Effect of Calcitonin on Experimental Osteolathyrism (II)
Action of Calcitonin on Collagen Metabolism

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Synopsis

In order to study the effect of calcitonin on the bone collagen metabolism in osteolathyrism, male Wistar rats were placed on 0.1% aminoacetonitrile diet for 9 days. Porcine calcitonin dissolved in gelatin was concurrently administered in a dose of 0.1 MRC U/100 g body weight for 8 days and on the 9th day 0.75 MRC U/100 g body weight was given 3, 2 and 1 hr respectively before sacrifice. Both bone collagen formation and maturation were examined by the determination of specific and total activities of \(^{3}H\)-hydroxyproline in bone collagen, and collagen resorption was examined by the estimation of the amount of hydroxyproline released into the incubation medium from metaphyses. Treatment of lathyritic rats with calcitonin diminished the concentration of serum calcium, phosphate, and rate of collagen resorption, but did not affect collagen formation and collagen maturation. Inhibitory effect of calcitonin on collagen resorption was more pronounced in osteolathyrism than under normal condition. It seemed that parathyroid glands did not play a vital role in the collagen resorption induced by osteolathyrism, since the resorption was not affected by parathyroidectomy. On the other hand, administration of calcitonin (every 0.8 MRC U/rat, 60 and 30 min before sacrifice) also counteracted collagen resorption which was induced by low calcium diet and treatment with parathyroid hormone (PTH, 70 USP units).

It is concluded that the effect of calcitonin on collagen metabolism resulted in primary inhibition of collagen resorption in osteolathyrism, and that calcitonin counteracted both PTH-dependent collagen resorption and PTH-independent collagen resorption induced by osteolathyrism.

Experimental osteolathyrism is one of the most well-investigated molecular diseases of connective tissue, manifested by abnormalities in the growth of cartilage and bones. The main effect of these changes is a marked increase in the content of soluble collagen in the tissue of animals (Tanzer, 1965).

β-Aminopropionitrile and aminoacetonitrile (AAN), and their analogs had been used widely to produce experimental osteolathyrism.

We have previously shown (Seyama et al., 1971) that AAN increases the amount of soluble collagen, accompanied by an increase in the degradation of bone collagen which then produces osteoporosis-like changes.

Recently, calcitonin, a hypocalcemic peptide hormone, was isolated and details of the effect of this hormone in mineral metabolism have been clarified (Friedman and Raisz, 1965; Aliapoulios et al., 1966; Johnston and Deiss, 1966; Wallach et al., 1967). However, the action of calcitonin in metabolism of bone organic matrix, especially under pathological condition, is not yet established.

Therefore, in the present investigation, experimental osteolathyrism was used as a representative of a disease in bone organic
matrix and the effect of calcitonin in metabolism of bone collagen under these diseased conditions was studied in vitro and in vivo.

Materials and Methods

Animals
Male Wistar rats weighing 70-80 g at the beginning of the experiments were used. In the 1st experiment rats were divided into two groups, both groups being placed on a diet (1.0% calcium: 1.0% phosphorus) containing 0.1% AAN sulfate for 9 days. In addition to AAN treatment, the 1st group of rats was given 0.1 MRC U of porcine calcitonin (dissolved in 15% gelatin) per 100 g body weight daily for 8 days by subcutaneous injection and on the 9th day, 0.75 MRC U of porcine calcitonin per 100 g body weight was injected subcutaneously 3, 2 and 1 hr respectively before the animals were sacrificed in order to examine their effects in vivo by an in vitro system. The 2nd group received the respective vehicles alone. On the 8th day of the experiment, 24 hr before killing, both groups of animals were given 100 µCi of 3H(G)-proline (specific activity, 29 Ci/m mole, Daiichi Kagaku Co.) per 100 g body weight intraperitoneally. In the 2nd experiment, two groups of intact rats were set up for the in vitro experiment. One group received the same amount of calcitonin as in the 1st experiment and the other group only the vehicle. In the 3rd experiment, intact and lathyritic rats were parathyroidectomized surgically on 2 days before sacrifice. In the 4th experiment, animals in one group were fed a normal diet (1.0% calcium: 1.0% phosphorus) for 4 days, those in the 2nd group were given a low-calcium diet (0% calcium: 1.6% phosphorus) prepared according to Hirsch et al., (1963) for 4 days and received subcutaneous injection of 70 USP unit of parathroid hormone (PTH) 3 hr before sacrifice, and those in the 3rd group, in addition to the treatment with low-calcium diet and PTH injection, received subcutaneous injection of calcitonin in a dose of 0.8 MRC U/rat, 60 and 30 min before sacrifice.

Hormone preparations
Porcine calcitonin was prepared from the thyroids according to the procedure of Tenenhouse et al. (1965). The potency of the preparation was 2.0 MRC U/mg as determined by the method of Morii et al. (1970). Parathyroid Injection from Eli Lilly and Co. was used as PTH.

Analytical procedure
Immediately after the rats were killed, blood samples were obtained for the assay of serum calcium and phosphate concentration, calcium was determined by atomic absorption spectrophotometry, and phosphate was measured by the method of Chen et al. (1956). The upper tibia, lower femur, and calvaria were removed, freed of soft tissue. Metaphyses were harvested from the upper tibia and lower femur according to the method of Borle et al. (1960). Metaphyses were divided into two portions, one portion was used for the incubation and the other for the incorporation of 3H-proline.

Metaphyses were diced into pieces of approximately 2 mm³ and washed thoroughly three times in chilled Krebs-Ringer bicarbonate medium (pH 7.4) for incubation. The diced metaphyses were incubated in 3.0 ml of the same medium under 95% O₂-5% CO₂ at 37.5°C for 3 hr. After incubation, the reaction mixture was rapidly chilled by placing it in ice water. The decanted medium was centrifuged at 10,000 rpm at 0°C for 30 min to remove any cell and other debris. Metaphyses and supernatant of the incubation medium were hydrolyzed in 6 N HCl for 18 hr in a sealed glass tube at 110°C. Hydroxyproline in the hydrolyzate and free hydroxyproline in the medium were measured by the method of Kivirikko et al. (1967). The amount of hydroxyproline in the medium was expressed as µmol hydroxyproline in the medium/mg collagen of the bone according to the method of Flanagan and Nichols (1969). Radioactivity of 3H-hydroxyproline in metaphyses was measured using an Aloka Liquid Scintillation counter according to the method of Juva and Prockop (1966).

Calvaria were decalcified by extraction twice with 10 ml each of 10% EDTA-2Na solution (pH 7.4) per bone and homogenized in chilled 0.45 M NaCl in 0.02 M solution of Tris buffer (pH 7.4) at 4°C in a glass homogenizer. The homogenate was shaken twice at 4°C for 24 hr each time and centrifuged at 10,000 rpm for 30 min. The supernatant fraction obtained was combined and evaporated. This fraction was designated as neutral-salt soluble collagen (NSC) fraction and the residue as insoluble collagen (ISC) fraction. The amount of hydroxyproline and the radioactivity of 3H-hydroxyproline in each fraction were determined using the above method.

Results

Serum calcium and phosphate concentration
Serum calcium and phosphate showed a statistically significant fall in the calcitonin-treated lathyritic rats when compared with the non-treated lathyritic rats. In the intact
Table 1. Effect of calcitonin on serum calcium and phosphate

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Treatment</th>
<th>No. of rat</th>
<th>Serum Ca mg/100 ml Mean±S.E.</th>
<th>Serum P mg/100 ml Mean±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Intact</td>
<td>8</td>
<td>9.07 ± 0.10</td>
<td>10.83 ± 0.63</td>
</tr>
<tr>
<td></td>
<td>Calcitonin</td>
<td>7</td>
<td>7.65 ± 0.14***</td>
<td>8.11 ± 0.60**</td>
</tr>
<tr>
<td>II</td>
<td>AAN</td>
<td>8</td>
<td>9.03 ± 0.14</td>
<td>11.92 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>AAN-calcitonin</td>
<td>7</td>
<td>7.26 ± 0.14**</td>
<td>7.78 ± 0.36*</td>
</tr>
</tbody>
</table>

*P<0.05 **P<0.01 ***P<0.001
AAN: aminoacetonitrile

Group, serum calcium and phosphate also showed a significant decrease by calcitonin treatment (Table 1). Another experiment indicated that the serum phosphate concentration (14.20 ± 0.17 mg/100 ml, mean ± S. E., n = 8) was slightly increased by the administration of AAN as compared with the control group (12.72 ± 0.66 mg/100 ml, mean ± S. E., n = 8).

Collagen formation

The specific activity of hydroxyproline in metaphyses showed no significant difference between calcitonin-treated and non-treated lathyritic rats, as shown in Table 2. Calcitonin produced no significant change in the total activity of hydroxyproline in collagen fraction of calvaria as shown in Table 4.

Collagen maturation

Table 3 gives the amount of collagen isolated from the calvaria. In lathyritic rats, the amount of NSC was very much increased as was indicated in the previous report (Seyama et al., 1971), but in lathyritic rats treated with calcitonin, a proportion of NSC to total collagen was not significantly altered in comparison with non-treated lathyritic rats. Table 4 is a comparison of specific, partial,

Table 2. Effect of calcitonin on collagen formation in metaphyseal bone

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rat</th>
<th>Specific radioactivity of hydroxyproline of bone collagen dpm/µg Mean±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAN</td>
<td>7</td>
<td>147 ± 10</td>
</tr>
<tr>
<td>AAN-calcitonin</td>
<td>7</td>
<td>152 ± 7</td>
</tr>
</tbody>
</table>

AAN: aminoacetonitrile

Table 3. Concent of soluble and insoluble collagen hydroxyproline in calvaria

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AAN (7) Mean±S.E.</th>
<th>AAN-calcitonin (7) Mean±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC (% of total collagen)</td>
<td>8.45 ± 0.65</td>
<td>10.98 ± 1.31</td>
</tr>
<tr>
<td>NSC (Hypro µg/mg of tissue in wet wt.)</td>
<td>1.94 ± 0.10</td>
<td>2.50 ± 0.24</td>
</tr>
<tr>
<td>ISC (Hypro µg/mg of tissue in wet wt.)</td>
<td>20.62 ± 0.82</td>
<td>20.65 ± 0.91</td>
</tr>
<tr>
<td>Total collagen</td>
<td>22.50 ± 0.75</td>
<td>23.15 ± 0.75</td>
</tr>
</tbody>
</table>


(N): number of animals used.

Table 4. Specific, Partial and Total radioactivity of hydroxyproline in various collagen fractions of calvaria

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AAN (7) Mean±S.E.</th>
<th>AAN-calcitonin (7) Mean±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC Specific radioactivity (dpm/µg Hypro)</td>
<td>46.95 ± 3.46</td>
<td>38.12 ± 2.48</td>
</tr>
<tr>
<td>Partial radioactivity (dpm)</td>
<td>82.72 ± 8.05</td>
<td>95.94 ± 11.52</td>
</tr>
<tr>
<td>ISC Specific radioactivity (dpm/µg Hypro)</td>
<td>13.77 ± 1.23</td>
<td>15.15 ± 0.97</td>
</tr>
<tr>
<td>Partial radioactivity (dpm)</td>
<td>279.95 ± 21.64</td>
<td>313.71 ± 27.53</td>
</tr>
<tr>
<td>Total activity (dpm)</td>
<td>362.67 ± 24.47</td>
<td>409.65 ± 24.62</td>
</tr>
</tbody>
</table>


(N): number of animals used.
and total activities of collagen in the calcitonin-treated and non-treated lathyritic rats. The product of specific activity and the amount of hydroxyproline in each collagen fraction of calvaria was expressed as the partial activity value. Total activity is the sum of partial activity of NSC and ISC. The total and partial activities of NSC and ISC were slightly increased in the calcitonin treated group, although not significant in this experiment. However, the ratio of partial activity of ISC to total activity of lathyritic calvaria (0.77) was equal to that of calcitonin-treated calvaria (0.77).

Collagen resorption in lathyritic rats

Our previous experiment indicated that the resorption of bone collagen was enhanced by the AAN treatment (Seyama et al., 1971). Table 5 shows that the amount of total hydroxyproline released from metaphyses in lathyritic rats was decreased by calcitonin treatment but not the amount of free hydroxyproline. In intact rats, the amount of total and free hydroxyproline decreased only slightly by calcitonin treatment, but its value was not significant as indicated in Table 5.

Relationship between collagen resorption and parathyroid function

Total hydroxyproline in the medium decreased significantly in intact rats by parathyroidectomy, but not in lathyritic rats. This fact suggests that the increased resorption of bone collagen found in osteolathyrism was scarcely dependent on parathyroid function (Table 6).

Parathyroid hormone dependent collagen resorption

Effect of calcitonin on the resorption of bone collagen induced by low-calcium diet and PTH injection is shown in Table 7. From this result, calcitonin is known to counteract the PTH-dependent resorption of bone collagen.

Discussion

Currently accumulated evidence indicates that calcitonin lowers serum calcium by inhibiting bone resorption (Friedman and Raisz, 1965; Aliapoulios et al., 1966; Johnston
Moreover calcitonin inhibits urinary hydroxyproline excretion in rats (Martin et al., 1966; Aer, 1968). Because the rate of excretion of hydroxyproline is mainly in a measure of the rate of bone resorption, these findings suggest that calcitonin acts not only by preventing mineral removal from bone, but also by inhibiting bone collagen breakdown. In an experiment using cultured long bone, Raisz et al. (1968) found that calcitonin inhibited calcium removal but not the release of hydroxyproline. However, Heersche (1969) subsequently showed by using explanted calvaria of mouse embryo that calcitonin inhibits PTH-dependent resorption of the previously formed mineralized collagen but does not affect PTH-dependent resorption of newly formed collagen. These results indicate that calcitonin inhibits directly or indirectly the resorption of bone collagen.

It is now generally accepted that calcitonin is more effective in diseases in which there is an abnormally high rate of bone resorption; for example Paget's disease and hyperparathyroidism (Potts, 1970; Bell et al., 1970). Therefore, it is suggested that calcitonin becomes a possible agent in the treatment of disorders of bone in which resorption is increased. The effect of calcitonin on experimentally induced osteolysis has been studied by several investigators (Fujita et al., 1968; Jowesy, 1969; Singh and Jowesy 1970; Chiroff et al., 1970). However, the action of calcitonin in metabolism of bone organic matrix, especially under pathological condition, is not yet known. Thus, in the present experiment the effect of calcitonin on collagen metabolism was investigated under a condition designated to alter the rate of collagen degradation and maturation.

Administration of a lathyrogen increased the soluble collagen and hydroxyproline peptide excreted in the urine (Tanzer, 1965) and also free hydroxyproline in the chick embryo (Decker et al., 1959). These facts indicate that increase of soluble collagen in lathyritic rats is accompanied by a simultaneous degradation of collagen. Previous results (Seyama et al., 1971) showed that the breakdown of bone collagen in vitro was increased in lathyritic rats. Treatment of lathyritic rats with calcitonin showed a significant decrease in the amount of total hydroxyproline released from metaphyses suggesting that the breakdown of bone collagen induced by AAN was counteracted by calcitonin. These facts indicate that the effect of calcitonin on collagen formation and maturation was different from that of sodium salicylate, hydrocortisone, and 17β-estradiol, which inhibit excretion of urinary hydroxyproline, are known to be effective in depressing the increased solubility of collagen in lathyritic rats (Tranavský and Trnavská, 1968; Henneman, 1970). In the present study calcitonin did not alter the decreased rate of conversion of soluble collagen into insoluble collagen in lathyritic rats. This fact suggests that the effect of calcitonin on collagen formation and maturation was different from that of sodium salicylate, hydrocortisone, and 17β-estradiol.

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significant hypocalcemic and hypophosphatemic effects. These results indicate that the magnitude of the calcitonin inhibition is dependent on the rate of collagen resorption.

Although Selye (1958) already suggested that the development of osteolathyrism was not due to a primary lesion of an endocrine gland, it is not yet established whether the degradation of bone collagen is independent of the parathyroid function. PTH is well known to produce an increase in bone resorption. In our experimental system the increase of collagen resorption was observed by treatment with a low-calcium diet and PTH. Accordingly, AAN might produce its effect by releasing PTH from the parathyroid gland. To test this possibility, parathyroidectomy was carried out on lathyritic and intact rats before incubation of their metaphyses. The rate of collagen resorption did not decrease by parathyroidectomy in lathyritic rats but did so significantly in intact rats. It seemed that parathyroid glands did not play a vital role in the collagen resorption of metaphyses induced by osteolathyism. This suggestion was also supported by the fact that serum phosphate tended to increase in lathyritic rats but decrease in rats treated with PTH.

Hirsch (1964) already found that calcitonin decreases serum calcium concentration in parathyroidectomized rats. It was also suggested from our experiment that the inhibition of bone resorption by calcitonin was not dependent on the cause of induction of bone resorption. Although we cannot as yet explain the mechanism by which calcitonin reduces PTH-independent resorption of bone collagen, it seems likely that calcitonin not only competes with PTH at the same site of action, but also acts to inhibit bone resorption at a different site.

Acknowledgment

The authors are indebted to Mr. E. Shimazawa for helpful advise on experimental technique and to Mr. H. Toyoda and Mr. S. Hashimoto for technical assistance.

References

Martin, T. J., C. J. Robinson and I. MacIntyre