Possible variation in bone resorption during the normal menstrual cycle

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In order to determine whether bone turnover varies during the normal menstrual cycle, we measured biochemical markers of bone resorption (serum pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen (sICTP), fasting urinary hydroxyproline/creatinine, fasting urinary pyridinoline/creatinine and fasting urinary deoxypyridinoline/creatinine) and bone formation (plasma osteocalcin, serum carboxy-terminal propeptide of type 1 procollagen and serum alkaline phosphatase) in ten healthy premenopausal women every two or three days for a complete menstrual cycle. A cyclic pattern was detected in sICTP, with its nadir during the follicular phase and its peak during the luteal phase, and an overall variation of 17% during the menstrual cycle (p=0.004). No cyclic changes were observed in the urinary parameters of bone resorption or in the biochemical markers of bone formation. We conclude that sICTP, a new biochemical marker of bone resorption, undergoes small variations during a normal menstrual cycle in premenopausal women, whereas the biochemical markers of bone formation remain constant.

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Hormone replacement therapy after the menopause has a major impact on bone metabolism (1, 2), although the exact mechanism is still unknown. Oestrogen and progestogen exhibit very characteristic changes during the menstrual cycle (3). Whether these cyclic changes have any impact on the bone turnover is still debated. A recent study by Nielsen et al. (4) indicated a cyclic variation in plasma osteocalcin (pOC), a marker of the bone formation, in premenopausal women, which reached a maximum during the luteal period. They found a similar variation in bone alkaline phosphatase, which did not reach significance. We have recently demonstrated a cyclic variation in the serum concentration of alkaline phosphatase (sAP) in postmenopausal women receiving sequential hormonal replacement therapy (5, 6).

Until now very few data have been available as to whether cyclic variations can be demonstrated in biochemical parameters of bone resorption (5–7). These studies used fasting urinary hydroxyproline/creatinine (fU-HPr/Cr) as the resorption parameter but when it is used as the sole measurement for the skeletal loss of collagen it is not, however, entirely valid because of its low specificity (8).

More specific markers of bone resorption have become available recently. The serum pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen (sICTP) seems to derive mainly from bone as a degradation product of the cleavage of old bone collagen into smaller fragments, which is released into the extracellular matrix. Furthermore, recent preliminary data showed that sICTP correlates with calcium balance and bone resorption as measured histomorphometrically (9). Pyridinoline (Pyr) and deoxypyridinoline (D-Pyr) are two mature cross-linking amino acids that have been identified in bone, cartilage and, to a lesser extent, other connective tissues except skin (10, 11). Both Pyr and D-Pyr have been found to be excreted non-metabolized by the kidney (12) and to be independent of the diet (13). Moreover, in a recent study we showed that urinary excretion of Pyr and D-Pyr correlates with histomorphometrically measured bone resorption in osteoporotic women (14). These new markers of bone resorption may therefore be superior to U-HPr/Cr.

The primary aim of the present study was to assess further the influence of the normal menstrual cycle on biochemical markers of bone turnover, with special emphasis on the new markers of bone resorption: sICTP and the urinary excretion of Pyr and D-Pyr.

Materials and methods

Participants

Ten healthy premenopausal women (aged 33±5 years; height 167±5 cm; weight 57±6 kg; mean±1 sd) participated in this cyclic study. None had any history of endocrine, renal or metabolic diseases or were taking any medication or oral contraceptive at the time of the study. For each woman, the study period started during the first 3 days following commencement of menstruation and ended within the first 3 days following com-
mencement of the next menstruation. All participants menstruated regularly.

Blood samples were taken and the urine collected every Monday, Wednesday and Friday during the study period, after 8 h of fasting and tobacco abstinence. Urine samples were collected about 1 h after the bladder had been emptied and the blood samples were taken at precisely the same time (08:00 h).

Informed consent was obtained from each participant according to the Helsinki Declaration II, and the trial was approved by the Ethical Committee of Copenhagen County.

Biochemistry

Urinary pyridinium cross-links. The fasting urine sample was hydrolysed in 6 mol/l HCl at 108°C for 24 h before extraction by cellulose CF1 partition column chromatography. The extraction product was then freeze-dried before separation on a reverse-phase C18 column by HPLC and identified by spectrofluorimetry. The results of U-Pyr and U-D-Pyr were expressed in accordance with a comparison with an external standard injected in an amount comparable to the expected sample concentration and are given as pmol/μmol creatinine. The intra-assay coefficients of variation were 4.1% and 6.0% for U-Pyr and U-D-Pyr, respectively. The interassay coefficients of variation were 9.1% for U-Pyr and 11.5% for U-D-Pyr. The recoveries from hydrolysis to identification were 82 ± 8% for U-Pyr and 85 ± 14% for U-D-Pyr (15). Both U-Pyr and U-D-Pyr were corrected for creatinine excretion (U-Pyr/Cr and U-D-Pyr/Cr).

The concentration of sICTP was measured by radioimmunoassay (16) (Orion Diagnostica, SF-90460 Oulunsalo, Finland). The antigen was isolated from insoluble type I collagen of decalcified human femoral bone after liberation by digestion with bacterial collagenase. The cross-linked telopeptide was purified by HPLC and its identity verified by amino-terminal amino acid sequence analysis. Polyclonal antibodies against ICTP were produced in rabbits and the peptide was labelled by the chloramine-T method with iodine-125. The intra-assay and interassay coefficients of variation were about 6% in the concentration range of this study. The preliminary reference interval for healthy adults (mean ± 2 SD) is 1.8–5.0 μg/l.

Urinary hydroxyproline was measured by spectrophotometry (17) and corrected for creatinine excretion (U-HPr/Cr). The intra-assay and interassay coefficients of variation of U-HPr were about 10% and 13%, respectively.

Plasma osteocalcin (pOC) was measured by radioimmunoassay (18). Antiserum was raised in rabbits immunized with purified intact calf OC, and homogeneous calf OC was used for standard and tracer. The sensitivity of the assay was 0.02 nmol/l. The intra-assay and interassay coefficients of variation were less than 7% and 12%, respectively.

The concentration of the carboxy-terminal propeptide of type I procollagen (sP1CP) was measured in duplicate 100 μl serum samples by radioimmunoassay (19) (Orion Diagnostica, SF-90460 Oulunsalo, Finland). The intra-assay and interassay coefficients of variation were about 5%.

Serum alkaline phosphatase (sAP) was measured enzymatically, according to Scandinavian recommendations (20).

Serum oestriadiol (E2) was measured with antisemur supplied by Milab (Sweden) and tritium-labelled E2 supplied by Amersham International (Amersham, UK). Standards were prepared with agents supplied by Merck (Darmstadt, Germany). The intra-assay and interassay coefficients of variation were 6% and 15%, respectively, and the sensitivity was 0.015 nmol/l.

Statistical evaluation

The procedures of the Statistical Analysis System were applied in the statistical analyses (21).

Each menstrual cycle was normalized to 28 days by commencement of menstruation (days 0 and 28) and time of ovulation (day 14), as estimated by the E2 peak. The model $Y = \beta_1 \sin(t) + \beta_2 \cos(t) + \alpha$, where $t = 2\pi t/28$, was used as an alternative to the hypothesis that no cyclic trend exists. The coefficients $\beta_1$ and $\beta_2$ were calculated for each woman by multiple regression analysis and compared to zero by MANOVA (21).

The data were transformed logarithmically to normalize variations and $p < 0.05$ was considered to be statistically significant. The estimated sinusoidal curves were transformed anti-logarithmically and presented in percentages.

We also tested the two phases (follicular and luteal) in the menstrual cycle by Student’s paired $t$-test. The follicular phase was given by the mean value of the 10 days before the oestradiol peak and the luteal phase by the mean value of the 10 days after the oestradiol peak.

Results

The mean length of the menstrual cycle was 29.2 days (range 25–35 days) in the ten women studied. The follicular phase was 9.9 days (range 7–17 days) and the luteal phase 15.5 days (range 11–19 days), separated by the $E_2$ peak. We standardized the duration of the cycles and synchronized by ovulation and phases. This eliminates interindividual variations in the lengths of the cycle and its phases.

Figure 1 shows the characteristic cyclic pattern of oestradiol and of the biochemical markers of bone formation, i.e. sP1CP, sAP and pOC, expressed in per cent of the mean individual value. Despite the well-known characteristic cyclic pattern in oestradiol, no cyclic changes were observed in the biochemical markers of bone formation.

Figure 2 shows the changes in the biochemical
markers of bone resorption, i.e. sICTP, fU-Pyr/Cr, fU-D-Pyr/Cr and fU-HPr/Cr during the menstrual cycle, expressed in per cent of the mean individual value. Serum ICTP showed a significant cyclic pattern during the menstrual cycle, with its nadir during the follicular phase and its peak during the luteal phase, and an overall change of 17% during the menstrual cycle (p = 0.004). No statistically significant cyclic changes were observed in the urinary biochemical markers of bone resorption. When testing the follicular phase against the luteal phase by Student's t-test, only sICTP gained significance (sICTP (follicular vs luteal) 3.7 ± 0.2 vs 4.3 ± 0.3, p = 0.0011), whereas the other parameters remained non-significant.

No significant cyclic variations were observed in serum total protein or in serum creatinine (data not shown), which indicates that the observed cyclic variations were not due to changes in vascular fluids during the menstrual cycle.

Discussion

We present here the first evidence of cyclic changes in bone resorption during the menstrual cycle. These were reflected in the serum concentration of ICTP but not revealed by the urinary markers of bone resorption, nor were there any corresponding changes in the biochemical markers of bone formation in the women studied.

The discrepancy between the observed cyclic changes in sICTP and the lack of any analogue tendency in the other marker of bone resorption is puzzling. The overall change in sICTP is relatively small, about 17% overall. Changes of this magnitude may be too small to be detected in the urinary excretion of pyridinium cross-links and hydroxyproline, where the day-to-day variations are large and thus may blur smaller cyclic variations in these parameters. Furthermore, sICTP may not be a specific marker of bone resorption, and may thus reflect other variables, such as soft-tissue components.
Although no change was observed in the biochemical markers of bone formation in the present study, other studies have suggested that oestrogens and gestagens may also affect bone formation. Our group has observed cyclic variations in sAP activity during a 28-day oestrogen/progestogen tablet-induced cycle in early postmenopausal women (5, 6). Another group has reported that the circulating osteocalcin concentration increases during the luteal phase of the menstrual cycle in normal premenopausal women (4). Oestrogen is considered a major regulator of bone metabolism, and generally believed to exert its effect mainly on the resorption side of the bone remodelling equation. However, the exact molecular and cellular mechanisms underlying this action are still not known. Specific receptors have been demonstrated in osteoblastic cells (22, 23), suggesting a direct action on these cells. The effect of progesterone on bone metabolism is still less clear. Moreover, observations from studies of the normal menstrual cycle and those of hormone replacement therapy after the menopause are not necessarily comparable, because the concentrations of oestrogen and progesterone in premenopausal women are much higher than the levels aimed at in the therapeutic use of these hormones in postmenopausal women. The higher oestrogen/progesterone concentration may thus blur cyclic changes in the biochemical markers of bone resorption.

In conclusion, a small cyclic variation was measured in serum ICTP but no cyclic variation could be demonstrated in either the two new urine resorption parameters, i.e. fU-Pyr/Cr and fU-D-Pyr/Cr, or in fU-HPr/Cr, sAP, pOC and sPICP in premenopausal women.

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