Epidermal growth factor in mice: changes during circadian and female reproductive cycles

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Abstract. To clarify the variation of mouse epidermal growth factor production during the 24-h and female reproductive cycles, we measured its concentrations in the plasma, submandibular salivary gland, urine, kidneys and liver of adult male and female mice during consecutive 12-h dark and 12-h light periods, different stages of pregnancy, and lactation. The concentration of epidermal growth factor in the submandibular gland showed no circadian changes. In plasma and in the liver it increased during the dark period, whereas in urine and in the kidneys it peaked in the daytime. In the submandibular gland the concentration decreased during early pregnancy, but it returned to the non-pregnant levels by mid-pregnancy. In plasma it decreased progressively during pregnancy and recovered during lactation. In urine and the kidneys the concentration of epidermal growth factor increased after early pregnancy; with a further great increase in the kidneys during lactation. In the liver the concentration was clearly below the non-pregnant levels during late pregnancy and lactation.

The concentrations of epidermal growth factor (EGF) in fluids and tissues of mice are influenced by sex steroids. Compared with the females, the adult males have higher EGF levels in the submandibular salivary gland (1-5) and plasma (3,6), and lower levels in the kidneys (2,4) and urine (2). Testosterone increases EGF in the submandibular gland and plasma (1,3,6-9), and estradiol increases it in the kidneys and urine (4,10). In the submandibular gland estradiol decreases EGF (10,11).

Kurachi & Oka (12) reported that the EGF concentrations in the submandibular gland and plasma increase during mouse pregnancy. The increase in plasma EGF was demonstrable only between 24.00 and 8.00 h, at the peak time of the female EGF concentration, which showed circadian variation. In contrast, Krieger et al. (13) observed circadian variation only for male and female submandibular gland EGF concentration, not for plasma EGF.

Knowledge of circadian variation of the EGF concentration in mouse tissues and fluids is important for the study of EGF physiology. Because circadian variation has been looked for only in the submandibular gland and plasma (12,13) and because the results are conflicting, we have examined a larger variety of tissues and fluids: submandibular gland, plasma, kidney, urine, and liver of adult male and female mice. We then followed these concentrations during different stages of pregnancy and during lactation.

Materials and Methods

Animals and samples

NMRI mice were bred by Bommice (Ry, Denmark). Adult male and female mice were maintained for several weeks in our mouse quarters under a cycle of 12 h of light (8.00-20.00) and 12 h of darkness (20.00-8.00). They had free access to food and water. Groups of 10 mice were killed at 8.00, 14.00, 20.00 and 2.00 h. Pregnant mice were killed between 24.00 and 2.00 h, during early pregnancy (7 days, N=6), midpregnancy (13 days, N=9), late pregnancy (20 days, N=10), and lactation (15 days post partum, N=10). They were killed by exposure to CO₂, which resulted in death within 30 s.

Urine samples were obtained by spontaneous voiding just before death. Immediately after death the abdomens
was opened and blood was collected from the inferior vena cava with a heparinized needle and syringe (3), cooled in an ice-water bath and centrifuged at 10,000 g for 2 min to separate plasma. The submandibular gland, the kidneys and the liver were dissected free of connective tissue. All the samples were frozen immediately and stored at −20°C.

The tissue weights were lower in the females than in the males: 64.6±0.9 vs 108±1.8 mg (p<0.001) for the submandibular glands, 228±4.5 vs 364±7.0 mg (p<0.001) for the kidneys, and 1503±51.2 vs 2021±72.7 mg (p<0.001) for the livers. In the lactating mice the kidney weights were higher than in the corresponding non-pregnant mice: 303±14.2 vs 220±4.6 mg (p<0.001). The liver weights also increased during pregnancy: 1637±98.7 mg in the non-pregnant mice at 2.00 h vs 2150±151 mg (p<0.02) during midpregnancy, 2545±142 mg (p<0.001) during late pregnancy, and 2519±242 mg (p<0.005) during lactation.

**Measurements**
The tissues (about 200 mg) were homogenized in 1.0 mol/l acetic acid (4). The EGF concentration in all the samples was measured by radioimmunoassay (4). Urea was measured by the urease/GLDH reaction (CobasBio analyzer, F. Hoffman-La Roche Ltd, Basle, Switzerland). During late pregnancy and lactation urea levels were lower than in the non-pregnant mice: 101±7.4 vs 61.7±5.1 g/l (p<0.001) and vs 47.1±6.0 g/l (p<0.001).

**Statistics**
Because of skewness EGF data were log-transformed, and geometric mean and (mean ± SEM) interval values were calculated. Analysis of variance followed by Student's t-test was used for the evaluation of differences; p<0.05 was considered significant. Since six of ten late pregnancy plasma EGF concentrations were below the detection limit of our RIA (12 pmol/l), they were assigned the value of 10 pmol/l for calculations, and a non-parametric Mann-Whitney U-test was used for the evaluation of all the plasma EGF data.

**Results**

**Plasma**
Plasma levels of EGF were consistently significantly higher in the males than in the females (Fig. 1A).

Both sexes displayed a significant circadian variation which amounted to a mean difference between the highest and lowest values of 33% for the males and 20% for the females. The concentration was highest during the period of darkness (in the females at 2.00 h and in the males at 8.00 h) and lowest at 14.00 h.

During pregnancy plasma EGF decreased, reaching a minimum (42% mean decrease) at the late stage. The values returned to the non-pregnant levels during lactation.
Submandibular gland
The levels of EGF were higher in the males than in the females at all times (Fig. 1B). No significant circadian periodicity was observed in either sex.
There was a decrease during early pregnancy, with mean concentration being 44% lower than in the non-pregnant mice. Thereafter the levels returned to the non-pregnant values.

Urine
The mean urinary EGF concentration was higher (18%, p=0.02) in the males than in the females at 20.00 h (Fig. 2A). Otherwise the mean levels showed no significant sex difference.
Both sexes displayed a significant circadian variation which amounted to a mean difference between the highest and lowest values of 19% for the males and 28% for the females. In the females the mean concentration was highest at 14.00 h and lowest at 2.00 h. In the males it was highest at 20.00 h and lowest at 2.00 h.
Clear alterations appeared during pregnancy. During the middle and late stages the EGF levels were higher (1.5 and 1.6 times) than during the early stage. During lactation they returned to the non-pregnant values.

Kidneys
In the kidney EGF concentration, a sex difference appeared only at 14.00 h, with female levels above male levels (p=0.0003) (Fig. 2B).
Both sexes displayed some significant circadian changes, which amounted to a mean difference between the highest and lowest values of 29% for the males and 19% for the females. In the females the concentration was highest at 14.00 h and lowest at 8.00 h. In the males the highest level appeared at 20.00 h and the lowest at 14.00 h.
The levels increased slightly with advancing pregnancy. During lactation an additional great increase occurred (to 3.0-fold the non-pregnant levels).

Liver
Liver levels of EGF were higher in the males than in the females at all times except 8.00 h (Fig. 3).
Again, circadian changes were evident in both sexes, amounting to a mean difference between the highest and lowest values of 64% for the males and 77% for the females. In the females the peak levels occurred at 8.00 h and the trough levels at 20.00 h.

In the males the levels were highest at 14.00 h, but almost as high at 8.00, and lowest at 2.00 h.
During pregnancy the levels decreased (by 42% by late pregnancy) and remained low during lactation.
Discussion

We observed significant circadian periodicity of the EGF concentration in the plasma, urine, kidney and liver of male and female mice. In mice EGF is a circulating compound (3) although blood EGF in humans is associated in platelets (14,15). Our present results may explain some previous conflicting findings on sex difference. In the kidneys a female over male difference has been repeatedly observed (2,4); when examined more closely, it appeared only in the afternoon. In urine, in contrast to reports of a small female over male difference (2) or no difference (4,5), we now observed a small male over female difference after 12 h of light. That male mice have higher EGF levels in the submandibular gland and plasma has been firmly established (1-6). Our finding of a significant male over female difference in the liver is in harmony with the difference in plasma and observations suggesting that the liver extracts EGF from plasma (20,21).

There are two earlier reports of circadian periodicity of the EGF concentration in the mouse, in the submandibular gland and plasma (12,13). We now also studied daily variation of the EGF concentration in the kidneys, urine and the liver. We found no circadian changes in the submandibular gland. This contrasts with the observation of Krieger et al. (13) who reported that the submandibular gland EGF was highest at 20.00 h (12 hours of light) in male mice and at 12.00 h (4 hours of light) in female mice. In plasma and in the liver the EGF concentration increased during darkness in both sexes. Our plasma finding is in concert with the results of Kurachi & Oka (12), but again in conflict with those of Krieger et al. (13).

In intact mice α-adrenergic stimulation releases EGF from the submandibular gland into the blood (1,16). In sialoadenectomized mice plasma levels of EGF also increase after phenylephrine (α-adrenergic agent) indicating release from tissues other than the submandibular gland (16). During the dark phase, the release of norepinephrine from sympathetic nerve terminals increases (17). Therefore one could expect increased release of submandibular gland EGF during darkness. Indeed, plasma levels of EGF increased during the dark hours, although the submandibular gland EGF levels did not change significantly. High testosterone levels at night (18,19) may stimulate the synthesis of EGF, compensating for the increased release caused by the enhanced α-adrenergic activity.

The increase in kidney EGF during the light period appears to be due to stimulated synthesis rather than inhibited release, because urinary EGF also increased during the same period. The increased levels of liver EGF during darkness might be due to extraction from plasma (20,21), although we cannot exclude the possibility of accelerated synthesis of EGF by the liver. Plasma levels of corticosterone and progesterone increase during the light period and decrease during the dark period (19,22-24). Furthermore, serum estradiol levels tend to be higher in the morning than in the evening (25). These steroids may contribute to the circadian periodicity observed in the fluids and tissues of adult mice.

During pregnancy, plasma levels of EGF decreased, which agrees with some (26) but disagrees with other data (12). Our finding of a temporary decrease in the submandibular gland EGF concentration during early pregnancy disagrees with the results of Kurachi & Oka (12), but agrees with those of Perheentupa et al. (26). Urinary EGF increased during middle and late pregnancy and returned to the non-pregnant levels during lactation. A new
observation is the slight increase in the kidney EGF during pregnancy followed by the great increase during lactation. In the liver, the EGF concentration had decreased by late pregnancy and remained low during lactation.

During pregnancy, the levels of various sex steroids increase. Testosterone increases submandibular gland and plasma EGF (1,3,6-9). In contrast, estradiol increases kidney and urinary EGF and decreases submandibular gland EGF (4,10,11). Progesterone has an increasing effect on plasma and kidney EGF and a decreasing effect on urinary EGF (4). The changes in the EGF levels during pregnancy are best attributed to the action of estradiol, but other hormones are likely to contribute.

Acknowledgments

This study was supported by the Academy of Finland, the Sigrid Jusélius Foundation, and the Foundation for Pediatric Research, Helsinki, Finland.

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Received July 9th, 1990.
Accepted September 21st, 1990.

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