

# Immunolocalization of estrogen receptor $\alpha$ and $\beta$ in gastric epithelium and enteric neurons

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## Abstract

A sexual dimorphism in gastric acid secretion has been known for many years, with women secreting less acid (~40%) than men. The mechanisms mediating this sex difference are unknown, but a role for estrogens is suggested from animal models. Two estrogen receptor (ER) subtypes, ER $\alpha$  and ER $\beta$ , mediate genomic effects of estrogens, and mRNA for both subtypes has been detected in the rat stomach. The objective of this study was to determine the cellular distribution of ER $\alpha$  and ER $\beta$  proteins in the rat stomach. ER $\alpha$  and ER $\beta$  proteins were detected in nuclei of fundic parietal cells and epithelial cells in the progenitor zone. In the antrum, several cells were immunoreactive for ER $\beta$  in regions containing stem and neuroendocrine cell types but ER $\alpha$  protein was not detected in antral glands. Both ER $\alpha$  and ER $\beta$  proteins were expressed in enteric neurons within the nucleus and

cytoplasm, with specific punctate staining for ER $\alpha$  in cell bodies and fibers. These studies are the first to show differences between ER $\alpha$  and ER $\beta$  proteins in the epithelial cellular distribution in the fundus and antrum and to detect co-expression in enteric neurons. These results suggest that estrogens may inhibit gastric acid secretion via genomic effects in fundic parietal cells through either ER subtype and in antral neuroendocrine cells via ER $\beta$ . Moreover, co-expression of ER $\alpha$  and ER $\beta$  in enteric neurons indicates that estrogenic effects could also be mediated through neurogenic reflexes. Our findings imply that direct regulation of multiple cell types by estrogens may contribute to the modulation of gastric functions that have been recognized during the estrous cycle and between the sexes.

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## Introduction

Gastric acid secretion has a significant role in the integrated responses to digestion of a meal. A striking sexual dimorphism in gastric acid secretion has been known for many years with women secreting significantly less acid (~40%) than men (Feldman *et al.* 1983, Prewett *et al.* 1991). Reciprocal changes in gastric acid secretion versus serum estradiol concentrations were reported during the menstrual cycle (Sakaguchi *et al.* 1991). In rats, basal gastric acid output is similar in females and males, whereas histamine- and pentagastrin-stimulated gastric acid output can be decreased by 17 $\beta$ -estradiol administration (Omole 1972, Limlomwongse & Piyachaturawat 1982, Aguwa 1984, Adeniyi 1991, Girma *et al.* 1997).

At least two estrogen receptor (ER) subtypes, ER $\alpha$  and ER $\beta$ , mediate the genomic actions of estrogens (reviewed in Gustafsson 1999, Muramatsu & Inoue 2000). ER $\alpha$  and ER $\beta$  share homologous regions in the DNA and ligand binding domains (~96% and ~58% amino acid homology respectively). These domains confer similar binding affinities for transcriptional activation of estrogen response elements (ERE) and estradiol respectively.

However, there are major differences between ER $\alpha$  and ER $\beta$  in their tissue distribution, the phenotype of the corresponding knockout mice, and their ligand-dependent transcriptional activities (Barkhem *et al.* 1998). For example, 17 $\beta$ -estradiol can stimulate transcriptional activity at an estrogen response element via either ER $\alpha$  or ER $\beta$ , yet can activate an AP-1 element only via ER $\beta$  (Paech *et al.* 1997). Tamoxifen and raloxifene, selective estrogen receptor modulators, stimulate AP-1 activity through ER $\beta$ , yet do not activate gene transcription via ER $\alpha$  or ER $\beta$  at an ERE. These interactions are further compounded by dimerization of ER as either homodimers ( $\alpha/\alpha$ ,  $\beta/\beta$ ) or heterodimers ( $\alpha/\beta$ ) (Cowley *et al.* 1997, Pace *et al.* 1997). Cell-specific gene regulation is also dependent on differential expression of certain ER coactivators and corepressors. Studies in mice with gene deletions for ER $\alpha$  and ER $\beta$  have determined that ER $\alpha$  is critical for fertility and bone density in both sexes, whereas ER $\beta$  has critical roles in follicular development in females and possibly in prostate and bladder development in males (Korach 2000). These findings underscore the complexity and diversity of estrogenic effects and the mechanisms involved and demonstrate the necessity for knowledge of

cellular distribution of the ER subtypes in each target tissue.

We reported that ER $\alpha$  and ER $\beta$  mRNA were expressed in the epithelium of the rat upper gastrointestinal (GI) tract and that ER $\beta$  mRNA was expressed in greater abundance than ER $\alpha$  (Campbell-Thompson 1997). Similar findings were reported in human intestinal tissues, and ER $\beta$  mRNA was localized throughout the GI mucosa (Brandenberger *et al.* 1997, Enmark *et al.* 1997). To date, immunolocalization of ER $\alpha$  has been studied in the stomach because ER $\beta$  antibodies have only recently become available. However, studies have been conflicting regarding ER $\alpha$  expression, with either low concentrations of ER $\alpha$  protein detected in normal human gastric mucosa (Cameron *et al.* 1992, Ciocca & Vargus Roig 1995) or a lack of expression (Rio & Chambon 1990, Kojima *et al.* 1991).

The specific mechanisms by which estrogens may alter gastric acid secretion are unknown. Direct effects on gastric epithelial cells and secondary neuroendocrine interactions regulate secretion rates. We propose that estrogens acting via nuclear ER directly regulate gastric parietal cell function. The aims of the present study were to determine the cellular distribution of ER $\alpha$  and ER $\beta$  genes in the gastric epithelium and to determine the effects of chronic 17 $\beta$ -estradiol administration on gastric acid secretion and gastric ER subtype distribution.

## Materials and Methods

### Animals and procedures

Young adult female Sprague-Dawley rats (210–270 g,  $n=31$ , Harlan, Indianapolis, IN, USA) were used. All experiments were approved by the Institutional Animal Care Committee of the University of Florida using procedures of the NIH Guide for the Care and Use of Laboratory Animals. Rats had *ad libitum* access to rat chow and water and were housed individually in wire-bottom cages (14-h light:10-h darkness cycle). Rats were acclimated to conditions for 7 days and divided into groups of ovariectomized and intact females. Bilateral ovariectomy was performed under general anesthesia using sterile techniques. Rats were allowed to recover from surgery for 5 days and then received daily s.c. injections of corn oil vehicle (Ovx) or 17 $\beta$ -estradiol (Ovx-E2; 1, 10, 20, 50 and 100  $\mu\text{g}/\text{kg}$ ) for 1 week. Ovx and Ovx-E2 rats were randomly assigned into pairs. The daily food consumption of each Ovx-E2 rat was calculated and the same amount of food was given to the Ovx rat. Without pair-feeding, Ovx-E2 rats ( $\geq 10 \mu\text{g}/\text{kg}$ ) ate 3–8 g food/day less than Ovx controls (data from four pairs monitored for 1 week). Body weights were determined at arrival, surgery and end of the studies. For immunolocalization and *in situ* hybridization studies, Ovx-E2 rats received 20  $\mu\text{g}/\text{kg}$  17 $\beta$ -estradiol.

For serum gastrin analysis, venous blood was obtained by cardiac puncture and stored on ice for 30 min. Samples were spun at 3000 g and serum collected and stored at  $-75^\circ\text{C}$ . Samples were analyzed in triplicate in a single assay using RIA methods established in the laboratory (McGuigan & Wolfe 1982). Human gastrin-17 was used as a standard and results were expressed as pg/ml (sensitivity 10 pg/ml).

### Tissue preparation

For immunohistochemistry and *in situ* hybridization, rats were fasted overnight with free access to water and anesthetized with sodium pentobarbital (60 mg/kg i.p.) between 0900 and 1100 h, to avoid diurnal variation. Rats were perfused through the abdominal aorta with Tyrodes's buffer and 2% paraformaldehyde-lysine-periodate fixative (PLP) (McLean & Nakane 1974). Stomach and uterus were dissected and mesentery and fat removed. The stomach was opened along the greater curvature and contents rinsed off with phosphate buffer (PBS, 10 mM KPO<sub>4</sub>, 150 mM NaCl, pH 7.4). Tissues were stored in fixative overnight at  $4^\circ\text{C}$ . Full-thickness sections of stomach, including proximal fundus and distal antrum, and uterine horn were embedded in a single paraffin block for each rat. Sections 5  $\mu\text{m}$  thick were placed on Probe-on-Plus slides (Fisher, Pittsburgh, PA, USA) and stored at  $-20^\circ\text{C}$ . For RNA analysis, rats were killed and uteri were removed and drained of fluid before freezing and weighing. Uterine total RNA was analyzed as previously reported (Campbell-Thompson 1997).

### Antibodies

The specificity of the polyclonal ER $\alpha$  and ER $\beta$  antibodies (Table 1) was verified by immunolocalization in the uterus which has been well characterized (Saunders *et al.* 1997, Weihua *et al.* 2000). To detect ER $\alpha$ , we used primarily antisera ER-21 and ER-715, which have no counterparts in ER $\beta$  (Kuiper *et al.* 1996), and have been extensively characterized (Table 1). To detect ER $\beta$ , we used two antibodies from Affinity BioReagents (Golden, CO, USA) (Table 1). PA1-311 was directed against a synthetic peptide corresponding to the N-terminal amino acids of rat ER $\beta$ . This peptide has three of 18 (19%) amino acids in common with rat ER $\alpha$ . PA1-310 was generated against amino acids in the C-terminal region of the rat ER $\beta$  and has only 6% amino acid homology with rat ER $\alpha$ . Both ER $\beta$  antibodies were affinity-purified by column chromatography and their immunolocalization reported in other tissues (Table 1).

### Immunohistochemistry

Tissue sections were heated at  $55^\circ\text{C}$  for 30 min and immediately dewaxed and hydrated using xylenes, graded ethanols, water and PBS. Antigen retrieval methods using

**Table 1** Affinity-purified rabbit polyclonal anti-rat ER antibodies used for immunochemistry

Antibody	Receptor domain <sup>a</sup>	Amino acids <sup>a</sup>	Source	Immunolocalization in endometrium <sup>b</sup>		Reference
				Nuclear	Cytoplasmic	
<b>ER<math>\alpha</math></b>						
ER-21	A/B	1–21	Dr G Greene	++++	+	Greene <i>et al.</i> 1980
ER-715	D	270–284	NIH <sup>c</sup>	++	–	Hess <i>et al.</i> 1997
MC-20	F	580–599	Santa Cruz <sup>d</sup>	+++	++	Furlow <i>et al.</i> 1990
<b>ER<math>\beta</math></b>						
PA1-311	A/B	55–70	Affinity BioReagents	+++	++	Rosenfeld <i>et al.</i> 1998
PA1-310	F	467–485	Affinity BioReagents	–	+++	Li <i>et al.</i> 1997

<sup>a</sup>ER $\alpha$  and ER $\beta$  domains and amino acid numbers from Koike *et al.* (1987) and Kuiper *et al.* (1996) respectively. <sup>b</sup>Immunoreactivity intensity and numbers of positive endometrial epithelial cells grades as: –, no signal; +, barely detectable; ++, minimum; +++, medium; +++++, maximum. <sup>c</sup>NIH/NIDDK Hormone and Pituitary Program, Rockville, MD, USA. <sup>d</sup>Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA.

microwave treatment in citrate buffer and trypsin digestion were also tested with each antibody. For microwave antigen retrieval, sections were immersed in 10 mM citrate solution, pH 6, and heated for four intervals of 2.5 min. The citrate buffer was replenished after each heating. The slides were cooled to room temperature for 20 min and washed in PBS for 5 min. For trypsin digestion, sections were immersed in trypsin solution (0.1 mg/ml trypsin, 200 mM Tris HCl, 4 mM CaCl<sub>2</sub>, pH 7.7, Sigma Chemical, St Louis, MO, USA) for 12 min at room temperature, followed by washing with PBS containing soybean trypsin inhibitor (10  $\mu$ g/ml, Sigma) for 5 min. After dehydration or antigen retrieval, sections were incubated with 10% normal donkey serum for 10 min in a humidified chamber, then incubated overnight at 10 °C with antibodies to rat ER $\alpha$  (ER-21, 10  $\mu$ g/ml) or ER $\beta$  (PA1-311, 20  $\mu$ g/ml) diluted in PBS with 1% BSA. After successive PBS washes (twice, 5 min each), slides were blocked for endogenous peroxidase activity with 3% H<sub>2</sub>O<sub>2</sub> in PBS for 10 min and incubated with donkey anti-rabbit peroxidase-conjugated antibodies (1:100, Amersham Life Science, Arlington Heights, IL, USA) for 10 min followed by PBS washes. The sections were reacted with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide for up to 10 min and washed with distilled water. Sections were lightly counterstained with hematoxylin, dehydrated in graded ethanols through xylene, and mounted. Controls included 1) replacement of the primary antibody with normal rabbit serum or PBS and 2) pre-absorption of the antiserum with peptide antigen using serial sections. Antiserum dilutions were incubated with peptide (1  $\mu$ g/ml (ER-21) or 20  $\mu$ g/ml (PA1-311) in PBS, 1% BSA) overnight at 4 °C, followed by centrifugation at 12 000 g before application to serial sections. Sections were observed with an Axiophot microscope (Carl Zeiss, Oberkochen, Germany) and

photographed with 35 mm color slide film (Ektachrome 160T, Kodak). Slides were scanned and compiled using Adobe Photoshop 3.0 before printing.

#### In situ hybridization

Non-radioactive *in situ* hybridization was performed as previously reported (Campbell-Thompson *et al.* 1995). The transcription plasmids contained sequence-verified 334-bp rat ER $\alpha$  (Campbell-Thompson 1997), 349-bp rat ER $\beta$  (Campbell-Thompson 1997), and 570-bp rabbit H,K-ATPase (Campbell-Thompson *et al.* 1995) cDNA fragments. A 262-bp rat gastrin cDNA was cloned by RT-PCR from antral total RNA into pCRII vector (Invitrogen, Carlsbad, CA, USA) as reported (Campbell-Thompson 1997). The PCR primer pair included a sense primer (5'-ATGCCT CGACTGTGTG) and antisense primer (5'-CGTATGC TTCCTCTTC) (nucleotides 46–307, Genbank accession M38653). Antisense and sense riboprobes were prepared by *in vitro* transcription using either T7 or SP6 RNA polymerases with digoxigenin-11-UTP (MegaShortScript, Ambion, Austin, TX, USA). Specificity of the cDNA fragments for each gene was evaluated by Northern analysis using total RNA samples from rat uterus or stomach as previously described (Campbell-Thompson 1997).

Gastric and uterine tissue sections were deparaffinized and treated with 0.2 M HCl and proteinase K (1  $\mu$ g/ml) for 20 min each with washes in wash buffer (TBS; 25 mM Tris-HCl, pH 7.6 and 150 mM NaCl) (Campbell-Thompson *et al.* 1995). Sections were post-fixed in 4% paraformaldehyde and incubated in pre-hybridization buffer for 30 min. Sections were hybridized with 0.1–0.5 ng riboprobe/ $\mu$ l hybridization buffer overnight at 42 °C. Sections were washed in three changes of 2  $\times$  saline sodium citrate–50% formamide at 55 °C for 1 h.

Riboprobe hybridization was detected by incubation with sheep anti-digoxigenin antibodies (1:500), followed by an alkaline phosphatase color reaction using nitroblue tetrazolium, bromochloroindoyl phosphate. Levamisole (24  $\mu$ g/ml) was included to inhibit endogenous alkaline phosphatase activity. Sections were mounted without counterstaining and photographed using black and white print film (T-MAX 100, Kodak). Photographs were scanned and processed as for immunohistochemistry. Control reactions included 1) incubation with hybridization buffer alone, and 2) incubation with a heterologous antisense rat H,K-ATPase  $\beta$  subunit or gastrin riboprobe. Adjacent serial sections were observed for differences in labeling between the antisense ER $\alpha$  and ER $\beta$  riboprobes and control conditions.

#### Stimulated gastric acid secretion

Rats were fasted overnight with access to water and anesthetized with 0.08 ml/100 g body weight of a 1:1 mixture of xylazine (20 mg/ml) and ketamine (100 mg/ml). The pylorus was ligated according to the method of Shay *et al.* (1954) and the abdominal incision closed with suture. Pentagastrin (50  $\mu$ g/kg i.p.; Ayerst Laboratories, New York, NY, USA) was administered to stimulate gastric acid secretion. Rats were placed in a warm cage and all regained consciousness within 20 min. One hour after pentagastrin treatment, rats were anesthetized with sodium pentobarbital, the stomachs removed and gastric contents collected. Contents were spun at 3000 g for 10 min. Gastric fluid volume was measured and acid concentration was determined by titration with 0.01 M NaOH to pH 7.0. Acid output was calculated by the product of gastric fluid volume and acid concentration and was normalized to body weight ( $\mu$ Eq H<sup>+</sup>/h per kg).

#### Statistical analysis

Data are expressed as means  $\pm$  s.d. The mean values were analyzed by one-way ANOVA with *post-hoc* Tukey's test. A *P* value < 0.05 was considered significant.

## Results

#### ER $\alpha$ and ER $\beta$ distribution in uterus

In this study, ER $\alpha$  was detected in nuclei of the luminal and glandular endometrium and stromal smooth muscle cells of the endometrium and myometrium (not shown). Using PA1-311 for ER $\beta$ , a distinct nuclear staining of glandular endometrial cells with slight cytoplasmic staining was observed (not shown). Maximum staining intensity for ER $\beta$  was less than for ER $\alpha$  in endometrial cells as reported by others (Weihua *et al.* 2000). ER $\beta$  immunoreactivity was also detected in nuclei of vascular smooth muscle cells,

but not in stromal smooth muscle cells (not shown). Using PA1-310, ER $\beta$  immunoreactivity was detected within the same endometrial cells as with PA1-311, but the immunoreactivity was distributed throughout the cytoplasm, in addition to the nucleus. The intensity of ER $\alpha$  and ER $\beta$  immunoreactivity in the epithelial cells of the endometrium was reduced in Ovx-E2 rats compared with Ovx rats, in agreement with the findings of others (Weihua *et al.* 2000). Antigen retrieval procedures did not improve immunoreactivity for these antisera using PLP-fixed tissues and immunoperoxidase detection. Nuclear staining of glandular endometrial cells with ER-21 or ER-715 was essentially eliminated by pre-absorption of the antisera (Fig. 1g, h) and nuclear immunoreactivity was eliminated by absorption of ER $\beta$  PA1-311. Cytoplasmic staining with PA1-311 was decreased by  $\sim$ 50% by peptide absorption. Cytoplasmic staining with PA1-310 was not reduced by peptide absorption (40  $\mu$ g/ml). Control conditions using substitution of the primary antisera with normal rabbit serum or antibody diluent did not produce cell staining.

#### Expression of ER $\alpha$ in gastric epithelium

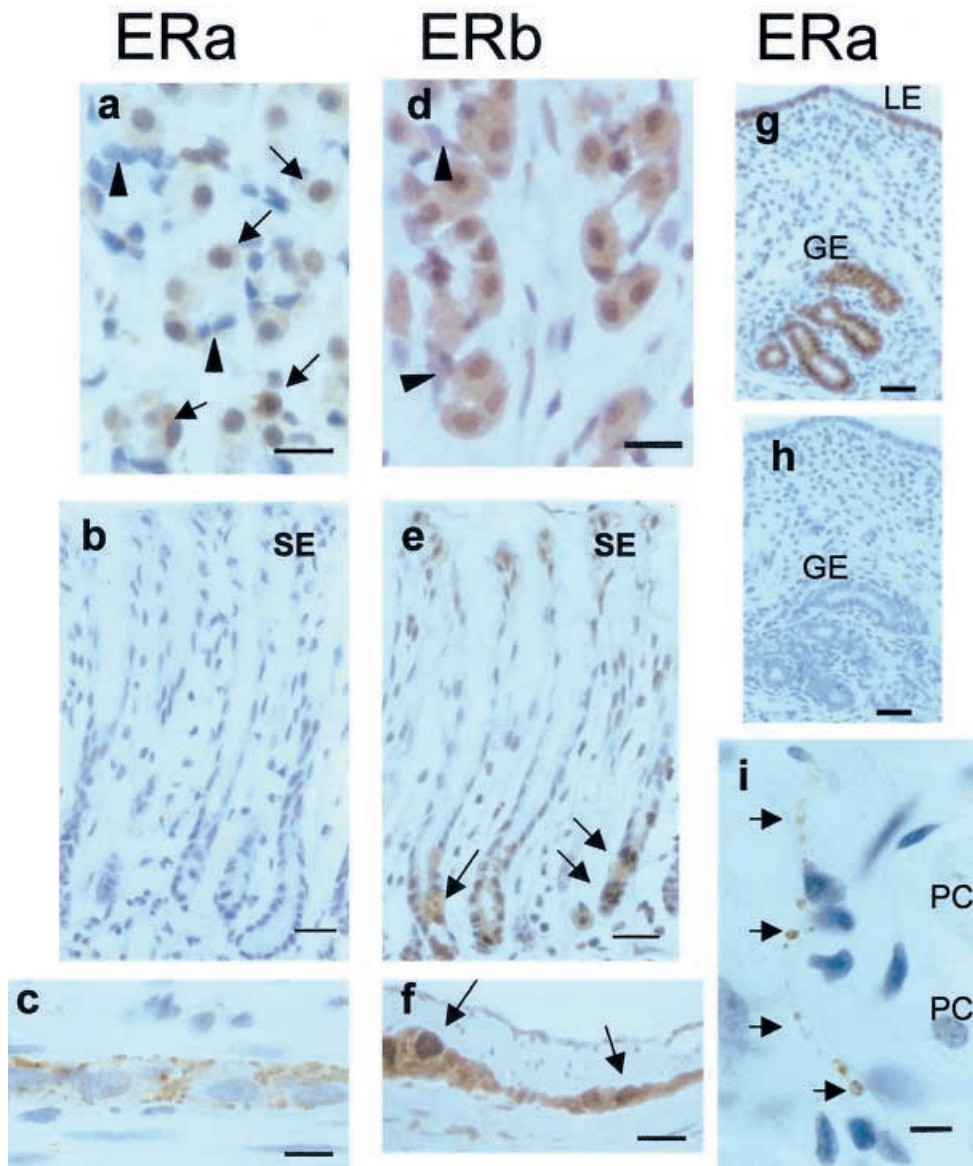
ER $\alpha$  immunoreactivity, detected with ER-21, was localized in fundic epithelial cells within the progenitor zone and in parietal cells throughout the glands (Fig. 1a). Superficial epithelial mucous cells were slightly positive for ER $\alpha$ , whereas chief cells were essentially negative (Fig. 1a). ER $\alpha$  antisera ER-715 showed similar nuclear immunolocalization in the progenitor zone of the fundus, but fewer parietal cells were positive than with ER-21. ER $\alpha$  immunoreactivity was not detected in the antral epithelium (Fig. 1b). Nuclear staining in gastric cells with ER $\alpha$  antisera was eliminated by pre-absorption with the antigenic peptide (not shown).

#### Expression of ER $\beta$ in gastric epithelium

ER $\beta$  immunoreactivity, detected with PA1-311, was found in the same fundic epithelial cells as for ER $\alpha$  with both nuclear and cytoplasmic distributions (Fig. 1d). In the antrum, ER $\beta$  nuclear immunoreactivity was detected in several cells located in the lower one-third of glands, with fainter staining in the superficial epithelium (Fig. 1e). Cytoplasmic immunoreactivity for ER $\beta$  was partially decreased by peptide pre-absorption ( $\sim$ 50%) of the antiserum, suggesting that a portion of the cytoplasmic staining may be non-specific.

#### Expression of ER $\alpha$ and ER $\beta$ proteins in enteric neurons

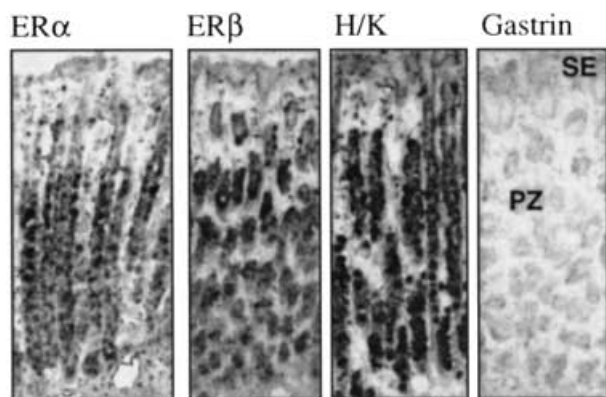
Enteric neurons in myenteric and submucosal plexi of fundus and antrum were immunopositive for both ER $\alpha$  and ER $\beta$  (Fig. 1c, f). Within each plexus, more neurons



**Figure 1** Immunolocalization of ER $\alpha$  and ER $\beta$  in the rat stomach. Representative photomicrographs are shown for ER $\alpha$  (a–c, g–i: ER-21) and ER $\beta$  (d–f: PA1–311) antisera. In the fundus, ER $\alpha$  (a) and ER $\beta$  (d) staining was detected in parietal cells (arrows), but not in chief cells (arrowheads). Cells were not positive for ER $\alpha$  (b) immunoreactivity in the antrum, ER $\beta$  (d) was detected in cells located at the base of antral glands (arrows), with fainter staining in the superficial epithelium. Myenteric neurons were stained for ER $\alpha$  (c) in the cytoplasm and for ER $\beta$  (f) in nuclei (arrows) and cytoplasm. ER $\alpha$ -positive nerve fibers (i, arrows) were detected near parietal cells at the base of glands. Serial sections of rat uterus show specific ER $\alpha$  (g) protein expression detected using the ER-21 antiserum in glandular and luminal epithelium. This staining was eliminated by peptide pre-absorption (h). LE, luminal endometrium; GE, glandular endometrium; PC, parietal cell; SE, superficial epithelium. Scale bars represent 40  $\mu$ m (b, e, g, h); 20  $\mu$ m (a, d); 10  $\mu$ m (c, f); 5  $\mu$ m (i).

were positive for ER $\beta$  than for ER $\alpha$ . In contrast to the predominantly nuclear localization in endometrium and fundic epithelium, ER $\alpha$  immunoreactivity using ER-21 antisera appeared as punctate foci in the cytoplasm of enteric neurons (Fig. 1c). Specific ER $\alpha$  immunoreactivity

could be traced within nerve fibers to submucosal plexi and near parietal cells at the base of gastric glands (Fig. 1i). This punctate staining pattern was detected with both ER-21 and ER-715 antisera and was completely eliminated by peptide pre-absorption. Nuclear and diffuse

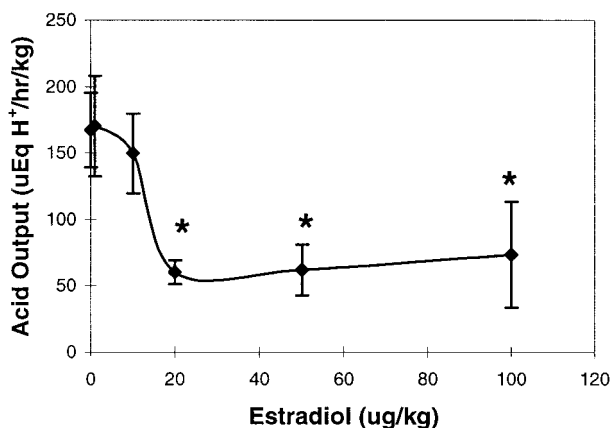


**Figure 2** *In situ* hybridization of ER $\alpha$  mRNA in rat fundus. Tissue sections were hybridized with digoxigenin-labeled riboprobes for ER $\alpha$ , ER $\beta$ , H/K-ATPase (H/K) and gastrin, as described in Methods. Color reactions were stopped after 15 min for H/K-ATPase and after 60 min for the other probes. ER $\alpha$  and ER $\beta$  mRNA were detected in the cytoplasm and peri-nuclear regions of fundic epithelium throughout the progenitor zone and lower glands. H/K-ATPase was detected in parietal cells. A gastrin riboprobe was used as a negative control and showed no staining in epithelium or lamina propria. SE, superficial epithelium; PZ, progenitor zone.

cytoplasmic staining for ER $\beta$  was detected in myenteric neurons using PA1-311 (Fig. 1f).

#### *In situ hybridization for ER $\alpha$ and ER $\beta$ mRNA expression in fundic mucosa*

Studies were also performed using *in situ* hybridization to verify cell-specific expression of ER $\alpha$  and ER $\beta$  mRNA in fundic glands. Identical cellular distribution of ER $\alpha$  and ER $\beta$  mRNA and protein in the rat uterus was observed (not shown). In the fundic epithelium, ER $\alpha$  and ER $\beta$  mRNA signals were detected in cells distributed throughout the progenitor zone and lower glands (Fig. 2). Superficial epithelial cells were not positive, in contrast to results for protein expression. The reason for this discrepancy is not known but may indicate cross-reactivity of the antisera with non-ER proteins expressed in the superficial epithelium. A similar pattern was observed using the



**Figure 3** Effects of 17 $\beta$ -estradiol treatment on pentagastrin-stimulated gastric acid output. Gastric contents were collected for 1 h after pentagastrin administration as described in Methods. Dose-dependent decreases in gastric acid output ( $\mu$ Eq H<sup>+</sup>/h per kg) were observed in Ovx-E2 rats. Values are means  $\pm$  S.D.;  $n=4-7$  rats/group). \* $P<0.05$  compared with Ovx.

H/K-ATPase riboprobe, indicating that parietal cells were the predominant cell type that expressed ER.

#### *17 $\beta$ -Estradiol inhibition of gastric acid output and gastrin concentrations*

The effects of chronic 17 $\beta$ -estradiol administration (1–100  $\mu$ g/kg) on gastric acid secretion were studied by means of a standard procedure using pyloric ligation. Treatment with 17 $\beta$ -estradiol significantly inhibited stimulated acid output at doses  $>10$   $\mu$ g/kg (Fig. 3). Gastric acid output was maximally inhibited to  $\sim 60\%$  of control values. Fasting serum gastrin concentrations were determined and a slight decrease in serum gastrin concentrations was observed after treatment with 100  $\mu$ g/kg ( $P=0.62$ ; Table 2). Similar body weights were maintained in Ovx and Ovx-E2 rats by pair-feeding (Table 2). Uterine weights increased  $\sim 2.4$ -fold in Ovx-E2 rats (Table 2).

**Table 2** Effects of 17 $\beta$ -estradiol on body weight, fasting serum gastrin levels, and uterine weights in Ovx rats. Values are means  $\pm$  S.D. (number of rats)

	Vehicle control	17 $\beta$ -Estradiol ( $\mu$ g/kg)	
		20	100
Body weight (g)	245 $\pm$ 12 (21)	231 $\pm$ 12 (6)	243 $\pm$ 14 (15)
Uterine weight (mg)	235 $\pm$ 22 (18)	525 $\pm$ 26 (8)*	600 $\pm$ 24 (10)*
Serum gastrin (pg/ml)	119 $\pm$ 35 (4)	124 $\pm$ 11 (6)	86 $\pm$ 30 (5)

Female rats were ovariectomized (Ovx) 5 days before receiving daily s.c. injections of 17 $\beta$ -estradiol or vehicle for 7 days. Final body weights and uterine weights are shown for pair-fed rats. Fasting serum samples were analyzed for gastrin concentrations. \* $P<0.05$  compared with Ovx control.

## Discussion

Gastric acid secretion by fundic parietal cells is regulated through a complex series of inter-related pathways mediated by paracrine and neuroendocrine mediators and neurotransmitters. Studies in both women and female rat models show that high serum estrogens inhibit gastric acid secretion (Omole 1972, Limlomwongse & Piyachaturawat 1982, Aguwa 1984, Adeniyi 1991, Sakaguchi *et al.* 1991, Girma *et al.* 1997). In the current study, we tested a range of physiological concentrations for 17 $\beta$ -estradiol replacement in Ovx rats and found dose-dependent inhibition up to ~60% of stimulated gastric acid output after 1 week of chronic 17 $\beta$ -estradiol therapy in pair-fed animals. The demonstration that both ER $\alpha$  and ER $\beta$  are expressed in gastric parietal cells suggests that estrogens may directly modulate the secretory activity of these cells. This proposal is supported by *in vitro* studies using isolated parietal cells from either female or male rats, wherein 17 $\beta$ -estradiol treatment (100 nM) decreased histamine-stimulated gastric acid production by ~20% (Girma *et al.* 1997). Our evidence for the presence of both ER $\alpha$  and ER $\beta$  in parietal cells suggests that ER heterodimers could mediate direct estrogenic effects on gastric acid secretion.

Others have reported that 17 $\beta$ -estradiol treatment in Ovx rats suppresses the characteristic ultrastructural changes associated with stimulated acid secretion in parietal cells (Piyachaturawat *et al.* 1983). However, they used extremely high doses of 17 $\beta$ -estradiol (800  $\mu$ g/kg per day for 7 days). These authors also reported that K<sup>+</sup>-ATPase activity was reduced in isolated gastric mucosa obtained from rats treated with this high dose of 17 $\beta$ -estradiol (Limlomwongse & Piyachaturawat 1982). In preliminary studies using our current model, we have been unable to detect differences in either basal or pentagastrin-stimulated parietal cell morphology by transmission electron microscopy between Ovx and Ovx-E2 rats (Campbell-Thompson & Eckard 1996). We also did not detect a difference by immunohistochemistry in gastric ER $\alpha$  and ER $\beta$  protein steady-state concentrations, although uterine concentrations of ER $\alpha$  and ER $\beta$  protein were regulated inversely with serum estrogen levels. Similarly, one study demonstrated that jejunal ER $\beta$  mRNA concentrations were not different between intact and ovariectomized females (Lim *et al.* 1999).

The role of neuroendocrine hormones in the control of gastric acid secretion has been studied for decades. Neuroendocrine hormones, such as gastrin, somatostatin and serotonin, are released in response to feeding and these hormones regulate secretory and motor functions in the stomach. By far the most studies have focused on roles for gastrin and histamine. Gastrin is synthesized in G-cells in antral glands and serum gastrin concentrations are regulated via complex feedback pathways involving other neuroendocrine hormones and neurotransmitters. Feedback stimulation of gastrin production, as reflected in

serum gastrin concentrations, is observed after chronic inhibition of gastric acid secretion. Although gastric acid secretion was significantly reduced in Ovx-E2 rats, fasting serum gastrin concentrations were not altered, in agreement with the findings of others (Lichtenberger *et al.* 1976). Other studies from our laboratory have shown that antral gastrin mRNA and protein concentrations are not altered by 17 $\beta$ -estradiol treatment (Campbell-Thompson *et al.* 1999). These results demonstrate that inhibition of stimulated gastric acid output by estradiol does not result from reduced gastrin production.

Gastric enteric neurons and their fibers were also immunoreactive for ER $\alpha$  and ER $\beta$  proteins. Specific ER $\alpha$  localization in enteric neurons was detected with two antisera directed to separate domains of ER $\alpha$ . Interestingly, ER $\alpha$  immunoreactivity was detected both in the cell body and within fibers. ER $\alpha$  localization has been described in other cell types in the central nervous system (reviewed in Levin 1999). The ER $\alpha$  antisera (ER-21) used in this study showed nerve fiber staining in the lateral habenula (Wagner *et al.* 1998) and ER $\beta$  immunoreactivity (PA1-310) in nerve fibers has been reported in regions of the hippocampus and lateral septum (Li *et al.* 1997). Localization of ER proteins in enteric neurons is provocative in the light of studies showing that estradiol administration delays gastric emptying in Ovx and male rats (Chen *et al.* 1995, Coskun *et al.* 1995). Our results suggest that ER $\alpha$  and ER $\beta$  could mediate estrogenic effects on gastric motility through the enteric nervous system.

In conclusion, the results detailed here show that ER $\alpha$  and ER $\beta$  proteins are expressed in both gastric epithelial and neuronal cells and administration of physiological concentrations of 17 $\beta$ -estradiol for at least 1 week results in decreased gastric acid output. Our findings indicate that fundic parietal cells and enteric neurons are potential targets for estrogens, and that direct regulation of these cells by estrogens may contribute to the modulation of gastric functions that have been recognized between the sexes and during estrous cycles and pregnancy.

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