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Posture, Age, Menopause, and Osteopenia Do Not Influence the Circadian Variation in the Urinary Excretion of Pyridinium Crosslinks

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ABSTRACT

This study was performed to investigate whether the circadian variation in urinary pyridinium crosslinks is related to physical activity, age, the menopause, and asymptomatic osteopenia. We measured urinary pyridinoline/creatinine (Pyr/Cr) and deoxypyridinoline/creatinine (D-Pyr/Cr) in 9 healthy premenopausal women in two 27 h studies, before and at the end of 5 days of total bed rest. Both Pyr/Cr and D-Pyr/Cr showed highly significant circadian variations, with the peak at night and the nadir during the day ($p < 0.001$). The 5 days of complete bed rest produced no changes in the circadian pattern, but a general increase of 28% was observed in pyridinium crosslinks. A group of 12 healthy, early postmenopausal women (aged 55 ± 2 years), 12 healthy, elderly postmenopausal women (aged 73 ± 1 years), and 12 elderly osteopenic but otherwise healthy women (aged 73 ± 1 years) were also studied for 27 h. All three groups showed highly significant ($p \leq 0.001$) circadian variations in the urinary excretion of pyridinium crosslinks. As expected, both Pyr/Cr ($p < 0.05$) and D-Pyr/Cr ($p < 0.001$) increased at the time of menopause, but the circadian variations in Pyr/Cr and D-Pyr/Cr were similar in all groups studied. We conclude that the circadian variation in the urinary excretion of pyridinium crosslinks is independent of physical factors. Furthermore, the circadian variation in pyridinium crosslinks was not related to age, menopausal status, or asymptomatic osteopenia.

INTRODUCTION

BONE IS UNDER CONSTANT RENEWAL, with the resorption of old bone by osteoclasts and the formation of new bone by osteoblasts. These processes appear to have a circadian rhythm, with increased activity at night. We recently showed that the urinary excretion of pyridinium crosslinks, a new specific marker of bone resorption, exhibits a marked circadian variation in premenopausal women, with almost twofold increased values at night compared with afternoon values.[1]

Biochemical markers of bone formation, such as plasma osteocalcin and serum carboxyl-terminal propeptide of type I procollagen, may also exhibit a circadian variation, although to a lesser degree.[2–5] We were unable to document this for plasma osteocalcin,[1] but others have found a nocturnal increase in plasma osteocalcin of 5–25%.[2–4]

The etiology of these circadian variations is unknown. Several hormones with known effects on bone metabolism, such as parathyroid hormone, growth hormone, and cortisol, also exhibit a circadian variation in vivo.[6,7] These hormones could thus be implicated in the circadian variation in bone metabolism, but direct proof of a causal relationship is lacking. Immobility is known to result in bone loss.[8] The marked increase in the urinary excretion of pyridinium crosslinks at night[1] could thus also be caused by physical factors, such as activity during the day and bed rest at night.

Two recent studies by Eastell et al.[9,10] showed that the urinary excretion of deoxypyridinoline is increased at night in both premenopausal and postmenopausal women, and the authors suggested that it may persist longer into the morning in osteoporotic women. In both studies 8-h urine samples were used, and therefore detailed investigation of the circadian variation in the urinary excretion of deoxypyridinoline was not possible.

The present study was designed to answer three questions: (1) Is the circadian variation in pyridinoline/creatinine (Pyr/Cr) and

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deoxypyridinoline/creatinine (D-Pyr/Cr) simply caused by activity during the day and bed rest at night? (2) Do age and menopause affect the circadian variation in Pyr/Cr and D-Pyr/Cr? (3) Does circadian variation in Pyr/Cr and D-Pyr/Cr differ in osteopenic women compared to healthy, age-matched women?

MATERIALS AND METHODS

Participants

There were 45 women, divided into four groups. Group I comprised 9 healthy premenopausal women (age 25 ± 3 years; height 170 ± 6 cm; weight 65 ± 11 kg; mean ± standard deviation, SD). These women participated in two circadian (27 h) studies, one before and one at the end of 5 days of total bed rest (including washing, emptying bladder and bowels, and eating).

In group II were 12 healthy postmenopausal women who had recently passed the menopause (age 55 ± 2 years; menopausal age 5.6 ± 0.6 years; height 164 ± 7 cm; weight 67 ± 8 kg; mean ± SD).

In group III were 12 healthy elderly postmenopausal women (age 73 ± 1 years; menopausal age 25.5 ± 3.5 years; height 162 ± 4 cm; weight 70 ± 12 kg; mean ± SD). These women were randomly selected from an initial group of 512 women, recruited by a questionnaire sent to 2009 women (see Ref. 11). The women were selected for the present group according to forearm bone mineral content (BMC), less than 2 SD below the mean premenopausal level).

Group IV included 12 healthy elderly postmenopausal women (age 73 ± 1 years; menopausal age 21.3 ± 2.1 years; height 157 ± 6 cm; weight 63 ± 10 kg; mean ± SD), with osteopenia defined by forearm BMC more than 2 SD below the mean premenopausal level. These women were randomly selected from the same initial group of 512 women as group III. Of the 512 women described in groups III and IV, 322 women (e.g., 63%) had a forearm BMC lower than 2 SD below premenopausal level; 2 women in group III and 2 women in group IV were found to have wedge fractures by x-ray of the spine. None were found to have crush fractures. Group III and IV were selected to be age matched.

All women started the study at 1400 and ended the next day at 1700. Urine was collected in 3 h aliquots, that is, a total of nine urine samples per participant. Meals (not controlled for gelatin content) were served at 0830, 1130–1230, and 1830–1930. Normal daytime activities and sleeping pattern were permitted, except in group I (see earlier) during the bed rest period.

None had any history of endocrine, renal, or metabolic diseases or were taking any medication or oral contraceptives at the time of the study.

The research protocol was approved by the Ethical Committee of Copenhagen County. In accordance with the Helsinki II Declaration, all participants gave written consent after receiving complete information.

Methods

The urine sample was hydrolyzed in 6 M HCl at 108°C for 24 h before extraction by cellulose CF1 participation column chromatography. The extraction product was then freeze dried before separation on a reversed-phase C18 column by high-performance liquid chromatography and identified by spectrofluorimetry with a modification of the method described by Eyre[12] and Black et al.[13] The Pyr and D-Pyr results were obtained by comparison with an external standard. The intraassay variations were 4.1% for Pyr and 6.0% for D-Pyr; the interassay variations were 9.1% for Pyr and 11.5% for D-Pyr. The recoveries from hydrolyzation to identification were 82 ± 8 and 85 ± 14%, respectively. The Pyr and D-Pyr values were corrected for creatinine excretion and given as pmol per μmol creatinine (for details, see Ref. 14).

Bone mineral content of the forearm was measured by single-photon absorptiometry[15,16] with a ^{125}I source (3.7 GBq) and photopeak at 27 keV.

Bone mineral density (BMD) of the lumbar spine was measured by dual-energy x-ray absorptiometry (Hologic, Inc., Waltham, MA; Model QDR-1000 bone densitometer and software Version 3.10).[17]

Statistical assessment

The procedures of the Statistical Analyses System were applied in the statistical analyses.[18] To obtain normality and homogeneity of variance, Pyr/Cr and D-Pyr/Cr were logarithmically transformed (log concentration) before analysis. The serial measurements were analyzed according to concepts described by Matthews et al.[19] The 24 h cyclic changes in bone turnover parameters were calculated for each woman by multiple-regression analysis, with sine and cosine as independent variables (e.g., $y = \beta_1 \sin(t) + \beta_2 \cos(t)$, where $t = 2\pi$time (h)/24). The coefficients (β_1 and β_2) to sine and cosine were then used as summary measures of the circadian variation and compared to zero by multiple analysis of variance.[18] Amplitudes were calculated as $\sqrt{\beta_1^2 + \beta_2^2}$.

Differences in circadian rhythms between groups were tested according to two-sample comparison of population rhythms, as described by Nelson et al.[20] No significant difference in amplitudes and/or acrophases were found between any groups, indicating similar circadian rhythms.

In the figures for comparison of the circadian pattern, each value was expressed as the difference between the individual value and the mean 24 h value for that individual ($X'_{i,t} = X_{i,t} - \bar{X}_i$, where i is the individual and t the time point).

In Table 1, Student's unpaired t-test was used for comparison between groups and Student's paired t-test for comparison within groups.

Correlations between pyridinium crosslinks amplitudes, 24 h pyridinium crosslink values, and bone mass were made by Pearson's correlation.[18]

RESULTS

Table 1 lists the mean 24 h values of Pyr/Cr and D-Pyr/Cr according to the four groups. As expected, both Pyr/Cr ($p < 0.05$) and D-Pyr/Cr ($p < 0.001$) increased highly significantly at the time of menopause (23–43%). After the menopause, both markers remained fairly constant, although a tendency toward a small decrease late in the menopause was seen.

TABLE 1. MEAN 24 H VALUES OF PYR/CR AND D-PYR/CR ACCORDING TO AGE, MENOPAUSAL STATUS, AND OSTEOPENIA[a]

	n	*Pyr/Cr (pmol/μmol)*	*D-Pyr/Cr (pmol/μmol)*
Premenopausal women	9	39 ± 3	5.4 ± 0.5
Early postmenopausal women	12	48 ± 10	7.7 ± 1.7
Late postmenopausal women	12	46 ± 4	6.4 ± 0.9
Osteopenic women	12	48 ± 9	6.9 ± 1.4

[a] Mean ± SD. Premenopausal versus early postmenopausal women: Pyr/Cr, $p < 0.05$; D-Pyr/Cr, $p < 0.001$.
Early postmenopausal versus late postmenopausal women: Pyr/Cr, NS; D-Pyr/Cr, $p < 0.05$.
Late postmenopausal versus osteopenic women: both Pyr/Cr and D-Pyr/Cr, NS.

Figures 1 through 3 show the highly significant ($p < 0.001$) circadian variations in Pyr/Cr and D-Pyr/Cr. No significant difference in amplitudes and/or acrophases were found. Figure 1 compares the urinary excretion of Pyr/Cr and D-Pyr/Cr throughout the 24 h period, before and at the end of 5 days of total bed rest. An overall increase of 28% (both Pyr/Cr and D-Pyr/Cr; $p < 0.01$) was observed in the mean 24 h values, but the pattern of the circadian rhythm was not affected significantly by the bed rest. Similar circadian patterns were observed in both Pyr and D-Pyr when expressed as absolute amount of crosslinks/3 h ($p < 0.05$–0.001; data not shown). The net 24 h urinary creatinine decreased 6% by bed rest (15.0 ± 2.6 versus 14.1 ± 2.1

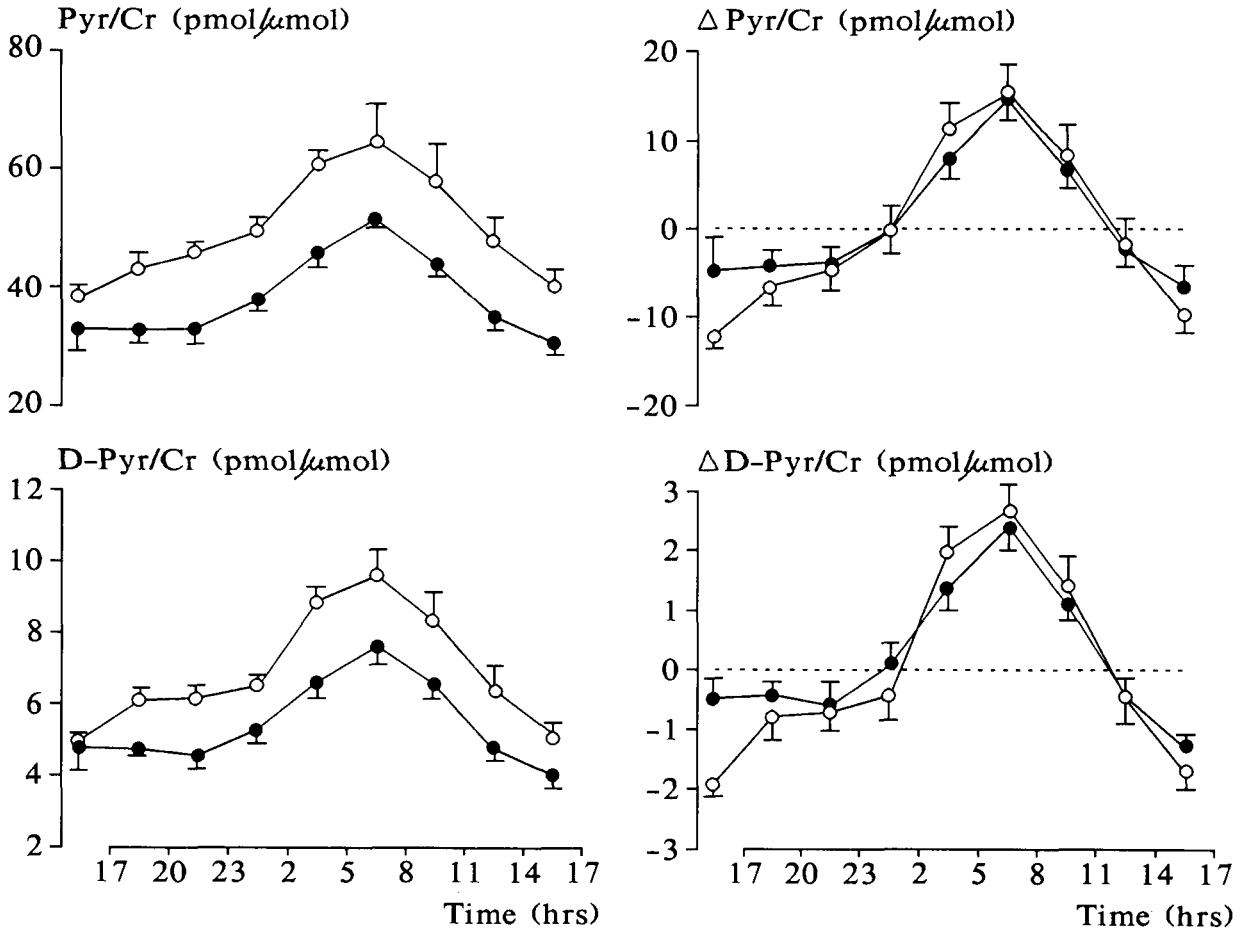

FIG. 1. (Left) The 24 h pattern in Pyr/Cr and D-Pyr/Cr according to normal daily activity (closed circles) and after 5 days of bed rest (open circles). (Right) The 24 h pattern in Pyr/Cr and D-Pyr/Cr expressed as the differences between the individual time values and the mean 24 h value for that individual, according to vertical (closed symbols) and 5 days supine position (open circles). Data given as mean ± standard error of the mean (SEM).

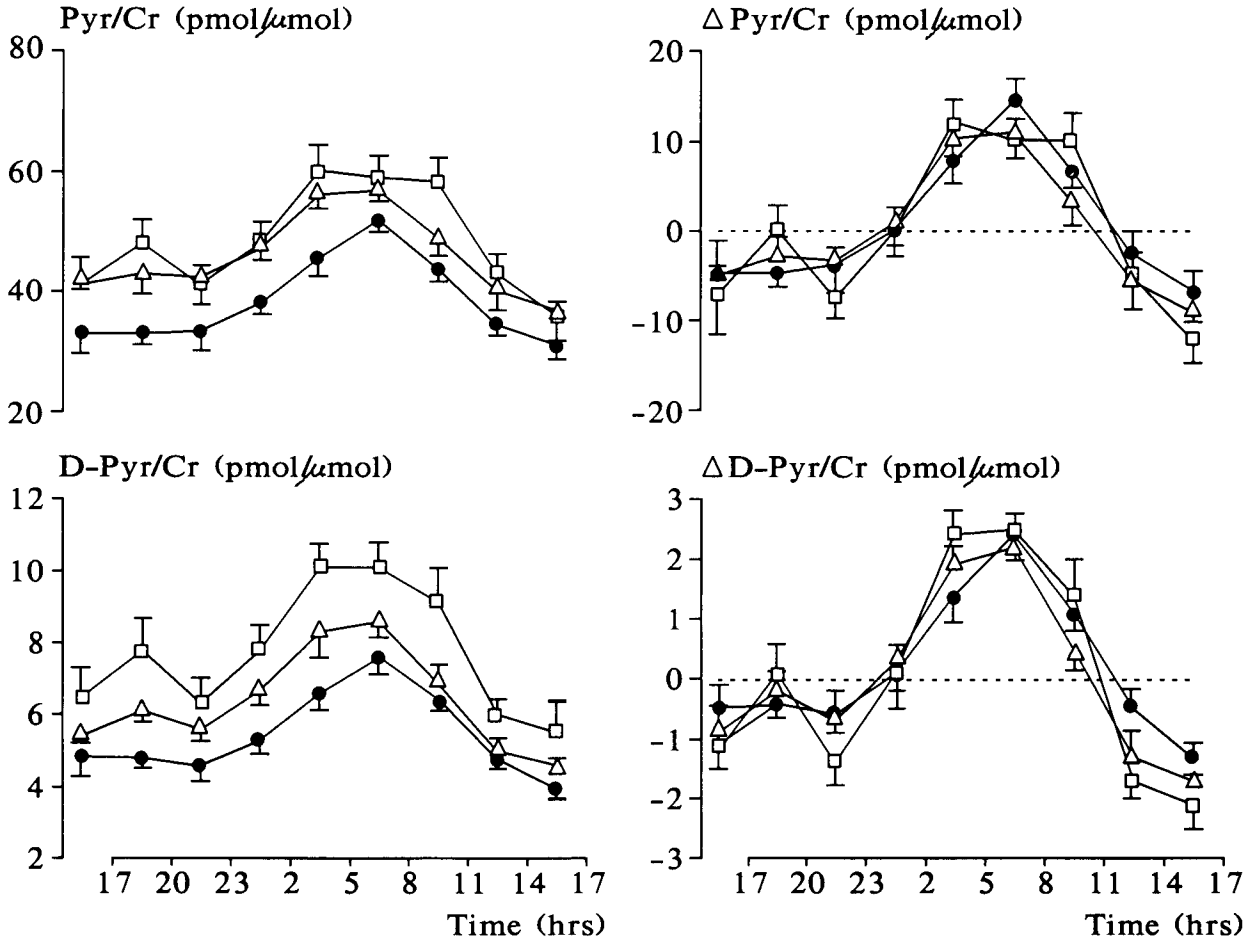

FIG. 2. (Left) The 24 h pattern and (right) the pattern when expressed as the difference between the individual time values and the mean 24 h value for that individual in Pyr/Cr and D-Pyr/Cr according to premenopausal (circles), early postmenopausal (squares), and late postmenopausal status (triangles). Data given as mean ± 1 SEM.

mmol, $p < 0.05$, before versus after bed rest), whereas the net 24 h urinary pyridinium crosslinks increased 18–20% by bed rest (Pyr, 568 ± 74 versus 684 ± 150 nmol, $p < 0.01$; D-Pyr, 80 ± 10 versus 95 ± 19 nmol, $p < 0.05$, before versus at the end of 5 days bed rest). The amplitudes calculated from the model given in the statistics were before bed rest (Pyr/Cr, 10 ± 3 pmol/μmol; D-Pyr/Cr, 1.5 ± 0.4 pmol/μmol) and at the end of 5 days of bed rest (Pyr/Cr, 14 ± 6 pmol/μmol; D-Pyr/Cr, 2.2 ± 0.9 pmol/μmol).

Figure 2 illustrates the circadian changes in the 24 h urinary excretion of Pyr/Cr and D-Pyr/Cr according to menopausal status and age. Similar circadian variations were observed in all three groups. The calculated amplitudes for the groups were premenopausal women (group I; Pyr/Cr, 10 ± 3 pmol/μmol; D-Pyr/Cr, 1.5 ± 0.4 pmol/μmol), early postmenopausal women (group II; Pyr/Cr, 12 ± 4 pmol/μmol; D-Pyr/Cr, 2.1 ± 0.8 pmol/μmol); and late postmenopausal women (group III; Pyr/Cr, 10 ± 4 pmol/μmol; D-Pyr/Cr, 1.7 ± 0.7 pmol/μmol).

Figure 3 compares the circadian variations in osteopenic and age-matched nonosteopenic women. No difference was observed between the two groups in the circadian pattern. The calculated amplitudes were, for the osteopenic women (group IV), (Pyr/Cr, 12 ± 4 pmol/μmol and D-Pyr/Cr, 2.1 ± 0.9 pmol/μmol and for the age-matched, nonosteopenic women (group III), Pyr/Cr, 10 ± 4 pmol/μmol and D-Pyr/Cr, 1.7 ± 0.7 pmol/μmol. There was no correlation between pyridinium crosslinks amplitude and forearm BMC or lumbar spine BMD in the combined group III and IV ($r = -0.34$ to -0.26; $n = 24$; $p > 0.1$).

DISCUSSION

Circadian variations in urinary pyridinium crosslinks in premenopausal women with peak values during the night and nadir during the day were reported previously.[1,9–10] The question was whether these observations were related to day- or nighttime activity or mediated merely by other biologic events.

The circadian pattern of both Pyr/Cr and D-Pyr/Cr at the end of 5 days of total bed rest was unchanged, which shows that the circadian pattern observed in bone resorption is not mediated by the change in gravity pattern (sleeping and walking, for example). It is possible, though, that the biochemical markers of bone

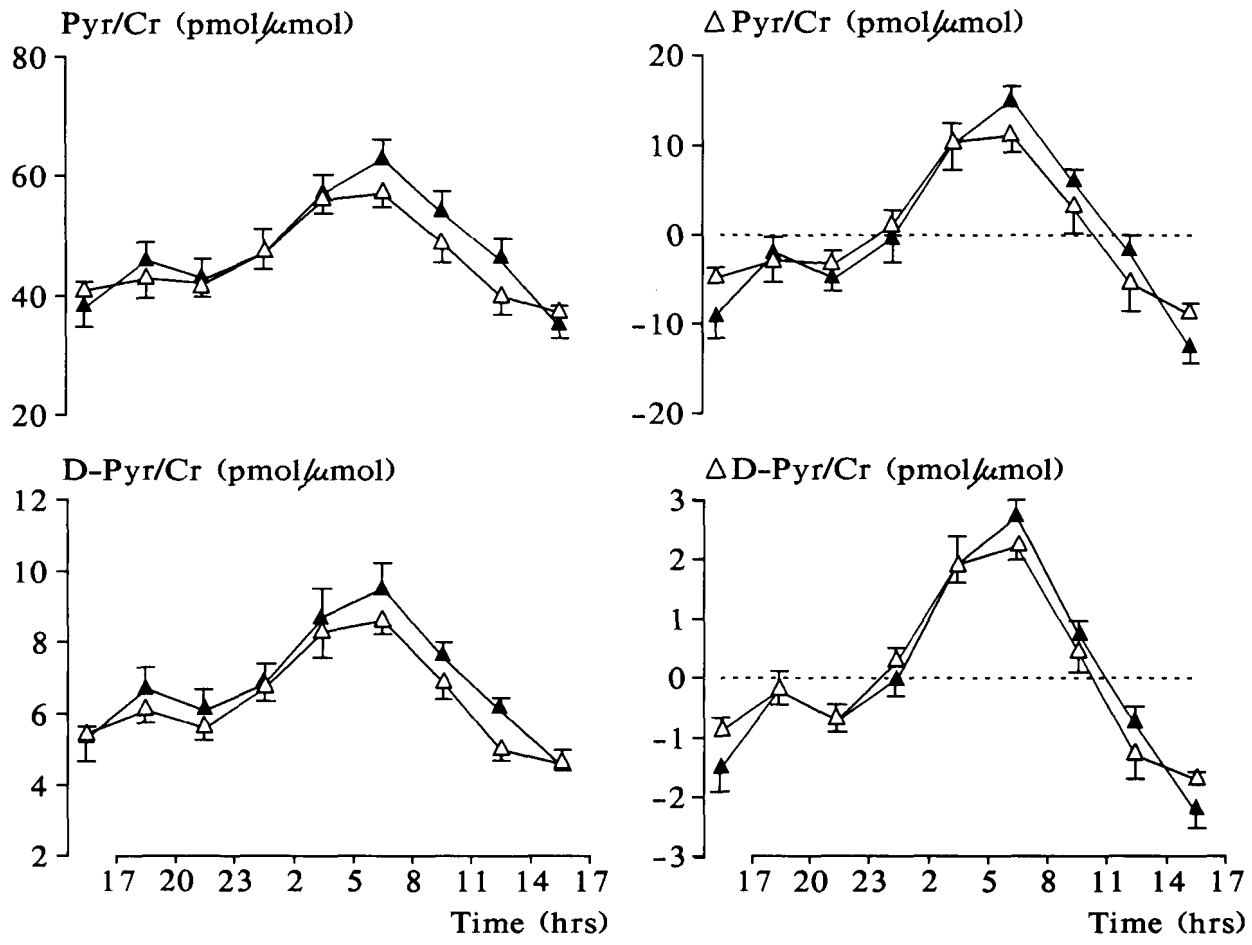

FIG. 3. (Left) The 24 h pattern and (right) the pattern when expressed as the difference between the individual time values and the mean 24 h value for that individual in Pyr/Cr and D-Pyr/Cr in osteopenic (closed triangles) and nonosteopenic (open triangles) women. Data given as mean ± 1 SEM.

resorption have a very low threshold for physical activity and that the minor movements the participants were permitted had an impact on the result, although we tried to minimize movement in bed by administering bedpans and washing utensils, for example. Furthermore, the average 24 h excretion of pyridinium crosslinks increased significantly at the end of the 5 days in bed, indicating that the bed rest had an impact on the bones and that a low threshold is not a likely explanation.

To assess further the physiology of the circadian variations in urinary pyridinium crosslinks, we investigated 33 nonosteoporotic women divided according to age and menopausal status. No difference was found between the groups in the circadian pattern of either parameter, although the levels of both Pyr/Cr and D-Pyr/Cr generally increased at the time of the menopause. The lack of effect of age or menopausal status on the circadian variation has also been reported by Eastell et al.[9] These observations suggest that sex hormones do not influence the circadian variations in biochemical markers of bone resorption.

The etiology of the circadian variations remains unknown. Several hormones known to influence bone turnover exhibit a circadian rhythm. Parathyroid hormone shows a circadian rhythm with the peak at night,[6] and it could therefore theoretically be a candidate for the etiology of the circadian variation in bone resorption. Glucocorticoid also exhibits circadian variations with the peak during night, and Nielsen et al. demonstrated that 10 mg prednisolone given in the evening is able to alter the circadian variation seen in osteocalcin.[21] Definite proof that glucocorticoid is the main mediator of the circadian variation in bone formation is still needed, and no data concerning the effect of glucocorticoid on circadian variation in pyridinium crosslinks have been published.

Eastell et al. previously reported that the night peak in D-Pyr/Cr tended to be prolonged further into the morning hours in osteoporotic women compared with nonosteoporotic women.[10] We were not able to confirm this. Our study showed identical circadian patterns in osteopenic and nonosteopenic women. This discrepancy may be a result of differences in study design. Eastell et al. defined osteoporosis on vertebral fractures, whereas in our study it was defined on low forearm BMC. None of our subjects had crush fractures.

We conclude that the urinary excretion of pyridinium crosslinks increases after bed rest and after the menopause. Neither bed rest nor the menopause affects the circadian rhythm.

Finally, asymptomatic osteopenic women have a normal circadian rhythm in urinary pyridinium crosslinks.

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