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The Metabolic Clearance Rate of Osteocalcin in Sheep

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Summary. Plasma osteocalcin has been proposed as a useful and convenient biochemical marker of bone formation. However, the effect on plasma osteocalcin due to variations in the rate of its removal from the circulation has been little investigated. We have measured the metabolic clearance rate of plasma osteocalcin in adult oophorectomized sheep. Two methods were used: intravenous bolus injection (six animals) and 6 hour constant intravenous infusion (four animals) of ^{125}I-ovine osteocalcin. Using the bolus injection method, the plasma clearance of osteocalcin was found to be 3.3 liters/h. With the constant infusion method, the calculated value was 2.8 liters/h; based on this value and the mean ovine plasma osteocalcin concentration of 26.9 ng/ml (N = 29), a plasma production rate of 1.8 mg/day was derived. Osteocalcin clearance was relatively constant among animals in a basal state. Hence, the present approach should permit us to evaluate the relative contributions to changes in circulating osteocalcin levels from altered osteocalcin plasma clearance and production under various physiological and pathological conditions.

Key words: Osteocalcin — Metabolic clearance — Sheep — Bone.

Osteocalcin in a component of the noncollagenous matrix of bone and contains the unusual amino acid gammacarboxyglutamic acid [1]. It has been found to circulate in the plasma, and can be measured by radioimmunoassay [2]. The levels of osteocalcin in plasma have been thought to reflect the rate of synthesis of bone matrix and have been used as an index of bone formation [3]. Levels are raised in conditions where there is increased turnover of bone such as Paget's disease and also where renal function is diminished, such as chronic renal failure [4]. Radioactive osteocalcin is rapidly cleared from the circulation in the rat and the human [5–7] mainly by destruction in the kidney [6]. However, no measure of the metabolic clearance rate of osteocalcin has yet been made and, if the plasma level is to be used as an index of bone formation, further information about its metabolism and production rate is required. In these studies the metabolic clearance rate of osteocalcin has been measured in sheep and the plasma production rate has been calculated.

Materials and Methods

Trained oophorectomized crossbred-merino ewes between 3 and 6 years old and weighing 30–56 kg were used. They were maintained on a diet of 800 g of lucerne chaff per day throughout. Oophorectomized sheep were used so that animals could be studied in a basal state hormonally and the effects of gonadal steroids could be evaluated in future studies. Plasma osteocalcin was similar in comparable oophorectomized and intact sheep (intact, 19.6 ± 1.5 ng/ml, mean ± SEM, n = 6; oophorectomized, 20.2 ± 2.0 ng/ml, n = 6). Catheters were inserted into the jugular vein for infusing material and into the carotid artery for sampling. The carotid artery was in an exteriorized skin loop [8]. Several animals were used in more than one study, but the studies were always at least 2 weeks apart and usually 4 or more weeks apart. All animals had normal renal function.

Sheep osteocalcin was prepared from bone obtained from a local abattoir. The bone was cleaned, powdered, and extracted with ethylenediaminetetraacetic acid (EDTA) as previously de-

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scribed [6]. The EDTA extract was processed by ion exchange chromatography on Trisacryl, the fractions containing peak immunoreactivity were passed through a Sephadex G-50 column, and finally purified using high-pressure liquid chromatography. Reversed phase chromatography was carried out in 0.1 M ammonium acetate/0.1% trifluoroacetic acid with a gradient of 0–50% acetonitrile, using a 30 cm μBondapak C18 column (Waters) operated at 1 ml/min. The final product gave one band on SDS-20% polyacrylamide gel electrophoresis and had an amino acid composition identical to that of sheep osteocalcin [9].

Antiserum to bovine osteocalcin was raised in rabbits [2]. Chemicals used were of the highest purity available. Hydroxyapatite (type 1: Gel [aged] calcium phosphate) was obtained from Sigma.

Labeling of Osteocalcin

Fifteen micrograms of sheep osteocalcin was iodinated using 5 μg Iodogen [10] in the presence of 1 mCi of ^{125}I, sheep plasma was added to the preparation, and the material was purified on a 20 ml column of Sephadex G-25. In all preparations, more than 98% of the radioactivity was precipitable by 10% trichloracetic acid (TCA).

Preparation of Infusion Solutions

For bolus injection studies, radioactive osteocalcin was mixed with plasma from the sheep to which it was to be administered, passed through a 0.22 μm Millipore filter, and made up to an appropriate volume, usually 3 or 4 ml. The syringe was weighed, the material was administered intravenously over 30 sec, the syringe was again weighed and the volume of material injected was calculated from the weight difference. The concentration of plasma in the injection was 10%. The specific activity of the material injected was 83 cpm/pg osteocalcin. The counts injected were approximately 30×10^6 cpm, giving a total amount of osteocalcin injected of 375 ng.

For constant infusion studies, radioactive osteocalcin was prepared in a similar fashion but the final volume was 75 ml. The rate of infusion was 12 ml/h using a calibrated syringe and Harvard infusion pump. The total material injected over 6 hours ranged from 190×10^6 cpm to 323×10^6 cpm (mean 246×10^6 cpm) and the amount of osteocalcin given was 2.9–4.5 μg (mean 3.5 μg). Thus, the hourly infusion rate varied from 32×10^6 to 54×10^6 cpm/h (mean 41×10^6 cpm/h) and the amount of osteocalcin given per hour from 0.48–0.75 μg (mean 0.59 μg).

Sampling of Blood

Blood was taken from animals on the day before the study and also prior to the injection or infusion being given. Samples were collected at the times indicated in the results. Ten milliliters of blood was drawn into a heparinized tube, placed on ice, and plasma was separated within 30 min. The samples were processed as soon as the experiment was completed, and unused plasma was frozen at −20°C. Previous studies have shown that osteocalcin in plasma is stable at 4°C for at least 12 hours (Melick RA, unpublished data).

Processing of Plasma Samples

Nonradioactive osteocalcin was measured in triplicate at two dilutions by radioimmunoassay [4]. The detection limit of the assay is 0.2 ng/tube (equivalent to 2 ng/ml plasma). Intraassay and inter-assay coefficients of variation at a measured concentration of approximately 20 ng/ml are 10 and 14%, respectively. Radioactive osteocalcin was measured in three ways: by TCA precipitation, by adsorption to hydroxyapatite, and by antibody precipitation. For TCA precipitation, 200 μl of 0.15 M sodium chloride was added to 300 μl of plasma, and then 500 μl of ice-cold 20% TCA was added. The mixture was vortex-mixed, allowed to stand for at least 30 min at 4°C, and centrifuged at 3,000 rpm at 4°C for 10 min. The precipitate was washed with 1 ml of cold 10% TCA, recentrifuged, the supernatant discarded, and the pellet counted in a Packard 5230 auto-gamma scintillation spectrometer.

Hydroxyapatite binding was measured by adding 30 mg of hydroxyapatite in 30 μl of aqueous slurry to 300 μl of plasma, the material was vortex-mixed, and then turned end-over-end at 4°C for at least 30 min; the mixture was centrifuged at 3,000 rpm for 10 min at 4°C, the supernatant was decanted and the pellets were counted. For antibody precipitation, the antiserum solution contained antibody at a 1:175 dilution, and the normal rabbit serum solution contained rabbit serum at a dilution of 1:400, both being made up in the osteocalcin immunoassay buffer. To 300 μl of diluted normal rabbit serum were added 300 μl of plasma and 900 μl of buffer solution. The blank solution contained normal rabbit serum, labeled osteocalcin, and 1.2 ml of buffer solution. These were incubated for 16 hours at room temperature. Goat antirabbit gamma globulin was added, this solution was left for 6 hours, and the precipitate was separated by centrifugation at 3,000 rpm for 30 min at 4°C. The supernatant was decanted and the pellets were counted. In later studies, the antibody precipitation was modified by using only two solutions—one containing antiserum at a dilution of 1:75 and normal rabbit serum at a dilution of 1:100—and adding 300 μl of this solution to 300 μl of plasma. Control solutions contained no antiserum. The incubation conditions and second antibody precipitation were the same as described above.

The radioactive solution infused or injected was processed at the same time as the plasma samples by adding a measured amount to the pre-injection plasma obtained from the same sheep and subjecting this plasma to the same precipitation tests. Thus, the dose of TCA-precipitable osteocalcin, hydroxyapatite-adsorbed osteocalcin, or antibody-precipitable osteocalcin could be measured. In the single injection studies, the data were expressed as the fraction of dose in plasma at the time the sample was obtained. In the constant infusion studies, the specific activity of osteocalcin was calculated using the control plasma osteocalcin concentration as the value at each time sampled. Evidence justifying this will be presented in the results.

Chromatography

Ten-milliliter plasma samples were subjected to chromatography on Sephadex G-100 columns (15 × 100 cm) (Pharmacia Fine Chemicals, Sweden) and high pressure liquid chromatography using a Waters C_{18} μBondapak column (Millipore Corporation, Milford, MA) as previously described [12].

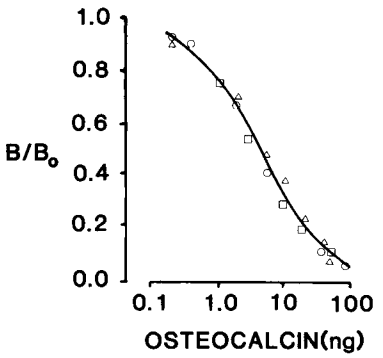

Fig. 1. Displacement of ^{125}I-ovine osteocalcin from antibovine osteocalcin by ovine (O), bovine (□), and human (△) osteocalcin. Incubation conditions were as previously described [4].

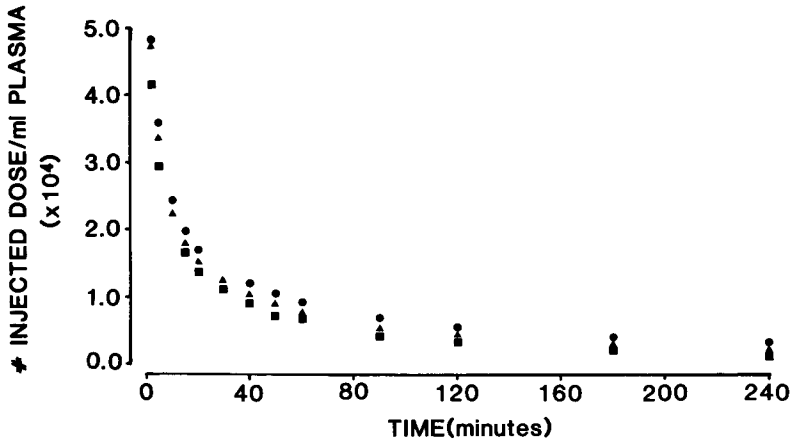

Fig. 2. Disappearance of ^{125}I-ovine osteocalcin after the bolus intravenous injection of 248 ng labeled osteocalcin (specific activity 83 cpm/pg) over 30 sec. ^{125}I-ovine osteocalcin concentration was measured by TCA precipitation (●), adsorption to hydroxyapatite (■), and immunoprecipitation (▲), and expressed as the fraction of the injected dose per ml of plasma.

Calculations

The metabolic clearance rates were calculated as described by Tait [11]. For the single injection studies, the area under the curve was determined using a Texas Instruments Calculator Model TI 59 and Simpson's approximator (TI programme ML-10). The production rate was determined by multiplying the metabolic clearance rate by the plasma concentration.

Results

The use of antiserum to bovine osteocalcin in the immunoassay of sheep osteocalcin was validated by showing that ovine and bovine osteocalcin gave superimposable standard curves (Fig. 1). Quantitation of sheep osteocalcin by amino acid analysis confirmed that ovine and bovine osteocalcin reacted identically in this assay. A 10 ml plasma sample was obtained at the termination of a 6 hour constant infusion of ^{125}I-ovine osteocalcin and chromatographed on Sephadex G-100, followed by HPLC of fractions containing radioactivity above background (excluding a small peak corresponding to ^{125}I), as previously described [12]. Only a single radioactive peak was detected by HPLC and this co-eluted with intact ovine osteocalcin.

The three methods of analyzing labeled osteocalcin—TCA precipitation, adsorption to hydroxyapatite, and antibody precipitation—all gave similar results (Fig. 2 and Table 4). Figure 2 shows a typical disappearance curve after a bolus injection of labeled sheep osteocalcin. When these data were analyzed as a double exponential, the half-time of the first exponential was 14 min and that of the second was 124 min. The volume of distribution of injected osteocalcin was 7.7 liters, slightly greater than the mean extracellular fluid volume of the sheep. The mean plasma clearance rate was 3.3 liters/h. Individual values calculated from bolus injection studies in six sheep are given in Table 1.

In an initial series of constant infusion studies, osteocalcin was infused for 3 hours. Although a plateau was reached in one animal (no. 6), analysis

Table 1. Plasma clearance rate of osteocalcin calculated from single bolus injections of labeled sheep osteocalcin in six animals (TCA precipitation method)

Sheep no.	Body wt/Kg	Plasma osteocalcin (ng/ml)	Clearance of osteocalcin (liters/h)
1	35	54.0	3.5
2	47	27.0	3.2
3	49	23.8	2.9
4	41	28.5	3.8
5	35	25.7	3.4
6	30	32.2	3.1
Mean	39.5	31.9	3.3
SD	7.5	11.2	0.32
SE	3.1	4.6	0.13

Table 2. Plasma levels of ^{125}I-osteocalcin (cpm/ml plasma) measured at 240–360 min of constant intravenous infusion of labeled sheep osteocalcin (TCA precipitation method)

Sheep no.	Time after infusion commenced (min)				
	240	270	300	330	360
1	10843	10877	11339	11229	11645
6	11103	11225	11644	11541	11635
8	16589	17234	17778	18484	16498
9	14083	15319	16372	17646	18445

of the other results showed that plasma ^{125}I-osteocalcin was still rising at the end of 3 hours. Accordingly, the studies were extended to 6 hours, and in all cases a plateau was reached (Table 2). The specific activity of labeled osteocalcin was calculated using the resting concentration. In the four animals sampled every 30 min for 4 hours, plasma osteocalcin values showed little variation; the mean and standard deviation of each are given in Table 3. The clearance rates calculated from the constant infusion studies are given in Table 4 and are similar to those derived from the single injection studies (3.3 ± 0.32 l/h (mean ± 1 SD) for single injection; 2.8 ± 0.17 l/h for constant infusion). For the constant infusion studies, the results are shown for each of the three precipitation methods. Because of the similarity and because TCA precipitation is the simplest method, the TCA values have been used in subsequent calculations.

The disappearance of radioactive osteocalcin after 3 hour infusions were ceased was studied in four animals (data not shown). In each, the decline in plasma radioactivity was linear when plotted semilogarithmically, the calculated half-times of disappearance being 80, 92, 92, and 100 min (91 ± 8.2 min, mean ± 1 SD).

Table 3. Variation in plasma osteocalcin determined by serial sampling at 30 min intervals over 4 hours in four sheep

Sheep no.	No. of samples	Range (ng/ml)	Mean (ng/ml)	SD	CV (%)
9	9	30.7–34.0	32.3	1.08	3.3
10	9	13.3–15.0	14.6	0.80	5.4
11	8	18.2–23.1	19.8	1.46	7.4
12	8	18.2–23.3	20.7	1.68	8.1

Table 4. Plasma clearance rate of osteocalcin calculated from 6 hour constant intravenous infusion of labeled sheep osteocalcin in four animals

Sheep no.	Body wt/Kg	Plasma osteocalcin (ng/ml)	Clearance of osteocalcin (1/h)		
			Antibody	Hydroxyapatite	TCA
1	35	25.3	2.4	2.6	2.7
6	30	25.7	2.4	3.1	2.7
8	56	16.2	3.7	3.3	3.0
9	47	15.3	3.7	2.7	2.6
Mean	42	20.6	3.1	2.9	2.8
SD	11.7	5.6	0.75	0.33	0.17
SE	5.9	2.8	0.38	0.17	0.09

The plasma osteocalcin concentration in 29 sheep was 26.9 ± 15.7 ng/ml (mean ± 1 SD). Taking this mean value and the mean clearance of 2.8 liters/h, the production rate can be calculated to be 1.8 mg per day.

Discussion

The identical dilution curves obtained with sheep osteocalcin and bovine osteocalcin, using the bovine antibody and the quantitation of sheep osteocalcin by amino acid analysis, validate the use of this radioimmunoassay for the measurement of sheep osteocalcin. The agreement between the various methods of measuring radioactive osteocalcin is strong evidence that they were measuring gamma-carboxylated intact osteocalcin. Although we have shown that TCA precipitation is not a reliable measure of labeled osteocalcin *in vitro* [6], possibly because of the precipitation of fragments, these fragments should be excreted by the intact animal with normal renal function. Weinstein and Gundberg [12] have described the presence of immunoreactive fragments of osteocalcin in the plasma of patients in renal failure but found none in normals. Using the same separation methods, we found no evidence of ^{125}I-labeled fragments in

plasma after a 6 hour constant infusion of ^{125}I-ovine osteocalcin. Moreover, after similar chromatographic analysis of plasma from normal and uremic sheep, we found only a single immunoreactive peak from high pressure liquid chromatography (HPLC) and this corresponded to intact ovine osteocalcin (data not shown). For the above reasons, we believe that the several methods used all measured intact osteocalcin and not fragments.

Because TCA precipitation was the easiest, cheapest, and least variable of the three techniques used, and because of the agreement between the three, we decided to use the TCA data for further calculations. The 3 hour infusions were obviously not long enough to reach steady-state conditions and therefore the clearance rates calculated were slightly higher than those obtained when the infusions were extended to 6 hours. The agreement between the bolus injections of radioactive osteocalcin and the 6 hour constant infusion studies strengthens the case that the results obtained reflect the plasma clearance rate of osteocalcin.

The data obtained give a plasma production rate of 1.8 mg of osteocalcin per day. To our knowledge, this is the first time that this calculation has been made. Price et al. [5] have calculated that the interval between new synthesis of osteocalcin and its complete turnover in serum is only 3 hours in the rat. They also observed that the time needed to clear from serum half of an intravenously administered dose of osteocalcin in the rat was less than 5 min. However, nontracer amounts of osteocalcin were injected in these studies and the findings are not comparable with our results. Our data, based on the disappearance of osteocalcin when the constant infusions were ceased, gave a mean half-time of 91 min in sheep. When radioactive bovine osteocalcin was injected into normal human subjects, the disappearance of TCA-precipitable radioactivity had several exponentials [7]; the half-time of the first was 10 min and of the second, 70 min (three studies in two normal human subjects; Melick, R.A., unpublished data).

The relationship between osteocalcin in plasma and osteocalcin in bone is not known. Price [13] has proposed that osteocalcin found in plasma is that which is secreted by the osteoblast but escapes binding to hydroxyapatite crystals and diffuses away from the site of mineralization into the blood. The data presented in this paper allow one to compare the plasma production rate of osteocalcin with the amount of osteocalcin that may be localized in bone. We have examined the osteocalcin content of sheep bone and find that it varies from 10% of noncollagenous matrix in young animals to 5% in older animals (unpublished data). These animals are older and one would expect to find approximately 1–1.2 mg of osteocalcin/g of sheep bone (noncollagenous matrix = 25 mg/g of bone). Based on the studies of Braithwaite in mature sheep [14], it can be estimated that the sheep used in our experiments may form approximately 2 g of bone daily. Therefore, if one assumes that the amount of osteocalcin deposited in bone is around 2–2.5 mg/day and the amount of osteocalcin released into plasma is 1.8 mg, then the amount of osteocalcin going to plasma is similar to that deposited in bone.

The use of plasma osteocalcin measurements to indicate bone formation rate depends upon the clearance of osteocalcin being constant and variations in the plasma concentration being due solely to variations in osteoblastic activity. The present data suggest that most of the variability in plasma osteocalcin in the basal state in oophorectomized sheep is attributable to variations in plasma production rate, since the plasma clearance rate was relatively constant (coefficient of variation = 6.1%). However, possible effects on plasma osteocalcin clearance associated with various perturbations of bone and mineral metabolism have yet to be investigated. The relative constancy of the plasma clearance rate basally will facilitate the detection of changes in clearance with various experimental manipulations.

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