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## SERUM GASTRIN AND GASTRIC ENTEROCHROMAFFIN-LIKE CELLS DURING ESTROUS CYCLE, PREGNANCY AND LACTATION, AND IN RESPONSE TO ESTROGEN-LIKE AGENTS IN RATS

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Histamine-containing enterochromaffin-like (ECL) cells are numerous in the gastric mucosa. They operate under the control of gastrin. ECL-cell tumors (gastric carcinoids) may arise as a consequence of sustained hypergastrinemia. For reasons unknown, such tumors have a female preponderance both in laboratory animals and humans. The present study consisted of four experiments exploring the possibility that gender-related factors might affect rat ECL cells. 1) A gender difference in terms of serum gastrin concentration and oxyntic mucosal histidine decarboxylase (HDC) activity appeared in Sprague-Dawley but not Wistar rats. Ultrastructural appearance of the ECL cells did not differ between genders. 2) During the different phases of the estrous cycle, the serum gastrin concentration, HDC activity and histamine concentration did not change. 3) During pregnancy, the serum gastrin concentration was suppressed, while it was increased during lactation. The HDC activity and the histamine concentration of the oxyntic mucosa were correlated with the levels of circulating gastrin. 4) Twelve-month treatment with estrogen-like agents, dieldrin and/or toxaphene (alone or in combination) was without any effect on the ECL cells neither in male nor in female rats. In conclusion, the ECL cells are under the control of gastrin, but probably not hormones that involve in the estrous cycle and pregnancy and lactation in rats. Possible gender-related factors behind the female preponderance of ECL-cell tumors remain unknown.

*Key words: dieldrin, histamine, enterochromaffin-like cells, gastrin, histidine decarboxylase, lactation, pregnancy, sex, toxaphene*

### INTRODUCTION

The term enterochromaffin-like cells has been used to describe many non-enterochromaffin endocrine cells that occur along the entire gastrointestinal tract. They all produce chromogranins and peptide hormones. Such cells are notably numerous in the oxyntic mucosa of stomach. One of the enterochromaffin-like cell populations in this location is known to produce and store histamine and chromogranin A. Histamine-producing enterochromaffin-like cells are unique for the oxyntic mucosa and are referred to as ECL cells (1). They operate under the control of gastrin with respect both to function and growth (2-4). Gastrin is released in response to food and to the consequent rise in gastric pH. Gastrin causes acid secretion by mobilizing ECL-cell histamine, and gastric acid causes feedback inhibition of gastrin release. The ECL cells respond to gastrin with secretion of histamine and chromogranin-derived peptides such as pancreastatin followed by activation of the histamine-forming enzyme histidine decarboxylase (HDC). Long-term hypergastrinemia is associated with ECL-cell hypertrophy and hyperplasia/neoplasia (5, 6). Potent antisecretagogues, such as histamine H<sub>2</sub> receptor antagonists and proton pump inhibitors, are widely used clinically to treat acid-related disorders. Whenever such drugs are used to block acid secretion

hypergastrinemia ensues. Not unexpectedly, an increased incidence of gastric carcinoids, identified as ECL-cell tumors or ECLomas, was noted in the murine stomach after long-term treatment with proton pump inhibitors (7). Interestingly, ECL-cell tumors turned out to be more frequent in females than in males, both in humans and rodents. After about two years of daily omeprazole ingestion, ECL-cell tumors occurred in 25-50% of the female rats, and in 1% of the male rats (7). In cotton rats, spontaneous ECL-cell tumors occurred in 50% of the females and less than 1% in males (8-10). The gender difference has been noted in humans; 65% of gastric carcinoids occur in females (11, 12). Although it is generally thought that gastrin promotes the development of ECLoma, the gender-related factors explaining the female preponderance of ECL-cell tumors remain unknown for many years. Much earlier studies showed that the difference between males and females reflected an insensitivity of female rats to gastrin during the estrous cycle, and pregnancy and lactation were associated with gastric acid hypersecretion and gastric mucosal hyperplasia (13-16).

In the present study, we compared ECL cells in male and female rats and explored the control of the ECL cells in terms of function and proliferation during estrous cycle, and in pregnant and lactating rats. In addition, we studied effects of estrogen-like agents on the ECL cells of both male and female rats.

## MATERIALS AND METHODS

### Study design

The study consisted of four experiments: 1) We compared male and female rats with respect to ECL-cell-related parameters (Table 1). 2) We examined ECL-cell-related parameters during the estrous cycle (Table 2). 3) We monitored ECL-cell-related parameters during pregnancy and lactation. 4) We examined the long-term effects of estrogen-like agents on ECL-cell-related parameters in female and male rats (Table 3).

### Animals

In the experiment comparing freely fed male and female rats, 10 females and 10 males (Sprague-Dawley strain at 2 months of age) were included. Female and male rats were housed separately.

In the experiments of the estrous cycle, and of pregnancy and lactation, 117 female Sprague-Dawley rats, weighing 220–270 g were used. The rats were housed in plastic cages, 4–5 rats in each before and during pregnancy. The rats were fed a standard rat pellet diet (ALAB, Stockholm, Sweden) and tap water. Vaginal smears were made between 3:00 and 4:00 P.M. and stained with methylene blue plus eosin in order to identify by histology the various phases of the estrous cycle (17–19). Non-pregnant rats were divided into the following groups: diestrus, metestrus, estrus and proestrus (each group consisted of 8–12 rats). In the experiment of pregnancy and lactation, the females were allowed to mate when in prooestrus. Two of them were transferred for 18 hours to a cage containing a male rat. The next morning another vaginal smear was taken. The female rats in which sperm was found were considered pregnant (day 0 of pregnancy). Pregnant rats were killed by decapitation at various times (5–6 rats at each time) during and after the pregnancy. Age-matched controls (5–6 nonpregnant rats at each time) were killed at the same time. After delivery, each mother was kept isolated with the litter. Lactating rats were killed at weekly intervals after delivery. The experiments above were approved by the local Animal Welfare Committee of Lund/Malmö, Sweden.

In the study of estrogen-like agents, 80 Wistar rats (40 females and 40 males) were divided into 4 groups of each sex (8 experimental groups): vehicle, dieldrin, toxaphene and dieldrin+toxaphene. Dieldrin and toxaphene were dissolved in corn oil and given to the rats *via* a gastric tube once daily for five days per week for twelve months. The doses were 7.5 µmol/kg for dieldrin and 30 µmol/kg for toxaphene. This treatment regimen was known to reduce the serum luteinizing hormone and follicle-stimulating hormone in male rats and increase the bone mineral density in both male and female rats in the same experiment (Syversen *et al.* unpublished observations). All rats were freely fed and killed between 9:00 and 12:00 AM by drawing blood from the aorta under the anesthesia and decapitation. Approval for this experiment was given by the Norwegian Animal Research Authority, Norway.

### Determination of serum gastrin concentration

The serum gastrin concentration was measured by radioimmunoassay (RIA) using rabbit gastrin antiserum (no. 4562, provided by Dr. J.F. Rehfeld, Copenhagen, Denmark) and with human synthetic 125 labeled gastrin-17 as tracer (Sigma, St Louis, MO, USA, code: G9020). Tracer, antiserum, and the sample to be analyzed were incubated for 48 h at 4°C. After 48 hours, donkey anti-rabbit serum was added, and the samples were incubated for another 24 h (4°C). The  $\gamma$ -counter COBRA II autogamma was used.

### Measurements of histidine decarboxylase activity and histamine concentration in the oxyntic mucosa

The stomachs were taken out and the oxyntic mucosa was scraped off the gastric wall, weighed and homogenized in cold (~4°C) 0.1 M sodium phosphate buffer, pH 7.0, to a final concentration of 100 mg wet weight/ml. Eighty µl aliquots of each homogenate were incubated with L<sup>14</sup>C-histidine (0.02 mCi/ml) (specific activity, 50 mCi/mmol) and 5x10<sup>-4</sup> M L-histidine in the presence of 10<sup>-5</sup> M pyridoxal-5-phosphate at 37°C for 1 hour under nitrogen. Total reaction volume was 160 µl. The amount of <sup>14</sup>CO<sub>2</sub> formed during the reaction was measured by liquid scintillation counting (20). The histamine concentration of the oxyntic mucosa was determined spectrofluorometrically using o-phthaldialdehyde as described by Ronnberg and Hakanson (21).

### Electron microscopy

Small specimens (<1 mm<sup>3</sup>) were collected from the oxyntic gland area, and immediately immersed in a mixture of glutaraldehyde (1%) and formaldehyde (3%) in 0.075 M sodium phosphate buffer, pH 7.2, for 6 hours. The specimens were post-fixed in 1% osmium tetroxide for 1 hour, dehydrated in graded acetone solutions and embedded in Epon 812. Ultrathin sections (60–80 nm) were cut on a LKB MK III Ultratome, routinely contrasted with uranyl acetate and lead citrate and examined in a transmission electron microscope (Joel CX200). ECL cells were identified based on their characteristic secretory organelles. ECL cells with visible nuclei were photographed at 6,000 x magnifications; the prints were enlarged to 20,000 x for planimetric analysis. The parameters included the cell profile area, nuclear area and volume density, cytoplasmic area, and the numbers and volume densities of granules, secretory vesicles, and microvesicles (22).

### Immunohistochemistry

The stomach was taken out, opened along the greater curvature, washed in 0.9% NaCl, and pinned flat to a glass board with the mucosal surface upwards. A standardized tissue specimen (5 mm diameter) was taken from a predetermined site in the oxyntic mucosa from each rat and frozen at the temperature of liquid nitrogen in propane-propylene and freeze-dried. The specimens were then exposed to diethylpyrocarbonate vapour and embedded in paraffin for histamine immunohistochemistry. Sections were cut at 6 µm, deparaffinized and exposed to histamine antiserum (code no. 8431, Milab, Malmö, Sweden), applied at a dilution of 1:6000 in phosphate buffered saline (PBS) for 20 hours at +4°C in a moist chamber. The sections were then rinsed in PBS and incubated for 2 hours with goat anti rabbit IgG (1:80 in PBS, Dako, Copenhagen, Denmark). For pancreastatin immunostaining, the specimens were fixed in 4% formaldehyde for 8–12 hours at 4°C, and then rinsed with 20% sucrose in 0.1 M phosphate buffer (pH 7.4). Pancreastatin antiserum (rabbit, code no. 9006/16) was used in a dilution of 1:1000. Histamine immunostaining demonstrates ECL cells and mast cells (the latter cells are few). Pancreastatin immunostaining demonstrates ECL cells (the majority endocrine cell population in the oxyntic mucosa), and other enterochromaffin-like cells, such as A-like cells and somatostatin cells (minority cell populations in the oxyntic mucosa). Histamine- or pancreastatin-immunoreactive cells were counted in transversely cut sections displaying the full thickness of the mucosa. The immunostained slides were examined in a light microscope (Zeiss Axioskop, Germany). A

positive immunoreactive cell was defined as a cell with a visible nucleus and positive staining in the cytoplasm. The results are expressed as number of immunoreactive cells per visual field (10x or 40x as stated).

#### Statistical analysis

Values are expressed as means±S.E.M. Independent *t* test or ANOVA was applied using SPSS v15. Statistical significance was defined as a *p* value of <0.05 (one- or two-ways as stated).

## RESULTS

#### Euterochromaffin-like cells of male and female rats

In Sprague-Dawley rats, freely fed females had lower serum gastrin concentration, HDC activity and ECL cell density (histamine immunostaining) than males, but displayed similar general appearance of the ECL cells and number and volume density of the granules and secretory vesicles (except for the microvesicles, the volume density being increased) as males (Fig. 1, Table 1). In Wistar rats, there was, however, a lack of gender difference in both serum gastrin concentration and HDC activity (Table 3).

#### Euterochromaffin-like - cell activity during the estrous cycle

The serum gastrin concentration and the ECL-cell parameters (HDC activity and histamine concentration in the oxyntic mucosa) did not change during the different phases of the estrous cycle (Table 2).

#### Euterochromaffin-like -cell activity during pregnancy and lactation

During pregnancy, the serum gastrin concentration was suppressed, while it was greatly increased during lactation. At the time of weaning, serum gastrin returned to normal. The ECL-cell parameters (HDC activity and histamine concentration in the oxyntic mucosa) were slightly reduced during the late period of pregnancy and greatly elevated during lactation (Fig. 2).

#### Effects of estrogen-like agents

Rats treated with dieldrin and/or toxaphene daily for 12 months did not differ with respect to the serum gastrin concentration, the oxyntic mucosal HDC activity and number of ECL cells (pancreastatin immunostaining) (Table 3).

## DISCUSSION

The present report includes four individual experiments, and the statistical analysis was performed within each experiment. The absolute values of oxyntic mucosal HDC activity and serum gastrin concentration varied between the experiments, which was most likely due to the technical rather than biological variations. However, it should be noticed that there was a gender difference in terms of serum gastrin concentration and oxyntic mucosal HDC activity in Sprague-Dawley rats but not in Wistar rats (Tables 1, 3). The strain difference has been reported previously (23, 24). In the present study, electron microscopic analysis revealed no difference in the ECL-cell ultrastructure between males and females, except that the volume density of microvesicles was greater in the ECL cells of male rats, which might suggest an increased endocytosis (22).

During the estrous cycle, the secretory response of parietal cells to gastrin was reported to be unaffected by the different phases of the estrous cycle (13). In one study, plasma gastrin was found to be lower in diestrus than in estrus or proestrus in rats (25). The results of the present study suggest that neither the serum gastrin concentration nor the ECL-cell-related parameters changed during the various phases of the estrous cycle in freely fed rats. Thus, the higher risk of developing ECL-tumors in female than in male rats is unlikely to be explained by mechanisms related to the estrous cycle.

Pregnancy and lactation have significant effects in gastrointestinal physiology, including gastric function (26-29). It

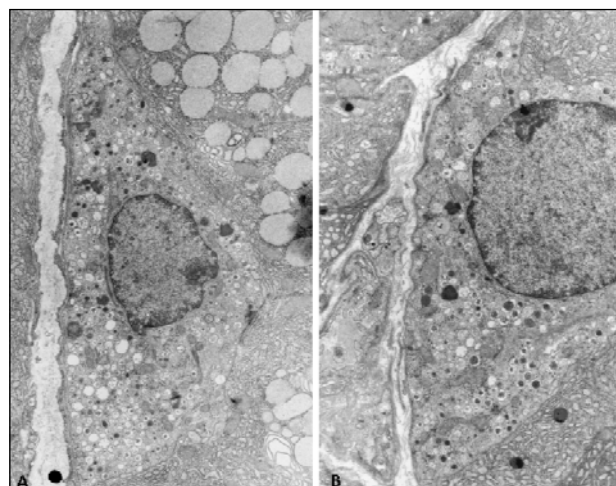


Fig. 1. Ultrastructure of typical ECL cells in the oxyntic mucosa of a female rat (A) and a male rat (B). 6000x.

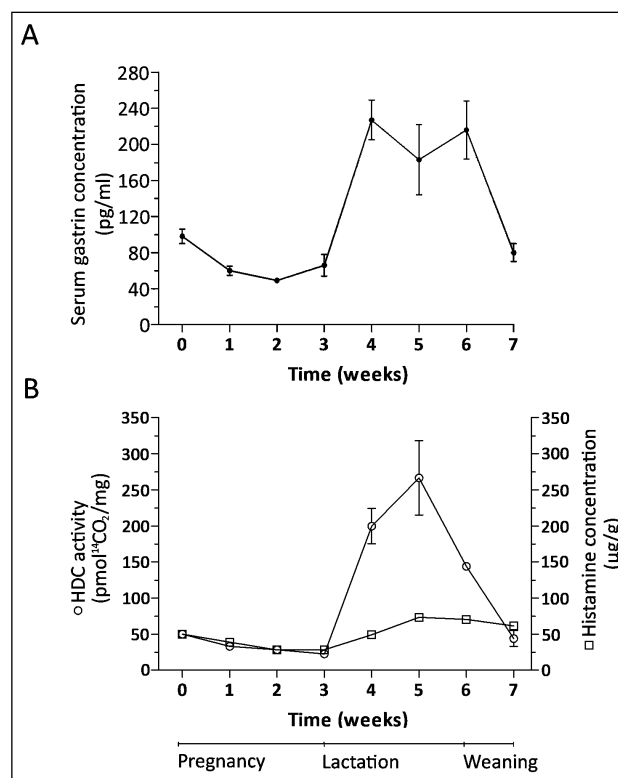


Fig. 2. Serum gastrin concentration (A), oxyntic mucosal HDC activity and histamine concentration (B) during pregnancy and lactation. Means±S.E.M. (n=5-6 rats).

Table 1. ECL-cell-related parameters in freely fed female vs. male Sprague-Dawley rats aged 8 weeks.

	Females	Males
Number of rats	10	10
Body weight (g)	192±27	279±34*
Serum gastrin concentration (pg/ml at freely feeding)	106.1±18.5	161.6±34.6*
Oxyntic mucosal HDC activity (pmol CO <sub>2</sub> /mg w. wt and hour)	28.1±11.2	44.9±16.9*
ECL cell density (no./visual field)	198.9±30.3	284.1±51.1*
Cell profile area (no. of cells examined)	38±3(35)	33±3(31) <sup>ns</sup>
Cytoplasm profile area	25±3	23±3 <sup>ns</sup>
Nuclear volume density (%)	38±3	37±4 <sup>ns</sup>
Secretory vesicles (no./cell profile)	42±6	36±5 <sup>ns</sup>
Secretory vesicles (%)	16±1	13±2 <sup>ns</sup>
Granules (no./cell profile)	2.4±0.4	3.0±0.9 <sup>ns</sup>
Granules (%)	0.4±0.1	1.1±0.4 <sup>ns</sup>
Microvesicles (no./cell profile)	17±2	15±2 <sup>ns</sup>
Microvesicles (%)	1.3±0.1	4.2±0.5*

Means±S.E.M. \* p<0.05. <sup>ns</sup>, no statistically significant differences between females and males (one-tailed).

Table 2. Serum gastrin concentration, oxyntic mucosal HDC activity and histamine concentration in freely fed. Sprague-Dawley rats during the estrous cycle.

Phase	Number of rats	Serum gastrin concentration (pg/ml)	HDC activity (pmol <sup>14</sup> CO <sub>2</sub> /mg)	Histamine (µg/g)
Diestrus	11	89±13	51±8	69±13
Metestrus	12	107±14	53±11	59±12
Estrus	9	109±20	46±7	59±5
Proestrus	8	119±25	61±7	56±11

Means±S.E.M. There were no statistically significant differences between the four groups (two-tailed).

Table 3. Effects of 12-month treatment with dieldrin and/or toxaphene on the serum gastrin concentration, HDC activity and number of pancreastatin-immunoreactive endocrine cells (mainly ECL cells) in the oxyntic mucosa of female and males Wistar rats.

Treatment	Number of rats	Serum gastrin concentration (pg/ml)	HDC activity (pmol <sup>14</sup> CO <sub>2</sub> /mg)	Pancreastatin immunoreactive cells (no./field)
Female rats				
Corn oil	7	59.1±6.9	19.0±2.0	26.6±1.6
Dieldrin	8	62.8±5.2	22.9±2.5	24.2±2.0
Toxaphene	7	42.0±0.9	20.9±1.8	24.3±2.0
Dieldrin + toxaphene	8	53.6±3.1	22.2±4.4	20.2±2.2
Male rats				
Corn oil	8	64.3±5.8	23.2±2.1	28.6±1.4
Dieldrin	8	67.0±4.0	28.5±3.4	27.3±1.2
Toxaphene	7	61.6±1.6	28.4±4.7	28.0±1.6
Dieldrin + toxaphene	6	59.7±8.3	27.0±4.4	32.3±3.7

Means±S.E.M. There were no statistically significant differences between the groups (two-tailed).

was reported a long time ago that the acid secretion was increased during pregnancy and lactation, and the chief cells seemed to be activated during lactation (16, 30, 31). The results of the present study suggest that while the ECL cells are slightly suppressed during pregnancy, they are greatly activated during

lactation, which was nicely correlated with circulating gastrin. These results agree with those reported by Takeuchi and Okabe (16) and partly with those reported by Lilja and Svensson (32). The latter authors reported that the histamine-forming capacity of the gastric mucosa was increased during the last days of

pregnancy and during lactation. The high gastrin levels during lactation are likely due to an increase in food intake (15, 16, 33). The increase food intake might also take place during the pregnancy, and thus it can not be excluded that sucking stimulus may be an additional factor. In addition to gastrin, prolactin could also be involved. However, the present observations do not explain the female preponderance of ECL-cell tumors in response to omeprazole-induced hypergastrinemia or spontaneously in cotton rats (7-10).

Together, the observations support the view that there is a close correlation between serum gastrin concentration and ECL-cell-related parameters in both male and female rats and that this is the case also during the estrous cycle, and during pregnancy and lactation. We conclude that rat ECL cells are activated during lactation, but not during pregnancy, that they are under the control of gastrin, and steroid sex hormones are unlikely to play a role.

We next examined long-term effects of environmental factors, such as dieldrin and toxaphene, pesticides with estrogenic effect. The experiment was based on an earlier report claiming that the two compounds had synergistic estrogenic effects. The paper was later retracted as the compounds were found to have additive effects by Ramamoorthy and colleagues (34) who compared the effect of 17 $\beta$ -estradiol with that of dieldrin, toxaphene, or the equimolar combination of the compounds, on uterine weight, peroxidase activity, and progesterone and estrogen receptor-binding assays (all markers for estrogen action) *in vivo*. The doses that were given were 2.5, 15, and 60  $\mu$ mol/kg daily for 3 days of both dieldrin and toxaphene, alone or in combination. These doses are comparable to the doses used in our study. In the present study, both male and female rats were treated with the estrogen-like agents for as long as 12 months and neither circulating gastrin nor ECL-cell-related parameters were changed.

In conclusion, male and female rats did not differ with respect to ECL-cell-related parameters. During lactation, the serum gastrin concentration was elevated and the ECL cells were activated. Neither the serum gastrin concentration nor the ECL-cell-related parameters changed during the various phases of the estrous cycle and during pregnancy or in response to long-term treatment of estrogen-like agents. The gender-related factors explaining the female preponderance of ECL-cell tumors remain unknown.

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