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ORIGINAL ARTICLE

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The effects of salmon calcitonin-induced hypocalcemia on bone metabolism in ovariectomized rats

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Abstract The ovariectomized rat has proved to be a most useful model for preclinical testing of potential therapies for osteoporosis. We describe the immediate effects of a single treatment with salmon calcitonin (sCT) on calcium homeostasis and bone turnover markers in 6-month-old sham and ovariectomized (ovx) rats at 15 days postovariectomy. Rats were fasted for 24 h prior to and following administration of 0.3 μg/kg body weight sCT. Blood specimens were collected at 0 (pretreatment), 2, 4, and 8 h. Urine samples were collected during the intervening periods. sCT treatment produced a decrease in blood ionized calcium at 2 h posttreatment in sham and ovx rats ($P < 0.001$), which was exaggerated in the ovx rats ($P < 0.001$). Increased parathyroid hormone (PTH) levels ($P < 0.001$) accompanied the hypocalcemia in ovx rats. Furthermore, PTH levels were significantly higher in ovx rats compared with sham rats for the same ionized calcium range of 1.275–1.300 mmol/l ($P < 0.05$). sCT treatment in sham rats increased urine hydroxyproline (UHyp) at 6 h posttreatment ($P < 0.01$). In conclusion, the calcitonin-induced hypocalcemia and secondary hyperparathyroidism was more pronounced in the ovariectomized rats, consistent with the actions of calcitonin in states of increased bone turnover induced by estrogen deficiency. This study highlights the importance of considering the actions of PTH and estrogen status when interpreting changes in calcium homeostasis and bone turnover following treatment with calcitonin in rodent models and provides further evidence for a potential role of estrogen in parathyroid function.

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Introductions

Calcitonin (CT) is a potent inhibitor of osteoclastic bone resorption and has been reported to exert analgesic effects [1], properties which have led to its use as a treatment for postmenopausal osteoporosis. The current form of therapy is salmon calcitonin (sCT), as it is more potent than CT from other species including that from humans [2]. sCT treatment in patients with established osteoporosis increases bone mineral density in the lumbar spine by 1%–2% following 1 year of treatment and reduces vertebral fracture risk after 2 years of treatment [3].

CT exerts its inhibitory action on osteoclasts via the calcitonin receptor (CTR), causing cytoplasmic contraction of the cell membrane [4] and promoting vacation of osteoclasts from the bone surface [5]. Furthermore, sCT treatment in intact rats decreases the number of nuclei per osteoclast by fission of preexisting osteoclasts into undistinguishable mononucleated cells and decreases the number of osteoclasts by decreasing the number of new cells entering the precursor pool [5]. CT also acts on osteoblasts to increase their proliferation and alkaline phosphatase (ALP) activity in vitro [6,7], which is associated with increased synthesis and deposition of bone matrix collagen [6,8]. These effects of CT on osteoblasts are dependent on the cell density and stage of differentiation at the time of treatment [7]. Recently, it has been postulated that the actions of CT on bone formation are, at least in part, mediated directly via CTRs located on osteoblasts [9] through the cAMP second messenger system [7].

The ovariectomized rat has been the most useful model for preclinical testing of osteoporosis therapies; however, rodents have relatively high rates of bone turnover compared with humans or larger mammals [10]. The effect of CT on bone structure in vivo appears to be dependent on the rate of bone turnover at the time of treatment. Treat-

ment of ovariectomized (ovx) rats immediately following the operation with sCT for 6 weeks prevented the development of osteopenia [11,12]. This suppressive effect of sCT in ovx rats was independent of whether it was administered intermittently or continuously [13]. In ovx rats with glucocorticoid-induced osteopenia and a bone turnover rate that does not differ from controls, sCT treatment suppressed bone resorption while stimulating bone formation, thereby preventing the progression of the glucocorticoid-induced osteopenia [14]. The anabolic action of sCT treatment on bone formation is believed to be, at least in part, mediated by the elevated parathyroid hormone (PTH) levels observed in these rats [14]. The bone-preserving effects of sCT therefore appear to be greater in states of low bone turnover. This view is supported by the observation that the prevention of ovariectomy-induced bone loss by sCT has a greater effect in the femoral neck, which has a slower rate of cancellous bone loss, than in the proximal tibia [15].

In states of normal bone turnover, treatment of intact rats with a high dose of sCT (20 IU/kg body weight) for 6 weeks decreases trabecular bone volume in the tibia by increasing osteoclast number and suppressing bone formation [16]. The effect of sCT in these animals seems to be mediated by hyperparathyroidism in response to the sCT-induced hypocalcemia, since bone loss was prevented by parathyroidectomy [16].

The majority of previous studies have investigated the effects of sCT on bone cell metabolism following 1 to 2 months of treatment [11–16]. Interpretation of the observed changes in bone structure following treatment, however, may be confounded by the hyperparathyroidism accompanying the CT-induced hypocalcemia. Therefore, the following study describes the immediate effects that occur in the biochemical markers of calcium homeostasis and bone cell metabolism following a single treatment with sCT in sham rats and in ovx rats at 15 days postoperation, when bone turnover is elevated as a result of estrogen deficiency [17,18].

Materials and methods

Animals

Twenty 6-month-old female Sprague Dawley rats (220–360 g) were obtained from the Gilles Plains Animal Resource Centre (Gilles Plains, Australia). The animals were fed commercial rat chow containing 0.7% calcium and 0.6% phosphorus (Milling Industries, Murray Bridge, Australia), and tap water was supplied ad libitum. The animals were housed at 26°C with a 12-h light–dark cycle. The Institute of Medical and Veterinary Science and the University of Adelaide Animal Ethics committees approved all procedures.

Experimental procedure and design

Twenty rats were randomly allocated to either an ovariectomy or sham operation performed under halothane anesthesia. At 15 days postoperation, rats underwent experimental protocol 1 and 2 (Fig. 1), which were separated by a 5-day recovery period. The order in which each rat under went protocols 1 and 2 was randomized. Food was withdrawn from the rats for 24 h, and a blood specimen was obtained from the tail vein while the rat was under halothane anesthesia to provide baseline serum biochemistry (time = 0 h). Immediately following blood collection, the rats were injected subcutaneously with sCT (Auspep, Parkville, Australia) at a dose of 0.3 μg/kg body weight (1.2 IU/kg body weight). No food was made available to the rats throughout each protocol. For experimental protocol 1, blood specimens from the tail vein were collected at 4 and 8 h following sCT administration. Immediately following sCT treatment (time = 0), urine was collected by placing the rats in metabolic cages for two 4-h periods between each blood specimen collection [i.e., a urine specimen was collected from 0 to 4 h post-sCT treatment and from 4 to 8 h post-sCT treatment (Fig. 1)]. For experimental protocol 2, a blood specimen was collected at 2 h post-sCT administration, after which the rats were placed in metabolic cages for 4 hours for urine specimen collection [i.e., a urine specimen was collected form 2 to 6 h post-sCT treatment (Fig. 1)]. After each blood sample was collected, the rats were administered an intraperitoneal injection of 2.0 ml of 0.9% saline to prevent hypovolemia.

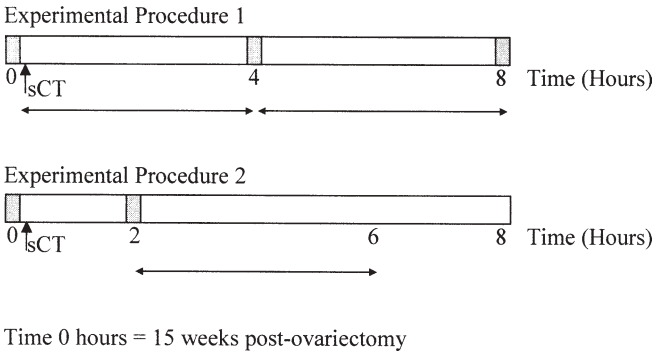

Fig. 1. Diagrammatic representation of experimental protocol. Salmon calcitonin (*sCT*) (0.3 μg/kg body weight) was administered 15 days postoperation (time = 0 h). *Shaded boxes* indicate fasting blood sample collection time and *arrows* indicate time periods for which fasting urine samples were collected following sCT treatment, as described in Materials and methods

Biochemical analyses

Blood ionized calcium was measured on a calcium-pH analyzer (Ciba-Corning 634, Australian Diagnostics, Scoresby, Australia). Serum intact and N-terminal parathyroid hormone (PTH) was analyzed by a two-site immunoradiometric assay (rat IRMA PTH, Nichols Institute, San Juan Capistrano, CA, USA). Urine creatinine, acidified urine calcium, and serum alkaline phosphatase (SALP), calcium, total protein, and creatinine were analyzed on a bichromatic analyzer (Abbott VP, Abbott Diagnostics, Irving, Texas, USA) using manufacturer-recommended chemical methods. Urine hydroxyproline (UHyp) was mea-

sured by the method of Bergman and Loxley [19]. Serum osteocalcin (SOC) was measured by radioimmunoassay as described by Morris et al. [20].

Calculations and statistical analyses

Urinary calcium and UHyp were expressed relative to creatinine (mmol/mmol). Ultrafiltrable calcium (UFCa) was calculated using the following formula described by Morris et al. [20].

$$\text{UFCa} = \text{Total Serum Calcium} - (0.02 \times \text{Total Protein})$$

This equation was derived by regression of total serum calcium on serum total protein in sham rats. Maximum renal tubular reabsorption of calcium (TmCa) was calculated by the formula described by Marshall [21].

The effect of ovariectomy on the biochemical analyses was determined in baseline samples collected at the 0-h time point using an unpaired Student's *t* test. The interaction between the effects of sCT and ovariectomy was tested by two-way analysis of variance, with ovariectomy as a fixed variable and time after sCT administration as a random variable. When the analysis of variance indicated a significant effect of treatment, the specific differences were identified by Tukey's post hoc test [22]. To further assess the relationship between PTH and ionized calcium in sham and ovx rats, ionized calcium levels were divided into six 0.025 mM intervals ranging from 1.25 mM to 1.40 mM ionized calcium. The 0.025 mM interval was chosen in an attempt to attain adequate and equal sample numbers within each interval for statistical analysis. Ionized calcium and corresponding PTH values were sorted into the appropriate 0.025 mM ionized calcium interval. Within each ionized calcium interval, the mean PTH levels were calculated for both sham and ovx rats. The effect of ovx on PTH levels at each 0.025 mM ionized calcium interval was determined by Student's unpaired *t* test. A value of $P < 0.05$ was considered significant.

Results

Effect of ovariectomy

The bone resorption marker, UHyp was increased in ovx rats compared to sham rats ($P < 0.005$) throughout the experiment (Table 1). The bone formation markers, SALP ($P < 0.05$) and SOC ($P < 0.001$), were increased as a result of ovariectomy prior to sCT administration at 0 h (Table 2). Baseline serum ionized calcium did not differ between

Table 1. Effect of salmon calcitonin (sCT) treatment on urine hydroxyproline/creatinine

Urine variable	Operation	Time posttreatment (h)		
		0–4	2–6	4–8
Hydroxyproline/creatinine (mmol/mmol)	Sham	7.3 ± 0.9 *n* = 5	17.4 ± 2.2** *n* = 2	11.6 ± 1.2 *n* = 8
	Ovx*	14.6 ± 1.5 *n* = 5	21.3 ± 0.3 *n* = 5	14.3 ± 1.4 *n* = 3

Values are mean ± SE. Fasting urine samples were collected for 4-h periods throughout the experiment as described in the methods
* $P < 0.005$, effect of ovariectomy
** $P < 0.05$ versus 0–4 h

Table 2. Effects of sCT on serum biochemical variasies

Serum variables	Operation	Time posttreatment (h)			
		0	2	4	8
Osteocalcin (μg/l)	Sham	11.4 ± 0.9 *n* = 10	8.4 ± 1.3 *n* = 9	10.5 ± 0.9 *n* = 9	10.8 ± 0.6 *n* = 9
	Ovx	19.2 ± 1.8** *n* = 10	12.5 ± 0.8**** *n* = 10	14.1 ± 0.8*** *n* = 10	16.6 ± 1.3 *n* = 10
Alkaline phosphatase (Units/l)	Sham	55.2 ± 6.4 *n* = 9	53.5 ± 15.6 *n* = 10	50.4 ± 5.8 *n* = 9	38.6 ± 4.9 *n* = 9
	Ovx	77.3 ± 8.4* *n* = 10	64.3 ± 6.6 *n* = 8	63.9 ± 5.0 *n* = 10	60.0 ± 5.3 *n* = 10
Calcium (mmol/l)	Sham	2.61 ± 0.10 *n* = 10	2.23 ± 0.15 *n* = 10	2.49 ± 0.08 *n* = 9	2.39 ± 0.08 *n* = 9
	Ovx	2.72 ± 0.09 *n* = 10	2.32 ± 0.05**** *n* = 10	2.36 ± 0.09**** *n* = 10	2.32 ± 0.04**** *n* = 10

Values are mean ± SE
* $P < 0.05$ versus sham
** $P < 0.001$ versus sham
*** $P < 0.05$ versus 0 h
**** $P < 0.005$ versus 0 h, within operation group

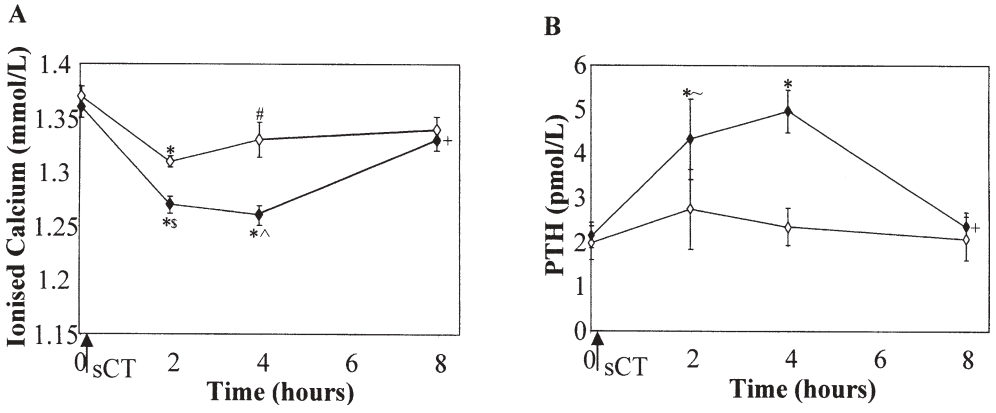

Fig. 2. Effect of sCT administered at 15 days postoperation on (**A**) blood ionized calcium (mmol/l) and (**B**) parathyroid hormone (*PTH*) (pmol/l) in sham (◇) and ovariectomized (ovx) (◆) rats. Values are mean ± SE, $n = 9$ or 10 at 2, 4, and 8 h. $^{+}P < 0.001$ effect of ovariectomy; $^{*}P < 0.001$ versus 0 h; $^{\#}P < 0.05$ versus 0 h; $^{\wedge}P < 0.001$ versus 8 h; $^{\sim}P < 0.005$ versus 8 h; and $^{\$}P < 0.01$ versus 8 h, within the operation group

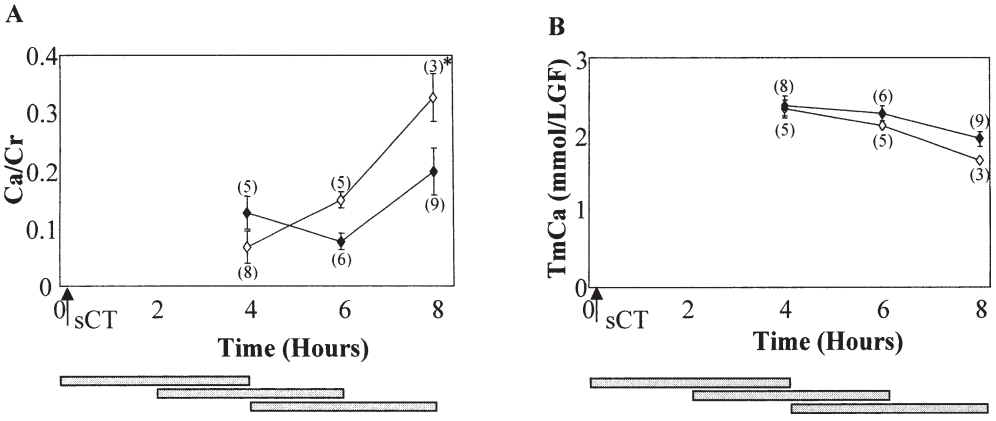

Fig. 3. Effect of sCT administered at 15 days postoperation on (**A**) urine calcium/creatinine (mmol/mmol) and (**B**) TmCa (mmol/l glomerular filtrate (*GF*)) in sham (◇) and ovx (◆) rats. Values are mean ± SE; *numbers in brackets* above each data point indicate number of observations. *Shaded bars* represent time periods for which fasting urine specimens were collected, as described in Materials and methods. $^{*}P < 0.05$ versus 0–4 h within the operation group

ovary intact and ovx rats in samples collected by experimental protocols 1 and 2 (time = 0 h, $n = 20$). Therefore, a random selection of ten baseline serum samples (one from each rat) was chosen for analysis to obtain equal numbers of analyses at each time point ($n = 10$). Baseline PTH, total serum calcium (Fig. 2b, Table 2), urine calcium/creatinine and TmCa (data not shown) were unaffected by ovariectomy.

Effect of salmon calcitonin treatment in sham rats

In sham rats, sCT treatment caused an immediate decrease in ionized calcium ($P < 0.001$) with maximal suppression occurring at 2 h posttreatment and returning to levels within the normal range by 8 h posttreatment (Fig. 2a). Small changes in PTH and total serum calcium levels were observed following sCT treatment; however, these were not statistically significant (Fig. 2b, Table 2).

In sham rats, the bone resorption marker, UHyp, changed significantly with time following sCT treatment ($P < 0.001$) such that UHyp levels were elevated at 6 h posttreatment ($P < 0.01$) but returned to normal levels by 8 h posttreatment (Table 1). Small nonsignificant changes were observed in the biochemical markers of bone formation SALP and SOC (Table 2).

sCT treatment in sham rats increased urine calcium/creatinine throughout the experiment ($P < 0.05$) (Fig. 3a), which was associated with a decrease in TmCa, although this change did not achieve statistical significance ($P = 0.059$) (Fig. 3b).

Effect of sCT treatment in ovx rats

In ovx rats, sCT treatment led to an immediate decrease in blood ionized calcium levels ($P < 0.001$) (Fig. 2a), which was associated with an increase in PTH ($P < 0.001$) (Fig. 2b). Ionized calcium and PTH levels normalized by completion of the experiment at 8 h posttreatment. sCT treatment decreased total serum calcium levels ($P < 0.001$), which mirrored the change in ionized calcium (Table 2).

There was a trend for UHyp to be increased at 6 h post-sCT treatment in ovx rats; however, this change did not reach statistical significance ($P = 0.007$) (Table 1). SALP was unaffected by sCT administration (Table 2). In contrast, SOC was decreased following sCT treatment, with the nadir occurring at 2 h posttreatment ($P < 0.01$) (Table 2).

No significant changes in urine calcium/creatinine or TmCa (Figs. 3a and b) were detected following sCT treatment in ovx rats.

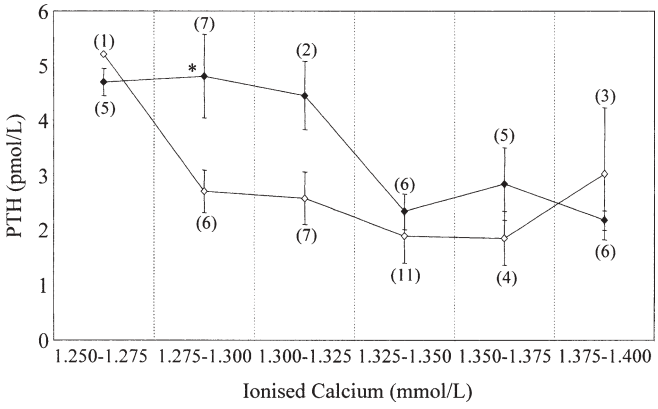

Fig. 4. The effect of ionized calcium (mmol/l) on PTH (pmol/l) in sham (◇) and ovx (◆) rats. Ionized calcium and corresponding PTH levels were sorted into 0.025 mM ionized calcium intervals, indicated by the dashed lines. The mean ± SE of PTH levels within each interval were calculated for both sham and ovx rats; *numbers in brackets* above each data point indicate the number of observations. $*P < 0.05$ versus sham for the same 0.25 mM ionized calcium range

Comparison of effects of sCT in sham versus ovx rats on the calcium–PTH axis

The decrease in blood ionized calcium and subsequent increase in PTH following sCT treatment were exaggerated in the ovx animals compared with that in the sham rats (interaction: $P < 0.001$, two-way analysis of variance) (Figs. 2a and b). Furthermore, the PTH levels were significantly higher in ovx rats compared with those in sham rats for the same ionized calcium range of 1.275 to 1.300 mmol/l ($P < 0.05$) (Fig. 4).

Discussion

Following continuous treatment of rodents with sCT for 1 to 2 months, effects on bone structure are often confounded by the hyperparathyroidism that accompanies the CT-induced hypocalcemia [16]. The present study describes the acute effects of sCT on the biochemical markers of calcium homeostasis and bone turnover.

A single dose of sCT resulted in a decrease in blood ionized calcium in both sham and ovx rats, evident at 2 h following treatment, and the levels returned to normal by 8 h posttreatment. The fall in ionized calcium levels in ovx rats was exaggerated compared with that observed in sham rats. In ovx rats, total serum calcium was also decreased by sCT, although the fall was undetectable in sham rats. The sCT-induced hypocalcemia is consistent with the inhibitory action of sCT on osteoclasts and bone resorption [4,5]. Our findings confirm a previous report of decreased ionized calcium levels at 4 h following treatment with a high dose of sCT (4 IU/kg body weight) in ovary-intact rats [16]. Previous studies measuring total serum calcium following CT treatment in ovx rats and dogs have reported conflicting findings of unchanged [11,23] and decreased [13] serum calcium levels at 6 weeks posttreatment. Total serum calcium levels are determined by ionized calcium levels plus the levels of calcium-binding agents, including albumin, bicarbonate, phosphate, and other anions. Variations in the levels of the binding agents often masks any changes in ionized calcium levels. Thus, measurements of ionized calcium as opposed to total serum calcium are more sensitive for detection of the inhibitory actions of CT on osteoclasts and the consequent decrease in flow of calcium from bone into the extracellular fluid.

The sCT-induced hypocalcemia was exaggerated in the ovx rats compared with that in the sham rats, which is consistent with the greater calcium-lowering effect of sCT observed in animals with higher rates of bone resorption [24,25]. The elevation of the bone biochemical markers in the present study is consistent with the marked increase in bone turnover following estrogen deficiency. Furthermore, it has been proposed that the differences observed in bone turnover in ovx and sham dogs following CT treatment may also be attributed to differences in the sensitivities of osteoclasts to CT in estrogen-deficient versus estrogen-sufficient states [23]. The hypocalcemia in the ovx rats was accompanied by an acute increase in PTH secretion, which returned to baseline levels by 8 h following sCT administration, when ionized calcium levels had also normalized. Apparently the sCT-induced fall in ionized calcium in the sham rats was of insufficient magnitude to invoke secondary hyperparathyroidism.

PTH levels were significantly elevated in ovx rats compared with those in sham rats at the same range of ionized calcium levels of 1.275–1.300 mmol/l. This elevation may reflect a change in the set point in ionized calcium levels for PTH secretion in the absence of estrogen [26,27]. Measurements of the parathyroid gland set point, that is, the calcium concentration that results in 50% of the maximal PTH stimulation, demonstrate a shift of the parathyroid gland set point with ovariectomy in humans [26]. Following Na_2 ethylenediaminetetraacetate-induced hypocalcemia, the regression slope between PTH and blood calcium levels was significantly higher than that observed prior to ovariectomy [26]. Furthermore, estrogen treatment in normal postmenopausal women decreases the set point of PTH stimulation by serum total and ionized calcium [27]. Caution, however, must be taken when assessing the PTH-ionized calcium relationship in vivo, as the set point for calcium-related PTH release is strongly dependent on baseline serum calcium levels [28,29]. In the present study, it is important to note that prior to sCT administration, the baseline levels of ionized calcium, total serum calcium, and PTH did not differ significantly between sham and ovx rats. The data from the present study provides further confirmation that in the absence of estrogen, the parathyroid glands have a higher sensitivity to serum calcium levels, resulting in increased PTH levels, which in turn may increase bone resorption. Further investigation into the effect of ovariectomy on the PTH set point in rodents is required, along with a complete study of parathyroid function.

The mechanism for the change in the PTH set point observed with estrogen deficiency in previous studies is

unclear [26,27]. There is a strong body of evidence that suggests that estradiol modulates calcium homeostatic mechanisms [30]. Estradiol has been demonstrated to directly stimulate PTH secretion from parathyroid cells in vitro [31] and to increase PTH mRNA levels in ovx rats [32]. In postmenopausal women, the evidence for the effect of estrogen on serum PTH levels is conflicting, with reports of increased [33] and unchanged [34] PTH secretion. Changes in PTH secretion following estrogen treatment, however, can be attributed to the antiresorptive actions of estrogen, thereby lowering ionized calcium and stimulating PTH secretion [33]. It is possible that estrogen may also act indirectly to modulate PTH secretion. The PTH set point is determined by activation of the calcium-sensing receptor (CaSR) of the parathyroid gland, which activates phospholipases C, A, and D [35] and mitogen-activated protein kinases (MAPKs) in a range of cells [36]. It has been proposed that estradiol can modulate MAPK activity [37], which in turn, therefore, can modulate CaSR activity.

In sham rats, UHyp increased between 2 to 6 h post-sCT treatment. There was a trend for UHyp to be elevated in ovx rats at this time; however, this increase was not statistically significant. As the predominant action of sCT is to suppress bone resorption, this increase in bone resorption in sham rats is somewhat surprising. Such a rise in bone resorption is most likely due to the action of PTH, which is well known to stimulate osteoclastogenesis via the receptor activator of nuclear factor κB ligand pathway [30]. Perhaps, the slight nonsignificant increase in PTH in sham rats at 2 h posttreatment was sufficient to stimulate bone resorption. The inability to detect a significant rise in UHyp in ovx rats despite the marked increase in PTH levels at this time may be attributed to the high level of bone turnover in these animals at the time of treatment. In sham rats, UHyp rose by 238% over baseline, whereas in ovx rats a 146% rise was observed over baseline. Therefore, the absolute level of osteoclast activity reached in the ovx rats may have been maximal.

There was a trend for the levels of the serum osteoblast markers SALP and SOC to vary following sCT treatment in both sham and ovx rats. SOC in ovx rats, with the higher basal levels, was the only marker to demonstrate a statistically significant decrease. Such a decrease in SOC could reflect actions to increase the renal clearance of SOC or the action of sCT to acutely downregulate bone cell activity. Further studies are required to identify the mechanism of this change.

The urinary excretion of calcium was increased in sham rats following sCT treatment and was associated with a decrease in the tubular reabsorption of calcium in the kidney, although the latter did not reach statistical significance. CT and PTH have opposing actions on the kidney, such that CT decreases the tubular resorption of calcium and increases calcium excretion [25], while PTH stimulates calcium conservation by the kidney [38]. The effect observed in the present study, therefore, appears to be mediated by sCT and not by the elevated PTH observed at this time. In patients with Paget's disease, hyperparathyroidism, or carcinoma of the breast, CT treatment results in increased calcium excretion [39] and is used clinically to treat hypercalcemia in patients with metastatic bone disease [25]. The effects of sCT on the kidney are most likely mediated via CTRs, which have been identified in the cortical thick ascending Loop of Henle and the distal tubule [40], where 25% and 10% of calcium is reabsorbed, respectively [41]. In ovx rats, the reduced effect of sCT on renal tubular reabsorption, combined with the exaggerated effect of hypocalcemia, produced no significant changes in urine calcium excretion. It appears, therefore, that the actions of sCT in the kidney dominate over those of PTH.

In conclusion, sCT treatment produces an immediate decrease in ionized calcium in sham and ovx rats. The sCT-induced hypocalcemia was more pronounced in the ovx rats and was accompanied by an increase in PTH secretion, consistent with the actions of CT in states of high bone turnover induced by estrogen deficiency. Of interest was the observation that PTH levels were elevated in ovariectomized rats compared with those in sham rats for the same ionized calcium range. These data provide further supportive evidence for a role of estrogen in parathyroid function and calcium homeostatic mechanisms. The results from this study further highlight the importance of considering the actions of PTH and estrogen status when interpreting the changes in calcium homeostasis and bone turnover following treatment with CT in rodents and may have implications for sCT treatment in humans.

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