h per 10^6 cells)	Palmitate oxidation (pmol/16 h per 10^6 cells)	Lactate production ($\mu\text{mol}/16$ h per 10^6 cells)
I	451 $\pm$ 20 (47)	3.90 $\pm$ 0.25 (47)	422 $\pm$ 28 (30)	4.24 $\pm$ 0.35 (30)
II	377 $\pm$ 42 (10)	4.09 $\pm$ 0.71 (10)		
III	307 $\pm$ 13 (9)***	4.55 $\pm$ 0.47 (10)		
IV	248 $\pm$ 17 (22)***	4.84 $\pm$ 0.30 (22)*		
V	219 $\pm$ 10 (65)***	4.74 $\pm$ 0.28 (72)*	197 $\pm$ 12 (27)***	5.86 $\pm$ 0.59 (29)*
VI	329 $\pm$ 36 (17)**	4.36 $\pm$ 0.32 (17)		
VII	449 $\pm$ 34 (45)	4.57 $\pm$ 0.26 (44)	380 $\pm$ 38 (30)	5.25 $\pm$ 0.31 (29)*
VIII	472 $\pm$ 30 (51)	4.78 $\pm$ 0.19 (52)**	437 $\pm$ 51 (29)	4.82 $\pm$ 0.23 (30)

Table 6. Influence of parathyrin on fatty acid oxidation and lactate production in different calvaria-cell populations

Populations I, V, VII and VIII were subcultured and the cells were grown in glass ampoules to confluence. Parathyrin (74 nM) was added 8 h before and during the measurement period of 16 h. The absolute values for the control and the ratio treated/control are expressed as means $\pm$ S.E.M. ** $P < 0.01$, *** $P < 0.001$, significantly different from 1.

Cell population	No. of ampoules	Palmitate oxidation		Lactate production	
		Control (pmol/16 h per 10^6 cells)	Treated/control	Control ($\mu\text{mol}/16$ h per 10^6 cells)	Treated/control
I	14	359 $\pm$ 16	1.13 $\pm$ 0.06	4.19 $\pm$ 0.47	1.05 $\pm$ 0.08
V	13	179 $\pm$ 23	1.12 $\pm$ 0.06	5.13 $\pm$ 0.89	1.35 $\pm$ 0.10**
VII	15	403 $\pm$ 55	1.06 $\pm$ 0.09	5.20 $\pm$ 0.37	1.47 $\pm$ 0.13**
VIII	20	476 $\pm$ 58	1.22 $\pm$ 0.05***	5.07 $\pm$ 0.23	1.22 $\pm$ 0.06**

parathyrin. This increase in lactate found in control samples might conceivably be the result of tissue damage, owing to the long incubation of the tissue.

Bone cells

Characterization. Table 4 lists the results of measurements of alkaline phosphatase activity found to be associated with the confluent calvarial bone-cell populations. Population V showed the highest alkaline phosphatase activity, and populations IV and VI also had a relatively high amount of alkaline phosphatase. As for the ability to respond to parathyrin, population IV produced the largest amount of cyclic AMP after having been challenged with 20 nM-parathyrin, followed by population V.

Influence of O_2 . When calvaria cells were incubated at O_2 partial pressures of 20, 58 and 95%, the last value being used for the bone tissue (see above), no effect of changing O_2 on either palmitate oxidation or lactate production was detected (results not shown). Thus 20% O_2 was used in all subsequent experiments. This concentration seems to allow sufficient diffusion into a

monolayer of cells to provide optimal supply of O_2 , whereas, with whole calvaria, where more tissue is present, a concentration of at least 60% O_2 appears to be required for optimal conditions (Table 1).

Metabolism of different bone-cell populations. Table 5 presents data on palmitate oxidation and lactate production obtained by different bone cell populations. In Table 5(a), data from all experiments performed were pooled, whereby not all populations were tested in each experiment. Cell populations III–VI oxidized palmitate to a lesser degree than did all the other populations, but showed a slight tendency to higher lactate production.

In Table 5(b) each of populations I, V, VII and VIII was examined in each experiment. The results shown confirm those in Table 5(a), whereby population V showed a lower fatty acid oxidation and a higher lactate production than did populations I, VII and VIII.

To compare the ATP produced by fatty acid oxidation and by glucose metabolism, the following was found: for population V, at concentrations of 0.1 mM-palmitate and 5.5 mM-glucose, $19.0 \pm 1.3(4)$ nmol of this fatty acid/ 10^6 cells were oxidized in 16 h [means $\pm$ S.E.M. (n)]

Table 7. Effect of 1,25(OH)₂D₃ on fatty acid oxidation and lactate production in different calvaria-cell populations *in vitro*

The conditions of cell culture and measurement of palmitate oxidation and lactate production were the same as described in Table 5; 100 nM-1,25(OH)₂D₃ was added 48 h before and during the 16 h of incubation. The absolute values for the control and the ratio treated/control are expressed as means $\pm$ S.E.M. ****P* < 0.001, significantly different from 1.

Cell population	No. of ampoules	Palmitate oxidation		Lactate production	
		Control (pmol/16 h per 10 ⁶ cells)	Treated/control	Control (μ mol/16 h per 10 ⁶ cells)	Treated/control
I	15	503 $\pm$ 15	1.28 $\pm$ 0.05***	3.30 $\pm$ 0.29	1.10 $\pm$ 0.19
IV	11	238 $\pm$ 17	1.94 $\pm$ 0.22***	4.42 $\pm$ 0.24	1.10 $\pm$ 0.07
V	17	239 $\pm$ 10	1.59 $\pm$ 0.09***	4.49 $\pm$ 0.23	1.17 $\pm$ 0.10
VIII	9	514 $\pm$ 25	1.14 $\pm$ 0.09	4.91 $\pm$ 0.57	1.08 $\pm$ 0.16

Table 8. Time course of the effect of 1,25(OH)₂D₃ on fatty acid oxidation and lactate production in calvaria cells *in vitro*

Cells of population V were cultured in the presence of 100 nM-1,25(OH)₂D₃ for time periods given in the Table. Fatty acid oxidation and lactate production were determined during the last 16 h of incubation. The results are expressed as means $\pm$ S.E.M. for the numbers of ampoules shown in parentheses. ****P* < 0.001, significantly different from the cells without hormone (0 h).

Duration of treatment (h)	Palmitate oxidation (pmol/16 h per 10 ⁶ cells)	Lactate production (μ mol/16 h per 10 ⁶ cells)
0	219 $\pm$ 5 (6)	4.04 $\pm$ 0.11 (6)
16	259 $\pm$ 15 (5)	3.86 $\pm$ 0.24 (5)
40	452 $\pm$ 29 (5)***	5.00 $\pm$ 0.35 (5)
64	425 $\pm$ 33 (5)***	5.54 $\pm$ 0.55 (5)

and 3.8 $\pm$ 0.3(5) μ mol of lactate/10⁶ cells were formed in 16 h. When [U-¹⁴C]glucose was used under the same conditions, it was found that 57 $\pm$ 4(5) nmol of glucose/10⁶ cells were oxidized to CO₂ in 16 h. The ATP production, calculated from these values, is 2.5 μ mol for fatty acid oxidation, 3.8 μ mol for lactate production and 2.1 μ mol for glucose oxidation.

Effect of hormones. Parathyrin did not affect palmitate oxidation, except in population VIII, where a small, but significant, increase was noticed. However, the hormone enhanced lactate production in populations V, VII and VIII, but not in population I (Table 6), indicating that the hormone was biologically active.

Calcitonin did not appear to affect any of the bone-cell populations in any parameters tested (results not shown).

Palmitate oxidation was increased by 1,25(OH)₂D₃ in all populations, the strongest effect being registered with populations IV and V (Table 7). This effect occurred after a preincubation time of at least 24 h with the hormone (Table 8). Fig. 1, which presents the dose/response for 1,25(OH)₂D₃, shows that the metabolite

Fig. 1. Effect of 1,25(OH)₂D₃ and 24,25(OH)₂D₃ on palmitate oxidation and lactate production in calvaria cells *in vitro*

The cells of population V were cultured and incubated at the same basal conditions as described in Table 5. 1,25- and 24,25-(OH)₂D₃ were added 48 h before and during the 16 h of measurement. The results are expressed as means $\pm$ S.E.M. for five ampoules (hormone present) or eight ampoules (absence of hormone). $\circ$, $\square$, Palmitate oxidation; $\bullet$, $\blacksquare$, lactate production. $\circ$, $\bullet$, 1,25(OH)₂D₃; $\square$, $\blacksquare$, 24,25(OH)₂D₃. **P* < 0.05, ***P* < 0.01, significantly different from control (absence of hormone).

acted within the concentration range 1–100 nM. Another vitamin D metabolite, 24,25(OH)₂D₃ likewise exhibited some activity on fatty acid oxidation, but only at 100 nM.

Insulin at 1.6 μ M did not affect palmitate oxidation in population I, but inhibited it in populations V, VII and VIII (Table 9). A small but not significant inhibition was observed at 16 nM-insulin (results not shown). Table 10 shows that, when the oxidation of fatty acids was increased by raising palmitate concentration in the medium, the ratio treated/control did not change, indicating that the effect of insulin was independent of the palmitate concentration. Preincubation with the hormone for 8, 28 or 52 h did not augment the effect (results not shown). The action of insulin was also tested in the absence of both glucose and amino acids. Under these conditions, as depicted in Table 11, fatty acid oxidation was markedly increased in the control. The effect of insulin was, however, no longer statistically

Table 9. Effect of insulin on fatty acid oxidation and lactate production in different calvaria-cell populations *in vitro*

The basal cell culture and incubation were the same as described in Table 5; 1.6 μ M-insulin was added 8 h before and during the 16 h of measurement. The absolute values for the control and the comparison treated/control are expressed as means $\pm$ S.E.M. * $P < 0.05$, *** $P < 0.001$, significantly different from 1.

Cell population	No. of ampoules	Palmitate oxidation		Lactate production	
		Control (pmol/16 h per 10^6 cells)	Treated/control	Control (μ mol/16 h per 10^6 cells)	Treated/control
I	10	446 $\pm$ 23	1.03 $\pm$ 0.07	2.04 $\pm$ 0.20	0.85 $\pm$ 0.10
V	10	239 $\pm$ 41	0.76 $\pm$ 0.05***	4.23 $\pm$ 0.52	1.12 $\pm$ 0.07
VII	9	304 $\pm$ 35	0.58 $\pm$ 0.08***	4.17 $\pm$ 0.40	1.32 $\pm$ 0.10*
VIII	4	390 $\pm$ 9	0.65 $\pm$ 0.03***	5.44 $\pm$ 0.34	1.05 $\pm$ 0.08

Table 10. Effect of palmitate concentrations on the action of insulin on palmitate oxidation by calvaria cells *in vitro*

Subcultured cells of population V were grown in glass ampoules to confluence. The incubation media contained different concentrations of [U- 14 C]palmitate (molar ratio palmitate/albumin = 5) and 10% foetal-calf serum. Insulin (1.6 μ M) was added 8 h before and during the period of measurement. The values of the palmitate oxidation are expressed as means $\pm$ S.E.M. for the numbers of ampoules shown in parentheses. ** $P < 0.01$, significantly different from controls without hormone.

Palmitate (mM)	Palmitate oxidation (nmol/16 h per 10^6 cells)		Insulin/control
	Control	Insulin	
0.01	0.24 $\pm$ 0.01 (5)	0.19 $\pm$ 0.01 (5)**	0.79
0.11	9.30 $\pm$ 0.21 (4)	7.22 $\pm$ 0.22 (5)**	0.78
0.51	18.31 $\pm$ 1.01 (4)	12.70 $\pm$ 0.97 (5)**	0.69

significant, suggesting that the effect of this hormone on fatty acid oxidation might be indirect.

Finally, glucagon, given 8 h before and during the 16 h measurement concentration of 16 nM–1.6 μ M, influenced neither palmitate oxidation nor lactate production (results not shown).

DISCUSSION

Ever since the early studies by Borle *et al.* (1960a,b), glucose has been considered as the major fuel source from which bone and other connective-tissue cells generate their energy. Evidence provided by the present study, however, indicates that, in bone tissue and cells, fatty acids appear to contribute considerably to the energy demand of these cells, namely about 40–80% of the energy estimated to originate from glucose metabolism. Supported by the fact that fatty acid oxidation occurred in the presence of glucose and at a fatty acid concentration of 1 mM, a value which is well within the physiological range of circulating non-esterified fatty acids in plasma of newborn rats (Snell & Walker, 1973;

Foster & Bailey, 1976) suggest that fatty acid oxidation plays an important role in the energy metabolism of bone cells.

The [14 C]palmitate was shown to become catabolized to similar amounts of 14 CO₂ and water-soluble radioactive components. With the aid of h.p.l.c., the water-soluble components were resolved and identified as citrate and lactate. Radiolabelled C₄ intermediates were therefore converted into lactate. Such a conversion has also been found in cultured fibroblasts (Zielke *et al.*, 1980). The detection of lactate and citrate is consistent with previous reports, where it was shown that bone tissue as well as bone cells release these two substances into their respective culture medium (Vaes, 1968; Price *et al.*, 1984).

The question of whether the different bone cells which collectively make up the osteogenic layer of bone (Owen, 1980) catabolize fatty acids in a comparable or differential manner was answered with the different cell populations obtained from calvaria by a modified time-dependent enzymic-digestion scheme (Luben *et al.*, 1976; Guenther *et al.*, 1984), which yielded eight bone-cell populations. On the basis of analytical data such as cyclic AMP production after a challenge by parathyryn, and alkaline phosphatase activity, it was concluded that cell populations IV and V bear characteristics that resemble osteoblast-like cells. Owing to the lack of specific cell markers, it is still rather difficult at the present time to know what each of the remaining cell populations represent, except to state that they are derived from calvarial tissue. The early populations (I and II) probably contain mainly fibroblast-like cells (Nijweide *et al.*, 1981). Previously, it was demonstrated that treatment of growing rats with dichloromethylenebisphosphonate caused a marked decrease in the osteoprogenitor cell pool (Miller & Jee, 1977). Later it was shown that exposure of various bone-cell populations to this particular compound resulted in a drastic growth inhibition of cell populations I and II. These observations, along with the discovery that these bisphosphonates induced populations I and II to become alkaline phosphatase-positive (Guenther *et al.*, 1984), lend some support to the notion that these cell populations appear to be enriched with cells that could conceivably belong to the osteoprogenitor cell pool. Microscopic observation of the tissue after each respective digestion step showed that after the fifth digestion period only cells embedded in the bone

Table 11. Influence of amino acids and glucose on palmitate oxidation and lactate production of calvaria cells in the presence or absence of insulin

Subcultured cells of population V were grown in glass ampoules to confluence. The incubation media contained 0.1 mM-[U-¹⁴C]palmitate plus 0.02 mM-albumin and alternatively or combined 5.6 mM-glucose and MEM amino acids; 1.6 μ M-insulin was added 8 h before and during the 16 h of measurement. The results are expressed as means $\pm$ S.E.M. for the numbers of ampoules shown in parentheses. *** P < 0.001, significantly different from palmitate alone. ** P < 0.01, *** P < 0.001, significantly different from I.

	Palmitate oxidation			Lactate production		
	(nmol/16 h per 10 ⁶ cells)			(μ mol/16 h per 10 ⁶ cells)		
	Control	Insulin	Insulin/control	Control	Insulin	Insulin/control
Palmitate	30.3 $\pm$ 2.5 (10)	25.5 $\pm$ 8.0 (10)	0.84 $\pm$ 0.11 (10)	0.17 $\pm$ 0.04 (10)	0.23 $\pm$ 0.06 (10)	1.34 $\pm$ 0.48 (10)
Palmitate + amino acids	32.8 $\pm$ 2.2 (10)	20.9 $\pm$ 1.1 (10)	0.64 $\pm$ 0.05 (10)***	0.24 $\pm$ 0.02 (10)	0.26 $\pm$ 0.02 (10)	1.10 $\pm$ 0.12 (10)
Palmitate + glucose	16.8 $\pm$ 0.7 (9)†††	13.8 $\pm$ 0.8 (10)	0.82 $\pm$ 0.06 (9)**	3.00 $\pm$ 0.33 (9)†††	3.72 $\pm$ 0.13 (10)	1.24 $\pm$ 0.14 (9)
Palmitate + amino acids + glucose	19.2 $\pm$ 0.8 (9)†††	12.4 $\pm$ 0.7 (8)	0.64 $\pm$ 0.05 (8)***	3.06 $\pm$ 0.30 (10)†††	3.32 $\pm$ 0.25 (9)	1.09 $\pm$ 0.13 (9)

matrix remained (results not shown). This observation, and the fact that cell population VII was still shown to respond to some extent to parathyryn, conceivably could assign this cell population to the osteoblast/osteocyte-like cell pool. Previous work by other investigators would seem to support the above proposed cell classification, as they have come to a similar conclusion on this cell type (Nijweide *et al.*, 1981).

It is generally accepted that energy metabolism in organisms is mainly regulated by insulin. Though few cells in the organism escape the effect of insulin, the principal targets are liver, muscle and adipose tissues. Our results with bone cells have shown that insulin acts as an inhibitor of fatty acid oxidation. This finding, in conjunction with the observations by Hahn *et al.* (1971), Schmid *et al.* (1982) and Ituarte *et al.* (1985) that insulin stimulates glucose oxidation and the synthesis of glycogen in bone tissue and bone cells, appears to indicate that the hormone plays an active role in regulating the energy metabolism of bone cells. A significant effect of insulin on fatty acid oxidation became apparent only after the addition of glucose and/or amino acids to the medium (Table 11), indicating that the effect may be indirect, stemming from the action of insulin on glucose and amino acid metabolism. Previously it has been shown by Hahn *et al.* (1971) that insulin increases amino acid uptake in bone tissue. Whether the inhibitory action on fatty acid oxidation occurs by malonyl-CoA, as described to be the case for the liver (McGarry *et al.*, 1978; McGarry & Foster, 1980), is not known.

It has been known for some time that parathyryn increases the consumption of glucose by bone tissue (Borle *et al.*, 1960a,b; Cohn & Forscher, 1962; Hekkelman *et al.*, 1974; Neuman *et al.*, 1978). More recently, it was also observed that it augments the synthesis of glycogen (Schmid *et al.*, 1982). In our study, although lactate production was increased by this hormone, fatty acid oxidation was not influenced, neither in calvaria nor in the separated cell populations, suggesting that parathyryn seems not to influence this latter pathway in bone tissue.

Calcitonin likewise did not act on the fatty acid oxidation. Conclusive evidence now shows that the target cell for this hormone is the osteoclast (Chambers & Moore, 1983; Nicholson *et al.*, 1986). In our calvaria-cell cultures osteoclasts do not survive, and in the calvaria their number probably is much too small to contribute significantly to the observed fatty acid oxidation. Hence an effect on osteoclasts, if present, might well be undetectable.

Osteoblast-like cells possess receptors for 1,25(OH)₂D₃ (Chen *et al.*, 1983), and it is well documented that this vitamin D metabolite exerts profound effects on alkaline phosphatase, collagen synthesis and citrate decarboxylase in these cells (Wong *et al.*, 1977). In our work fatty acid oxidation was stimulated within the range 1–100 nM. These are similar concentrations where others also have found bone cells to respond (Wong *et al.*, 1977). Treatment of rat bone cells with 1,25(OH)₂D₃ for 1 day induced a moderate inhibition of collagen synthesis, namely about 20% at the optimal concentration of 10 nM (Chen *et al.*, 1986). After 2 days' incubation with the hormone, the effect increased to 40% at 10 nM, and some inhibition could be observed at 0.1 nM. Comparing the action of 1,25(OH)₂D₃ on fatty acid oxidation with that on collagen synthesis, a similar dose/response and

time course are observed. Whether the effect on fatty acid oxidation is direct or indirect cannot be answered at present. However, the possibility of fatty acid oxidation being interrelated with the action of the metabolite on collagen synthesis is highly unlikely, since the observed inhibition of the latter would result in a smaller demand for energy. This in turn would probably lead to a lower fatty acid oxidation, a situation opposite to what we observed. $24,25(\text{OH})_2\text{D}_3$ was 100 times less active than $1,25(\text{OH})_2\text{D}_3$, which has also been observed previously (Stern *et al.*, 1975).

In conclusion, this study has demonstrated that palmitate may be regarded, at least in tissue and cell culture, as an important substrate for bone tissue and bone cells to satisfy their energy requirement. Insulin, the hormone known to influence energy metabolism in many tissues, but not glucagon, was shown to act on bone. With respect to the hormones which are known to regulate calcium metabolism, only $1,25(\text{OH})_2\text{D}_3$, and at high concentration $24,25(\text{OH})_2\text{D}_3$, but not parathyrin or calcitonin, acted on fatty acid oxidation. Further studies are needed to show whether the data are also valid *in vivo*.

We thank Ms. M.-L. Aebersold and Ms. J. Portenier for excellent technical assistance, and Ms. B. Meier and Ms. B. Järnmann for typing the manuscript. This work has been supported by the Swiss National Science Foundation (grant 3.937.82).

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Received 5 May 1987; accepted 5 August 1987