

# Circadian variations in serum eosinophil cationic protein, and serum and urine eosinophil protein X

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Biochemical evaluation of inflammation may be a useful adjunct to measures of pulmonary function and symptoms in children with asthma. However, little data have been provided to validate the markers in children. The aim of the present study was to assess circadian variations in serum eosinophil cationic protein (ECP), and serum and urine eosinophil protein X (EPX) in children. Five girls and two boys aged 10–14 years were studied. The first sample of urine consisted of urine collected from 24.00 hours the night before until 08.00 hours on the morning of the day of investigation. Thereafter urine was collected at 4-h intervals until 24.00 hours and in another 8-h interval from 24.00 to 08.00 hours. Blood samples for assessment of serum ECP and serum EPX were collected every 2 h during the 24 h. Statistically significant circadian variations in serum ECP ( $F = 3.2$ ,  $p = 0.002$ ), serum EPX ( $F = 3.1$ ,  $p = 0.002$ ) and in urine EPX/creatinine ( $F = 5.4$ ,  $p = 0.003$ ) were detected. The concentrations were higher during the night compared to daytime. Peak levels of serum ECP (mean  $\pm$  SEM) were found at 06.00 hours (16.3 [5.3]  $\mu\text{g/l}$ ), trough levels at 08.00 hours (3.9 [0.7]  $\mu\text{g/l}$ ) ( $p = 0.01$ ). Peak levels of serum EPX were seen at 06.00 (43.7 [9.5]  $\mu\text{g/l}$ ) with trough levels at 12.00 hours (22.0 [3.5]  $\mu\text{g/l}$ ) ( $p = 0.01$ ). Peak levels of urine EPX/creatinine occurred in urine collected from 24.00 to 08.00 hours (90.0 [27.7]  $\mu\text{g/mmol}$ ), trough levels in the 16.00–20.00 hours sample (29.7 [8.9]  $\mu\text{g/mmol}$ ) ( $p = 0.02$ ). Serum ECP, serum EPX and urine EPX exhibit a circadian variation in children with nocturnal and early morning peak levels. To avoid confounding influence from circadian variations in ECP and EPX in clinical studies blood or urine should be sampled at consistent times.

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Eosinophil proteins such as eosinophil cationic protein (ECP) in serum and eosinophil protein X (EPX) in serum and urine are measures of eosinophil activation anywhere in the body. In children with asthma evaluation of airway inflammation by assessment of the eosinophil markers may be a useful adjunct to measures of pulmonary function and symptoms (1). Serum ECP, serum EPX and urine EPX may reflect asthma severity (2, 3) and may be used for monitoring of anti-inflammatory effects of systemic and topical glucocorticoids (4–6). However, little data have been provided to validate the markers in children. Because eosinophils

exhibit increased serum levels during the night an important aspect to consider is the possibility of circadian variations (7). In adults nocturnal increases in serum ECP have been reported (8). A study of urine EPX in children suggested that urine EPX levels may be higher in the morning than in the evening (9). The aim of the present study was to assess circadian variations in serum ECP, serum EPX and urine EPX in children.

## **Subjects and methods**

Two boys and five girls aged 10.4–14.4 years (mean: 12.2 years) were studied. The subjects

were screened by medical examination and proved healthy without any history of chronic disease. Height at study entry varied from 152 to 173 cm (mean: 157 cm), height standard deviation score from -2.5 to 2.2 (mean: 0.6), weight from 30.5 to 53.0 kg (mean: 44.5 kg) and weight standard deviation score from -1.2 to 1.5 (mean: -0.4). The study was approved by the local ethics committee, and informed consent was obtained from all children and their parents. After an overnight fast, the children reported at 07.30 hours. The first sample of urine consisted of urine collected from 24.00 hours the night before until 08.00 hours on the morning of the day of investigation. Thereafter urine was collected in 4-h intervals until 24.00 hours and in another 8-h interval from 24.00 to 08.00 hours. Through an indwelling venous catheter placed in an antecubital vein blood samples were taken at 08.00 hours and thereafter every 2 h during the 24 h. After 1 h  $\pm$  10 min blood samples were centrifuged at 2750 *g* for 10 min. After centrifugation the samples were stored at -80°C and batch assayed at the completion of the study. Each subject received breakfast at 8.15 hours, lunch at 13.00 hours, an ice-cream at 16.30 hours and dinner at 19.00 hours. All children were studied during the month of July.

The serum concentrations of ECP and EPX, and EPX in urine were determined using specific radio immunoassays as previously described (Pharmacia & Upjohn, Sweden) (4, 10). Intra- and interassay variations were 6 and 7%, 6 and 9%, and 5 and 9%, respectively. Creatinine in urine was determined by standard laboratory methods.

### Statistics

Data are described as a percentage of the overall daymean  $\pm$  1 standard error of the mean in the 24-h profiles. To evaluate the 24-h profiles one-way analysis of variance (ANOVA) for repeated measurements was performed followed by Student-Newman-Keuls method for all pairwise multiple comparisons. The 5% level of significance was used.

### Results

Hemolysis occurred in 02.00 and 06.00 hour blood samples in one subject, in 10.00 and 08.00 hour samples in one, in 24.00 and 02.00 hours samples in one, and in the 04.00 hour sample in one subject. These samples were withdrawn from analysis.

Figure 1 shows the 24-h profiles of serum ECP and serum EPX, and of urine EPX standardized

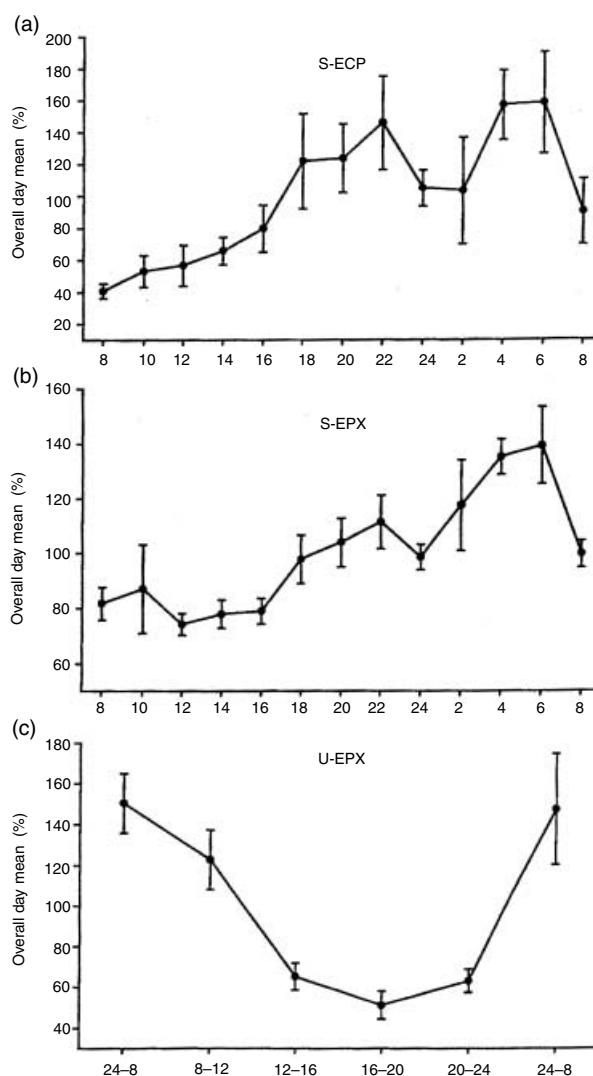


Fig. 1. Diurnal concentrations of serum ECP (S-ECP) (a) and EPX (S-EPX) (b) and of urine EPX/creatinine (U-EPX) (c) in seven healthy children, expressed as a percentage of overall day mean  $\pm$  SEM.

for creatinine expressed as a percentage of the overall day mean. A statistically significant circadian variation in all parameters was detected (serum ECP:  $F = 3.2$ ,  $p = 0.002$ ; serum EPX:  $F = 3.1$ ,  $p = 0.002$ ; urine EPX/creatinine:  $F = 5.4$ ,  $p = 0.003$ ). Generally, the concentrations were relatively low during the day, whereas increased levels occurred during the night and early morning.

Peak levels of serum ECP (mean  $\pm$  SEM) occurred at 06.00 hours (16.3 [3.5]  $\mu\text{g/l}$ ), trough levels at 08.00 hours (3.9 [0.7]  $\mu\text{g/l}$ ) ( $p = 0.01$ ) in the first morning of the study. Mean peak levels at 06.00 hours also differed from the 10.00–16.00 hour levels ( $p = 0.03$  for all comparisons). Furthermore, mean ECP at 04.00 hours (13.9

[2.0]  $\mu\text{g/l}$ ) differed from 08.00–16.00 hour levels ( $p = 0.03$  for all comparisons).

Peak levels of serum EPX were seen at 06.00 hours (43.7 [5.9]  $\mu\text{g/l}$ ), trough levels at 12.00 hours (22.0 [3.5]  $\mu\text{g/l}$ ) ( $p = 0.01$ ). The mean peak level at 06.00 hours also differed from the 14.00 ( $p = 0.01$ ) and 16.00 ( $p = 0.01$ ) hour levels. Mean EPX at 04.00 hours (40.0 [5.9]  $\mu\text{g/l}$ ) differed from the 12.00–16.00 hour levels ( $p = 0.03$  for all comparisons).

Peak levels of urine EPX/creatinine occurred in urine collected from 24.00 to 08.00 hours during the first night of the study (90.0 [27.7]  $\mu\text{g/mmol}$ ), trough levels in the 16.00–20.00 hour sample (29.7 [8.9]  $\mu\text{g/mmol}$ ) ( $p = 0.02$ ). EPX/creatinine in the two 24.00–08.00 hour samples did not vary ( $p = 0.90$ ), whereas both samples varied statistically significantly from the 12.00–16.00, 16.00–20.00 and 20.00–24.00 hour samples ( $p = 0.03$  for all comparisons). Otherwise, there were no statistically significant variations between any of the repeated measurements of serum ECP, serum EPX or urine EPX.

## Discussion

The present study is the first to assess circadian variations in serum ECP, serum EPX and urine EPX in children using frequent sampling of serum and fractioned urine. The rhythmicity characterized by higher levels during the night compared to daytime may depend on nocturnal increases in circulating eosinophils that relate to endogenous secretory patterns in cortisol and variations in adrenergic activity that again may depend on sleep patterns (11). To some extent this may explain why no variations were found in a study of children with allergic rhinitis and healthy young adults in whom serum ECP was assessed at 06.00 hours and 15.00 hours (12). The subjects were woken up very early to participate in stressful nasal provocation tests and normal sleep patterns may have been disturbed. Furthermore, assessment of serum ECP may be confounded by ECP released from eosinophils during hemolysis, which may cause falsely elevated levels (13). Studies in normal adults and adults with asthma have found nocturnal elevations in serum ECP, whereas serum EPX has not been assessed (8,14). In accordance with the present observations peak serum ECP levels were detected at 04.00 hours when blood was collected every 4 h during the 24 h (8).

ECP and EPX are closely related single chain proteins with similar molecular masses, and EPX especially is a very stable structure that is

excreted and can be reliably measured in urine (15). In children with asthma and in normal children spot urine samples have been taken between 07.00 and 09.00 hours and between 14.00 and 18.00 hours, respectively, for assessment of EPX. Urine EPX/creatinine was statistically significantly higher in the morning than in the evening (9). The observation was taken to suggest a circadian variation in EPX. In the present study we wanted to assess urine EPX profiles throughout a 24-h period by means of a more sensitive, yet practical design. Therefore, we applied 4-h fractioned urine sampling periods. Because we wanted to minimize the risk of interfering with sleep patterns, which may possibly confound the results, to avoid disturbing the children the sampling period was extended to 8 h during the night. The findings establish a circadian variation in urine EPX in children characterized by uniformly low levels from 12.00 to 24.00 hours and increased levels from 24.00 to 12.00 hours.

Serum ECP and EPX as well as urine EPX have been used in assessments of the anti-inflammatory efficacy of exogenous glucocorticoids in children (4–6, 13, 16–18). The data, however, should be interpreted with caution. Several of the studies gave no information about the time of serum or urine sampling (4–6, 16). Others used blood or spot urine sampling in the morning which, as evidenced by the present findings, may be associated with confounding due to circadian variations (13, 18). To some extent the discrepant results that have been reported may have been caused by differences in the timing of serum or urine sampling. To minimize influences from circadian variations in serum ECP, serum EPX, and in urine EPX it should be recommended to sample at consistent times in cross-sectional as well as in longitudinal comparisons. Being a non-invasive measure urine EPX may be the more attractive inflammation marker to apply in children.

As is the case with other dynamic markers that may be under endogenous hormonal control it may be expected that inflammation markers not only exhibit circadian variations but also may be influenced by spontaneous day-to-day and seasonal variations (19, 20). Day-to-day variations may explain why serum ECP and serum EPX on the two mornings at 08.00 hours showed a conspicuous although not a statistically significant variation. Such variations may confound both short and long-term studies. Finally, the eosinophil proteins are indirect measures of eosinophil activation in the body. To what extent they reflect airway inflammation in children with

asthma or other atopic diseases still remains to be established. Thus, further basic validation of serum and urine inflammation markers are needed.

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