INHIBITION OF DIPHOSPHONATE-BLOCKED BONE MINERALIZATION.
EVIDENCE THAT CALCITONIN PROMOTES MINERALIZATION

By

Alfred Boris, James F. Hurley, Thelma Trmal,
John P. Mallon and Diana S. Matuszewski

ABSTRACT

There is much experimental evidence which indicates that calcitonin inhibits bone mineral resorption, but there are few data available in support of the proposal that calcitonin may also promote mineralization. Ethane-1-hydroxy-1,1-diphosphonate (EHDP) administered to immature rats inhibited mineralization as evidenced by widened tibial epiphyseal plates and decreased bone ash to dry weight ratios. Concurrent dosing with salmon calcitonin (SCT) prevented or reversed the EHDP-blocked mineralization in a dose dependent manner. Administration of SCT during the period after EHDP treatment significantly improved mineralization of tibial epiphyseal plates as shown by plate width narrowing and increased uptake of radioactive calcium. These results suggest that SCT increased mineralization in EHDP-treated rats, and provide supportive evidence for the proposal that calcitonin may also promote mineralization, in addition to its well known ability to inhibit bone mineral resorption.

Although the existence of calcitonin was demonstrated (Copp et al. 1962; Hirsch et al. 1963), its structure determined (Potts et al. 1968; Neher et al. 1968; Bell et al. 1968), and its synthesis accomplished (Rittel et al. 1968; Guttmann et al. 1968) a decade ago, the physiological role of calcitonin in bone metabolism is still the subject of intense investigation.

Correspondence to: Dr. Alfred Boris, Research Division, Hoffmann-La Roche Inc., Nutley, New Jersey 07110, USA.
remains unclear. Its hypocalcaemic and hypophosphataemic effects have been
ascribed to the inhibition of bone resorption. Both in vivo (Johnston & Diess
1966; Klein et al. 1967; O'Riordan & Aurbach 1968) and in vitro (Friedman
& Raisz 1965; Aliapoulios et al. 1966) data are available in support of this
contention. There is also experimental evidence which suggests that calcitonin
may promote deposition of calcium in bone (Wase et al. 1966; Foster et al. 1966;
Macintyre et al. 1967). The currently accepted view for the action of calcitonin,
however, would appear to favour inhibition of bone resorption, rather than pro-
motion of bone deposition (Rasmussen & Bordier 1974; Munson 1976).

Ethane-1-hydroxy-1,1-disphosphonate (EHDP) is known to inhibit both bone
resorption (Russell et al. 1970; Cates et al. 1971; Michael et al. 1971; Muhlbauer
et al. 1971; Reynolds et al. 1973) and bone mineralization (Fleisch et al. 1970;
Jowsey et al. 1970; King et al. 1971; Strates et al. 1971; Russell et al. 1973;
Schenk et al. 1973). Since both EHDP and calcitonin are known to inhibit bone
resorption, and both compounds may also affect bone mineralization, it was
considered of interest to determine the possible interactions of these two agents
on bone. Talmage et al. (1974) examined the effect of EHDP on the hypo-
calcaemic and hypophosphataemic activity of salmon calcitonin (SCT). These
investigators reported that the hypophosphataemic action of SCT was produced
independently of the hypocalcaemic effect, and concluded that the effect on
plasma phosphate could not result solely from a reduction of phosphate release
from bone, but was due to an increased exit of phosphate from plasma. These
investigators did not report the effects of SCT on EHDP-induced changes in
bone incorporation of $^{45}$Ca and $^{32}$P, but suggested that the hypophosphataemia
in response to SCT resulted, at least in part, from an extrasosseous effect which
was not directly related to bone physiology, renal excretion of phosphate, or
parathyroid action. The possibility that SCT may have promoted mineraliza-
tion, which may have contributed to the hypocalcaemia and hypophosphataemia,
was apparently not considered.

The results obtained in the present experiments, with multiple doses of EHDP
and SCT, and measurement of bone parameters, suggest that SCT promoted
mineralization in EHDP-treated rats.

MATERIALS AND METHODS

Charles River CD, 21-22-day-old male rats were used. Rats were housed in temperature
and humidity controlled, air-conditioned animal rooms under normal white fluo-
rescent lighting. Purina rat chow and drinking water were supplied ad libitum. Ethane-
1-hydroxy-1,1-diphosphonate (EHDP), dipotassium salt, was administered subcuta-
neously (sc) in 0.2 ml/rat/day distilled water. Salmon calcitonin (SCT) was given sc in
1% gelatin in saline, 0.2 ml/rat/day. Commercially-available SCT was used in all
experiments (CALCIMAR, Armour – 4000 MRC units/mg). EHDP and SCT were
administered by separate injections at different body sites, and control animals received injections of vehicle(s). Tibias were processed by a modified von Kossa procedure based upon silver impregnation of bone salts (Edwards 1961), and epiphyseal plate widths were measured with a micrometer ocular using standard microscopic magnification (35×). Tibias and vertebrae (4th coccygeal) were placed in boiling water for 1 to 2 min to enable easy removal of adherent soft tissue and marrow, and were dried to constant weight at 102°C for determination of dry weight. Bones were ashed at 800°C for 24 h. Whole bones were analyzed, since preliminary experiments had established that the response to SCT occurred whether the ends of long bones (epiphysis and metaphysis) or the shafts (diaphysis) were analyzed separately or together.

Serum calcium was measured by the method of Gindler & King (1972), and serum inorganic phosphorus was determined by the standard Technicon Auto Analyzer procedure (N-4c I/I). Statistical differences were computed by Student's t-test.

Epiphyseal plate uptake of 45Ca was determined by a modification of the procedure of Anderson & Sajdera (1976). Epiphyseal plates were removed from the tibia, cut into 4 to 6 wedges, pooled by treatment group, and placed in ice cold calcifying buffer containing 85 mm NaCl, 15 mm KCl, 10 mm NaHCO3, 1.6 mm KH2PO4, 2.2 mm CaCl2, and 50 mm Tris-(hydroxymethyl) aminomethane, pH 7.4. Six epiphyseal wedges, matched by size, were weighed and placed in incubation flasks containing 0.2 ml/mg tissue of the calcifying buffer solution. Five flasks, each containing six wedges, were used for each control and treatment group. Flasks were incubated at 37°C in a water bath shaker for 2 h. 45CaCl2, 0.6 μCi/ml incubation solution, was then added to each flask and the incubation continued for 1 h. The epiphyseal plate wedges were then removed from each flask, washed 3 times with 20 ml of physiological saline, and digested with 1 ml of concentrated nitric acid (70%, Sp. Gr. 1.42). An 0.05 ml aliquot of the digest was then added to 10 ml of scintillation fluid and radioactivity was measured. Results were expressed as cpm/mg tissue.

RESULTS AND DISCUSSION

Administration of EHDP to immature rats resulted in a dose dependent widening of the tibial epiphyseal plate, as shown in Table 1. It is unlikely that the widened epiphyseal plates represented decalcification of prior mineralization, since EHDP inhibits bone resorption. Epiphyseal plate widening by EHDP has been ascribed to inhibition of mineralization, and resembles the plate widening which is observed in vitamin D deficiency rickets (Alcock 1972; Schenk et al. 1973). It is noteworthy that EHDP caused widening of the tibial epiphyseal plate while the compound depressed growth of the animals as indicated by decreased body weight gain.

Concurrent, but separate, injection of various doses of SCT with a single dose level of EHDP (2 mg/rat/day) resulted in a dose-dependent prevention or reversal of EHDP-induced tibial epiphyseal plate widening as shown in Fig. 1. Since both EHDP and calcitonin independently manifest effects on bone, the observed effect of calcitonin on EHDP-induced plate widening might have been the result of competition for a common bone receptor. If SCT blocked the access of EHDP to bone, then the mineralization block, and the consequent
Table 1.
Effects of EHDP on body weight and tibial epiphyseal plate width in rats after 10 days treatment.

<table>
<thead>
<tr>
<th>EHDP mg/rat/day, sc</th>
<th>No. of rats</th>
<th>Mean body weight (g)</th>
<th>Mean ± se plate width (μ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>0</td>
<td>9</td>
<td>49</td>
<td>105</td>
</tr>
<tr>
<td>0.5</td>
<td>10</td>
<td>45</td>
<td>97</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>49</td>
<td>101</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>48</td>
<td>78</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>49</td>
<td>77</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>50</td>
<td>63</td>
</tr>
</tbody>
</table>

* P < 0.05.  ** P < 0.01.  *** P < 0.001.

Fig. 1.
Effect of salmon calcitonin on EHDP-blocked tibial epiphyseal plate mineralization. Ten days treatment. Ten rats per group. Points and vertical bars represent means ± standard errors. EHDP, 2 mg/rat/day.
epiphyseal plate widening, would either not have occurred or would have been diminished. This would appear to be an unlikely explanation for the observed effect, since the molecular ratio of EHDP (MW 282) to SCT (MW 3427) was overwhelmingly in favour of EHDP, ranging from more than 24 000 to one at the highest SCT dose to almost 5 million to one at the lowest effective dose of SCT. Also, the relatively long duration (weeks) of EHDP in bone (King et al. 1971) compared to the short duration (hours) of SCT activity (Munson 1976) would be advantageous to EHDP rather than SCT in any competition at receptor sites in in vivo studies. The data obtained suggest that SCT stimulated mineralization and thus narrowed the EHDP-widened epiphyseal plates. The proposal that calcitonin promotes mineralization has been difficult to demonstrate unequivocally by direct effects on bone. The present indirect model would seem to be analogous to other pharmacological procedures in which agonist activity is sometimes more easily demonstrated by showing ability to overcome antagonists than by direct agonist-induced biological response.

Tables 2 and 3 present data from an experiment in which parameters in addition to epiphyseal plate widths were measured in rats treated with EHDP and/or SCT. EHDP significantly reduced body weight gain, serum phosphorus, and tibia and vertebra dry weight, ash, and ash/dry weight ratio when compared to controls. EHDP significantly increased serum calcium and tibial epiphyseal plate width in comparison to controls. SCT decreased serum calcium, and slightly elevated vertebral dry weight and ash without a significant effect on vertebral ash/dry weight ratio. When EHDP and SCT were both administered, SCT significantly inhibited EHDP effects on all bone parameters. There was no difference in serum calcium and phosphorus levels between EHDP-treated rats and EHDP plus SCT-treated animals. The increase in bone ash/dry weight ratios for both the tibia and vertebra in rats given SCT and EHDP lends further support to the proposal that SCT stimulated mineralization, and is consistent with the observed narrowing of the widened epiphyseal plate. In fact, the ash/dry weight ratios for both the tibia and the vertebra in the group given both compounds were not significantly different from the ratios for the vehicle-treated controls, indicating that SCT completely prevented or reversed the mineralization block induced with EHDP.

Since EHDP reduced body weight gain, its effects upon bone could have been due, at least in part, to interference with somatic growth, including bone growth. The prevention or reversal of EHDP effects on bone by SCT might have been the result of an improvement in growth as a result of SCT administration. Indeed, the data shown in Table 2 would seem to support such a conclusion, since SCT improved body weight gain in EHDP-treated rats in this experiment. This explanation for the observed prevention or reversal of diphosphonate effects on bone by calcitonin would appear to be unlikely, however, since, as shown in Figs. 2 and 3, narrowing of EHDP-widened epiphyseal plates

355
Table 2.
Effects of EHDP and/or SCT on body weight, serum calcium and phosphorus, and tibial epiphyseal plate width in rats after 10 days treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose/rat/day, sc</th>
<th>No. of rats</th>
<th>Mean body weight (g)</th>
<th>Mean ± se</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EHDP (mg)</td>
<td>SCT (units)</td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>48</td>
<td>103</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
<td>46</td>
<td>73</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0.4</td>
<td>47</td>
<td>102</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0.4</td>
<td>47</td>
<td>81</td>
</tr>
</tbody>
</table>

Groups | Body weight change | Ca | Ph | Plate width |
-------|-------------------|----|----|------------|
1 vs. 2 | *** | ** | *** | *** |
1 vs. 3 | ns | ** | ns | ns |
1 vs. 4 | *** | *** | *** | *** |
2 vs. 3 | *** | *** | *** | *** |
2 vs. 4 | ** | ns | ns | *** |
3 vs. 4 | *** | *** | *** | *** |
Table 3.
Effects of EHDP and/or SCT on bone dry weight, ash weight, and ash/dry weight ratio in rats after 10 days treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose/rat/day, sc</th>
<th>No. of rats</th>
<th>Mean ± se</th>
<th>Mean ± se</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EHD (mg)</td>
<td></td>
<td>Dry weight (mg)</td>
<td>Ash (mg)</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>20</td>
<td>129.1 ± 5.5</td>
<td>58.5 ± 1.7</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>19</td>
<td>76.7 ± 2.9</td>
<td>30.5 ± 0.7</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>20</td>
<td>123.9 ± 5.4</td>
<td>58.7 ± 1.7</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>20</td>
<td>91.4 ± 3.7</td>
<td>41.7 ± 1.6</td>
</tr>
</tbody>
</table>

1) 4th coccygeal vertebra.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tibia</th>
<th>Vertebra</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DW</td>
<td>A</td>
</tr>
<tr>
<td>1 vs. 2</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>1 vs. 3</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>1 vs. 4</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>2 vs. 3</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>2 vs. 4</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>3 vs. 4</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>
Effect of fixed dose of salmon calcitonin with graded doses of EHDP on mean body weight change. Open bars: EHDP alone. Cross-hatched bars: EHDP + SCT, 0.4 unit/rat/day. Both compounds given sc for 10 consecutive days. Nine to 11 rats per group. Vertical lines represent standard errors.

Effect of fixed dose of salmon calcitonin with graded doses of EHDP on mean increase above respective controls in tibial epiphyseal plate width. Open bars: EHDP alone. Cross-hatched bars: EHDP + SCT, 0.4 unit/rat/day. Both compounds given sc for 10 consecutive days. Nine to 11 rats per group. Vertical lines represent standard errors. *** $P < 0.001$. 

358
Mineralization of tibial epiphyseal plate after EHDP treatment with and without calcitonin administration.

<table>
<thead>
<tr>
<th>Days post EHDP</th>
<th>Mean plate width (μ) ± se</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>SCT</td>
</tr>
<tr>
<td>3</td>
<td>1363.6 ± 20.0</td>
<td>1361.6 ± 4.3</td>
</tr>
<tr>
<td>6</td>
<td>1153.5 ± 30.8</td>
<td>973.8 ± 31.3</td>
</tr>
<tr>
<td>10</td>
<td>939.0 ± 32.6</td>
<td>842.0 ± 18.1</td>
</tr>
<tr>
<td>13</td>
<td>755.5 ± 25.9</td>
<td>524.1 ± 21.8</td>
</tr>
<tr>
<td>18</td>
<td>597.5 ± 32.7</td>
<td>393.2 ± 20.4</td>
</tr>
<tr>
<td>21</td>
<td>427.7 ± 7.6</td>
<td>389.1 ± 12.3</td>
</tr>
<tr>
<td>26</td>
<td>430.6 ± 11.1</td>
<td>343.1 ± 13.1</td>
</tr>
</tbody>
</table>

Eleven rats per group. EHDP, 2 mg/rat/day sc × 10 days followed by vehicle or SCT, 0.4 unit/rat/day, sc for periods shown.

in response to SCT occurred in the absence of effects on body weight gain by SCT at several dose levels of EHDP. Inhibition of EHDP-induced plate widening by SCT was also seen at a dose of EHDP (1,2 mg) which had no effect on body weight gain when compared to vehicle-treated controls not given EHDP with both groups gaining 62 g.

Table 4 shows the post EHDP mineralization of the tibial epiphyseal plate

Effect of calcitonin on $^{45}$Ca uptake and width of tibial epiphyseal plates during recovery after EHDP treatment.

<table>
<thead>
<tr>
<th>Post EHDP treatment</th>
<th>No. of days treated</th>
<th>$^{45}$Ca uptake (CPM/mg tissue)</th>
<th>Plate width (micra)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>10</td>
<td>3165 ± 143¹</td>
<td>1333 ± 92</td>
</tr>
<tr>
<td>Calcitonin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>14</td>
<td>2846 ± 177</td>
<td>784 ± 16</td>
</tr>
<tr>
<td>Calcitonin</td>
<td></td>
<td>4354 ± 204²</td>
<td>584 ± 23²</td>
</tr>
</tbody>
</table>

¹ Mean ± se 5 flasks per group.
² Mean ± se 10 to 12 rats per group.

Rats administered EHDP, 2 mg/day sc, for 10 days prior to treatment with 1% gelatin in saline, 0.2 ml/day sc or calcitonin, 0.4 unit/day, sc. ** P < 0.01, *** P < 0.001.
with and without the administration of SCT. These data illustrate the long duration of the EHDP effect on bone, and also show that the recovery from the EHDP-induced mineralization block occurred more rapidly when calcitonin was administered.

The results obtained when the calcium uptake of epiphysal plates during recovery from EHDP-treatment was measured are shown in Table 5. SCT significantly improved $^{45}$Ca uptake by epiphysal plates, and significantly narrowed plate width when compared to vehicle-treated controls. These observations lend further support to the proposal that calcitonin promoted mineralization.

The utility of diphosphonate-treated animals for studies of basic mineralization mechanisms is already evident from the many publications on such studies currently available in the literature. The results obtained in the experiments reported here suggest that calcitonin promoted mineralization in EHDP-treated rats, and would appear to support the proposal that calcitonin also stimulates mineralization in addition to its more established role in blocking bone resorption. The EHDP-treated rat model could also prove useful for finding other compounds which stimulate mineralization. Such “EHDP antagonists” might possibly provide useful therapeutic agents for the treatment and/or prevention of bone disorders such as osteoporosis.

ACKNOWLEDGMENTS

The expert technical assistance of Mrs. E. Nelson, Mr. F. Nemeth, Sr. and Mr. I. Stolz is gratefully acknowledged.

REFERENCES


Received on December 7th, 1978.