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Submucosal microinfusion of endothelin and adrenaline mobilizes ECL-cell histamine in rat stomach, and causes mucosal damage: a microdialysis study

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1 Rat stomach ECL cells release histamine in response to gastrin. Submucosal microinfusion of endothelin or adrenaline, known to cause vasoconstriction and gastric lesions, mobilized striking amounts of histamine. While the histamine response to gastrin is sustainable for hours, that to endothelin and adrenaline was characteristically short-lasting (1–2 h).

2 The aims of this study were to identify the cellular source of histamine mobilized by endothelin and adrenaline, and examine the differences between the histamine-mobilizing effects of gastrin, and of endothelin and adrenaline.

3 Endothelin, adrenaline or gastrin were administered by submucosal microinfusion. Gastric histamine mobilization was monitored by microdialysis.

4 Local pretreatment with the H_1-receptor antagonist mepyramine and the H_2-receptor antagonist ranitidine did not prevent endothelin- or adrenaline-induced mucosal damage. Submucosal microinfusion of histamine did not cause damage. Acid blockade by ranitidine or omeprazole prevented the damage, suggesting that acid back diffusion contributes.

5 Gastrin raised histidine decarboxylase (HDC) activity close to the probe, without affecting the histamine concentration. Endothelin and adrenaline lowered histamine by 50–70%, without activating HDC. Histamine mobilization declined upon repeated administration. Endothelin reduced the number of histamine-immunoreactive ECL cells locally, and reduced the number of secretory vesicles. Thus, unlike gastrin, endothelin (and adrenaline) is capable of exhausting ECL-cell histamine.

6 Microinfusion of α-fluoromethylhistidine (known to deplete ECL cells but not mast cells of histamine) reduced the histamine-mobilizing effect of endothelin by 80%, while 1-week pretreatment with omeprazole enhanced it, supporting the involvement of ECL cells.

7 Somatostatin or the prostanoid misoprostol inhibited gastrin-, but not endothelin-stimulated histamine release, suggesting that endothelin and gastrin mobilize histamine *via* different mechanisms.

8 While gastrin effectively mobilized histamine from ECL cells in primary culture, endothelin had no effect, and adrenaline, a modest effect. Hence, the striking effects of endothelin and adrenaline on ECL cells *in situ* are probably indirect, possibly a consequence of ischemia.
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Keywords: ECL cells; microdialysis; histamine; gastrin; endothelin; adrenaline

Abbreviations: BSA, bovine serum albumin; CCK, cholecystokinin; α-FMH, α-fluoromethylhistidine; HDC, histidine decarboxylase; PACAP, pituitary adenylate cyclase-activating peptide; VIP, vasoactive intestinal peptide

Introduction

Histamine-producing ECL cells are numerous in the acid-producing part of the rat stomach. They release histamine and chromogranin A-derived peptides, such as pancreastatin, in response to circulating gastrin (Chen *et al*., 1994; Håkanson *et al*., 1995; Kitano *et al*., 2000). The histamine that is mobilized stimulates acid secretion from adjacent parietal cells (Sandvik *et al*., 1987; Andersson *et al*., 1996; Lindström *et al*., 2001a). Studies of isolated ECL cells in primary culture have revealed that several neuromessenger compounds, notably pituitary adenylate cyclase-activating peptide (PACAP), vasoactive intestinal peptide (VIP) and adrenaline/noradrenaline, stimulate ECL-cell histamine mobilization *via* a direct action (Lindström *et al*., 1997; Lindström & Håkanson, 2001), and that somatostatin and prostaglandin E_2 are powerful inhibitors of the exocytotic release of histamine from the ECL cells (Lindström *et al*., 1997; Lindström & Håkanson, 2001). Microdialysis studies confirmed the histamine-mobilizing effect of gastrin/cholecystokinin (CCK), PACAP/VIP and adrenaline/noradrenaline (Norlén *et al*., 2001). While gastrin and CCK induced sustained, long-lasting histamine secretion

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(approximately five-fold increase over basal for several hours), VIP and PACAP induced transient histamine mobilization with a peak response of the same magnitude as the plateau response to gastrin/CCK. Interestingly, endothelin and adrenaline induced a spectacular but short-lasting (1–2 h) release of histamine; the peak response was much greater than that induced by any other stimulus. In addition, we found the two agents to cause severe local damage to the mucosa (along the probe). Both agents are powerful vasoconstrictors known to generate local tissue damage in the gastric mucosa (Whittle & Esplugues, 1988; Tepperman & Whittle, 1991; see also Whittle, 1993). Conceivably, their ability to mobilize striking amounts of gastric histamine (Norlén *et al*., 2001) may contribute to the mucosal damage by stimulating acid secretion locally, thereby promoting acid back diffusion.

The purpose of the present study is to identify the source of gastric histamine mobilized by endothelin and adrenaline, to explain why the histamine response to endothelin and adrenaline is short-lasting, and to establish whether the effects of endothelin and adrenaline on the mucosal histamine stores are direct or indirect. Also, we address the question as to whether the spectacular local release of histamine contributes to the mucosal injury or *vice versa*.

Methods

Chemicals

Adrenaline hydrochloride and α-fluoromethylhistidine (α-FMH) were purchased from Sigma (St Louis, MO, U.S.A.). Endothelin-1 was from Bachem (Bubendorf, Switzerland). Human Leu^{15}-gastrin-17, rat gastrin-17 and somatostatin-14 were from Research Plus (South Plainfield, NJ, U.S.A.). Histamine dihydrochloride was from ICN Biomedicals (Aurora, OH, U.S.A.). The prostanoid misoprostol was a kind gift from Searle (Skokie, IL, U.S.A.). All agents were dissolved in 0.9% saline (unless otherwise stated). α-FMH, an irreversible inhibitor of HDC (Kollonitsch *et al*., 1978; Andersson *et al*., 1990; 1992), was administered continuously *via* osmotic minipumps (see below) for almost 24 h ($3\,mg\,kg^{-1}\,h^{-1}$) (Andersson *et al*., 1992). The proton pump inhibitor omeprazole was obtained from AstraZeneca, Mölndal, Sweden, and dissolved in 0.25% Methocel (Dow Corning, Midland, MI, U.S.A.). It was given daily by oral gavage between 0700 and 1000 hours, at a dose of $400\,\mu mol\,kg^{-1}$ (Larsson *et al*., 1986). Also, the histamine (H_2) receptor antagonist ranitidine hydrochloride was a gift from AstraZeneca. The H_1 receptor antagonist mepyramine maleate was obtained from Tocris (Ellisville, MO, U.S.A.). Bovine serum albumin (BSA) was from ICN Biomedicals (Aurora, OH, U.S.A.). Antiserum (rabbit) to histamine (code no. 8431) was generated in our own laboratory (Håkanson *et al*., 1986).

Pronase (lot 13960228-57) from Boehringer-Mannheim (Mannheim, Germany) was used for dispersion of cells from the oxyntic mucosa. The wells of the tissue culture plates were coated with Matrigel® (Collaborative Biomedical Products, Bedford, MA, U.S.A.). The culture medium was Dulbecco's modified Eagle's medium (DMEM)–Ham's F12 (Sigma). Streptomycin, penicillin and amphotericin B (Boehringer-Mannheim) were added to the medium together with hydrocortisone (Sigma), insulin–transferrin–sodium selenite (Becton Dickinson, Bedford, MA, U.S.A.) and fetal calf serum (HyClone, Logan, UT, U.S.A.).

Animals

Female (if not otherwise stated) Sprague–Dawley rats (250–300 g) were kept at a 12-h light/12-h dark cycle in plastic cages (2–3 in each cage), with free access to standard rat food pellets (Lactamin, Vadstena, Sweden) and tap water. Rats to be fasted were deprived of food (but not water) and housed in individual cages with wire mesh bottoms for at least 24 h. Starting at least 1 week prior to the experiments, all rats were familiarized with Bollman-type restraining cages for 1–2 h daily. They were kept in such cages throughout the microdialysis experiments. Osmotic minipumps (Alzet 2001 D, Alza, Palo Alto, CA, U.S.A.) for α-FMH administration were implanted subcutaneously in the neck under chloral hydrate anesthesia ($300\,mg\,kg^{-1}$ intraperitoneally). Before implantation, the minipumps were activated in 0.9% NaCl at 37°C for 4 h. The studies were approved by the local Animal Welfare Committee, Lund.

Studies of ECL cells in situ*: microdialysis of the gastric submucosa*

Implantation of the microdialysis probe and sampling of microdialysate Surgery was performed under chloral hydrate anesthesia, using clean, but not sterile, instruments. No antibiotics were used. A flexible microdialysis probe (MAB 3.8.10, AgnTho's AB, length 10 mm, outer diameter 0.57 mm, 35 kDa cutoff) was used. The abdomen was opened by a midline incision. The probe was placed in the submucosal layer of the dorsal aspect of the acid-producing part of the stomach, and kept in place with sutures. The inlet and outlet tubes were passed through the abdominal opening, and tunneled under the skin to a point at the nape of the neck, where they were secured with sutures (Kitano *et al*., 2000; Ericsson *et al*., 2003). The rats were fed freely or fasted before start of the microdialysis, which was performed 3 days after the implantation of the probe. All rats were awake during the experiments since anesthesia has been shown to suppress the mobilization of histamine from the ECL cells (Norlén *et al*., 2000). The inlet tube of the microdialysis probe was connected to a microinfusion pump (Model 361, Sage instrument, ATI Orion, Boston, U.S.A.), and the outlet tube was allowed to drain into 300 μl polyethylene vials. Perfusion of the microdialysis probes with saline ($1.2\,\mu l\,min^{-1}$) started at 0700 hours. Collection of microdialysate commenced after a 2-h equilibration period. First, samples were collected during 2 h of basal histamine secretion, after which perfusion with the various agents (see below) in saline was started (at time zero). Control rats received saline only. Samples were collected every 20 min during the first hour of stimulation, and then every hour. Infusions lasted for 3–4 h. Each rat was used only once (if not otherwise stated), and was killed by exsanguination (under chloral hydrate anesthesia). The position of the probe in the submucosa was verified at autopsy. The stomachs were rinsed with ice-cold saline, and the mucosa was inspected for injury and photographed. Samples of tissue surrounding the probe were collected for chemical analysis, histological examination and electron microscopy (for details see below).

Study design (exploring mucosal damage and histamine mobilization) Rats were fasted for at least 24 h unless otherwise stated.

1. Endothelin-1 and adrenaline were administered *via* microdialysis probes for 3 h, in doses specified in Results, to both fasted and fed rats. For comparison, human-17 gastrin was administered locally in various doses for 3 h to fasted rats. Each rat received only one dose.
2. Histamine (10 mmol l^{-1}) was administered *via* the microdialysis probe for 3 h, to assess its ability to induce gastric mucosal damage.
3. Endothelin (10 μmol l^{-1}) was administered *via* the microdialysis probe, after local pretreatment with 0.1 mmol l^{-1} mepyramine and 0.1 mmol l^{-1} ranitidine. The two drugs were coadministered for 2 h before administration of endothelin, in order to assess their ability to prevent the mucosal damage. Infusion of the two drugs was maintained throughout the experiment. In another experiment, ranitidine alone was given by subcutanous injection (40 mg kg^{-1}) 30 min before endothelin. This dose is known to produce maximum inhibition of acid secretion for several hours (Scarpignato *et al*., 1986).
4. Endothelin (10 μmol l^{-1}) and adrenaline (1 mmol l^{-1}) were infused twice with 2, 24, or 48 h intervals. The rats were fasted for 24–48 h before the first administration, and were allowed free access to food 4–5 h later.
5. Endothelin-induced mobilization of gastric histamine was monitored in freely fed rats pretreated with α-FMH (3 mg kg^{-1} h^{-1}) (continuous infusion from osmotic minipumps) or 0.9% saline (controls) before (20 h) and during (3 h) local administration of endothelin (10 μmol l^{-1}).
6. Freely fed rats received omeprazole or vehicle daily for 1 week prior to local administration of gastrin (0.1 μmol l^{-1}), endothelin (10 μmol l^{-1}) or adrenaline (1 mmol l^{-1}) for 3 h. The experiments were carried out 2 h after the last omeprazole dose.
7. Gastrin (3 nmol kg^{-1} h^{-1}) was given by intravenous infusion (dissolved in 0.9% NaCl, 1% BSA) for 4 h. After 2 h, misoprostol (0.1 mmol l^{-1}) or somatostatin (0.1 mmol l^{-1}) was administered *via* microdialysis probes. The doses of misoprostol and somatostatin have previously been shown to be near-maximally effective for inhibiting gastrin-evoked histamine mobilization (Norlén *et al*., 2001). Other rats received endothelin (10 μmol l^{-1}) together with misoprostol or somatostatin for 3 h *via* microdialysis probes.

Light and electron microscopy Stomachs were removed, opened along the major curvature, and rinsed in 0.9% saline. Small specimens (2 × 4 mm) were taken along and around the probe for light microscopy, fixed in 4% 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (Sigma) overnight at 4°C, and rinsed with 20% sucrose in 0.1 mol l^{-1} phosphate buffer (pH 7.4). Carbodiimide-fixed specimens were used for histamine immunostaining (Panula *et al*., 1988). The specimens were frozen at −80°C, and sections were cut in a cryostat (Bright, Huntington, U.K.) at 10 μm thickness. The sections had a transverse orientation, and were thawed onto gelatin-coated slides. The sections were incubated with the histamine antiserum (final dilution 1 : 1000) overnight at 4°C (Håkanson *et al*., 1986). After washing in buffer, the sections were incubated at room temperature for 1 h with fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (diluted 1 : 40) (Dako, Glostrup, Denmark). After rinsing, the sections were mounted in buffer : glycerol (1 : 4), and examined in a fluorescence microscope.

Minute mucosal specimens (0.5 mm cubes) were collected close to the probes for electron microscopy, and immersed in 2% glutaraldehyde in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.4) at room temperature overnight. The specimens were postfixed for 1 h in 2% osmium tetroxide before being dehydrated in graded ethanol solutions, and embedded in epoxy resin (Ladd, Wien, Austria). Sections were cut at 60 nm on an ultramicrotome (Ultracut E; Reichert-Jung, Burlington, VT, U.S.A.), contrasted with uranyl acetate and lead citrate, and examined in a JEM-100 CX electron microscope (Jeol, Tokyo, Japan). The ECL cells were recognized by their characteristic ultrastructure (Zhao *et al*., 1999): granules are membrane-enclosed organelles with a diameter of 25–200 nm, displaying an electron-dense core, the diameter of the dense core representing more than 50% of the diameter of the entire organelle. Vesicles are membrane-enclosed organelles, often electron-lucent, sometimes possessing a small eccentrically located dense core, smaller than 50% of the diameter of the vesicle. The vesicles can be classified into two subpopulations, based on profile size and the presence or absence of a dense core: (1) secretory vesicles (occasional dense core) with a diameter of 125–500 nm, and (2) microvesicles (no dense core) with a diameter of 25–125 nm (Zhao *et al*., 1999). The ECL cells (nucleated cell profiles only) were photographed at × 6000 magnification, and the prints were enlarged to × 20,000. The prints were subjected to point-counting methods to determine the cell profile area and the numbers and volume densities of granules, secretory vesicles and microvesicles (Zhao *et al*., 1999).

Studies of ECL cells in primary culture

Isolation of ECL cells ECL cells were isolated as described earlier (Lindström *et al*., 1997) with a few modifications. In each experiment, oxyntic mucosal cells from three male Sprague–Dawley rats (300–350 g) were dispersed using pronase digestion (0.9 mg ml^{-1}, Boehringer Mannheim, Mannheim, Germany) and calcium chelation (1 mmol l^{-1} EDTA). The ECL cells were enriched by repeated counterflow elutriation, using first a standard chamber and then a Sanderson chamber (Beckman, Palo Alto, CA, U.S.A.). The enriched cells from the standard chamber were collected at 25 ml min^{-1}, and at a speed of 2000 r.p.m. (380–560 g). They were purified further in a Sanderson chamber and collected at 18 ml min^{-1} and 2000 r.p.m. This fraction consisted of 1–2 million cells, about 80% being ECL cells. The purity of the cell preparation was determined by immunocytochemistry of smears from this fraction, using the antihistamine antiserum (1 : 1000) and FITC-conjugated secondary antibodies (Håkanson *et al*., 1986).

Primary cell culture The ECL cells were cultured in 96-well plates precoated with Matrigel® (diluted 1 : 10 in DMEM/Ham's F12, 20,000 cells $well^{-1}$, 100 μl volume) for secretion experiments. All cell cultures were incubated in a humid atmosphere with 5% CO_2/95% air at 37°C for 48 h, until the start of the experiments. The culture medium consisted of

DMEM–Ham's F12 (1 : 1) supplemented with 2% fetal calf serum, 2 mmol l^{-1} glutamine, 100 IU ml^{-1} penicillin, 100 $\mu g\,ml^{-1}$ streptomycin, 250 ng ml^{-1} amphotericin B, 10 $\mu g\,ml^{-1}$ insulin, 5.5 $\mu g\,ml^{-1}$ transferrin, 5 ng ml^{-1} selenious acid, 0.5 $\mu g\,ml^{-1}$ BSA, 15 mmol l^{-1} HEPES, 10 μmol l^{-1} pyridoxal-5-phosphate, 10 nmol l^{-1} hydrocortisone and 100 pmol l^{-1} gastrin-17.

Secretion experiments The cells were washed with serum-free and gastrin-free culture medium. After equilibration for about 2 h, the medium was aspirated and replaced with secretion medium (μmol l^{-1}): 150 NaCl, 5 KCl, 2 $CaCl_2$, 10 HEPES at pH 7.0, containing the various stimulatory compounds at concentrations varying from 1 pmol l^{-1} to 0.1 mmol l^{-1}. The histamine-mobilizing effects of gastrin, endothelin-1 and adrenaline were tested. Incubation lasted for 30 min at 37°C, and was interrupted by centrifuging the plates at $220 \times g$ for 1 min. The supernatants were collected and stored at −20°C until the measurement of histamine.

Chemical analysis

Determination of histamine in microdialysate and culture medium Histamine was measured in 10 μl microdialysate (diluted 1 : 10) and in 10 μl culture medium, using a commercially available radioimmunoassay kit (Immunotech, Marseille, France). The histamine concentration was expressed as nmol l^{-1} or pmol $well^{-1}$. All agents used in the infusion experiments were tested with respect to their interference with the radioimmunoassay of histamine. Only gastrin was found to interfere, precluding the use of doses higher than 0.1 mmol l^{-1} for local administration.

Determination of HDC activity and histamine concentration in tissue slices Tissue samples were collected from the stomach wall along the microdialysis probe (2×12 mm). The specimens were weighed and homogenized in ice-cold 0.1 mol l^{-1} sodium phosphate buffer, pH 7.4, to a concentration of 100 mg ml^{-1}. Aliquots (80 μl) of the oxyntic mucosal homogenates were incubated with L-[l-^{14}C]histidine (specific activity 50 mCi $mmol^{-1}$), 0.5 mmol l^{-1} L-histidine and 0.01 mmol l^{-1} pyridoxal-5-phosphate, in a total volume of 160 μl at 37°C for 1 h, as described previously (Larsson *et al*., 1986). HDC activity was expressed as pmol $^{14}CO_2\,mg^{-1}\,h^{-1}$. The homogenate was diluted 1 : 10 in 3% trichloroacetic acid, and placed in boiling water for 5 min. The precipitated material was spun down, and the clear supernatant was diluted 1 : 50 with redistilled water. Histamine was determined spectrophotofluorometrically, and expressed as $\mu g\,g^{-1}$ (Håkanson & Rönnberg, 1974; Rönnberg & Håkanson, 1984).

Serum gastrin determination Blood was drawn from the tail, and the serum gastrin concentration was determined as described previously (Stadil & Rehfeld, 1973). A volume of 20 μl serum was used in the assay. Rat gastrin-17 was used as standard. The concentration was expressed as pmol equivalents of rat gastrin-17 per liter.

Statistical analysis

Data are presented as mean values ± s.e.m. Differences were statistically analyzed by one-way analysis of variance (ANOVA), followed by the Dunnett multiple comparison test. Stimulatory effects are presented as peak values as well as integrated responses, that is, the rise in microdialysate histamine concentration above basal levels during the period of stimulation (usually 3 h). Dose–response curves were drawn by the use of a computer software-aided curve-fitter. EC_{50} values (i.e. the concentrations of agents that induced half-maximal stimulating effect) were not calculated, since they are affected by a variety of factors besides the kinetics of ligand–receptor interaction (for instance, rate of diffusion over the dialysis membrane, penetration to target, degradation in tissue), and consequently are not meaningful.

Results

Gastric mucosal damage in response to endothelin and adrenaline

Local submucosal administration of endothelin and adrenaline induced mucosal lesions along the probe (Figure 1a, b). Endothelin induced hemorrhagic damage at a concentration of 1 μmol l^{-1} in both fasted and fed rats; the corresponding adrenaline concentration was 1 mmol l^{-1}. Ulcers, surrounded by petechiae, were seen along the probe at endothelin doses of 10 μmol l^{-1}. Even at the highest concentration tested (100 μmol l^{-1}), gastrin had no such effect.

The possibility that histamine contributes to the lesions was evaluated by examining the effect of exogenous histamine *per se*. Also, the ability of histamine H_1 and H_2 receptor blockade

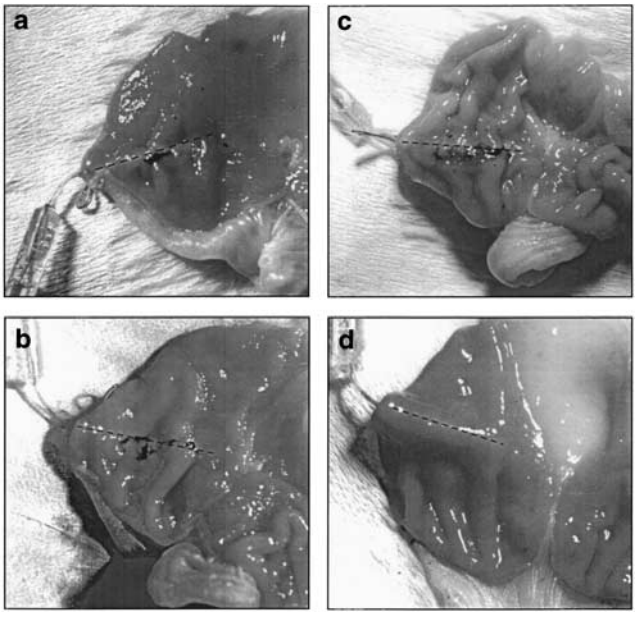

Figure 1 Gastric mucosal damage caused by microinfusion for 3 h of adrenaline (1 mmol l^{-1}) (a) or endothelin (10 μmol l^{-1}) (b) into the submucosa of the acid-producing part of the rat stomach *via* microdialysis probes. Local administration of mepyramine and ranitidine, 1 h prior to and during the local administration of adrenaline failed to prevent the damage (c). Daily dosing with omeprazole, (400 μmol kg^{-1}) for 1 week prevented the gastric mucosal damage caused by local administration of endothelin (d). The black dotted line shows the position of the microdialysis probe in the submucosa.

to prevent the mucosal damage was tested. Local administration of 10 mmol l^{-1} histamine failed to induce gastric mucosal damage (not shown in the figure), and local pretreatment with a mixture of the H_1 receptor antagonist mepyramine and the H_2 receptor antagonist ranitidine (administered *via* the microdialysis probe at a concentration of 0.1 mol l^{-1}) failed to prevent the mucosal damage caused by endothelin or adrenaline (Figure 1c).

Subcutaneous injection of ranitidine (40 mg kg^{-1}), a dose known to abolish acid secretion, prevented the mucosal damage induced by local endothelin (not shown). Also, acid blockade by omeprazole prevented endothelin from damaging the mucosa (Figure 1d).

Mobilization of gastric histamine in response to endothelin and adrenaline

Local submucosal administration (*via* microdialysis probes) of gastrin (0.1 mmol l^{-1}, a near-maximally effective dose) to fasted rats during a 3 h period produced a sustained five-fold increase in the microdialysate histamine concentration (Figure 2a). The histamine-mobilizing effect was characteristically dose-dependent (Figure 3a, b). The local histamine concentration in the tissue along the probe was not much affected by 3 h of gastrin infusion, whereas the local HDC activity was elevated almost 10-fold (Figure 4).

Local submucosal administration of a high dose of endothelin (30 μmol l^{-1}) produced a striking but short-lasting mobilization of histamine (Figure 2b). The response was dose-dependent (Figure 3c, d). The peak response to a near-maximal dose of endothelin (after 40–60 min) was >30 times the basal microdialysate histamine concentration, and 10 times greater than the response to gastrin. The integrated response (3-h histamine mobilization) was >5 times higher than the response to gastrin (Figure 3). The tissue histamine concentration was reduced by almost 50% compared to control. The HDC activity was not affected (Figure 4).

Local submucosal administration of adrenaline at a near-maximally effective dose (1 mmol l^{-1}) mobilized large amounts of histamine (Figure 2c). The response was characteristically short-lasting – it peaked after 20–40 min. The decline of the response was apparent 1 h after the start of infusion, and the microdialysate histamine concentration was almost back to basal after 3 h. The dose–response relationship is illustrated in Figure 3. The peak response to a maximal dose of adrenaline was >20 times the basal histamine concentration, while the integrated response was only slightly higher than the response to gastrin (Figure 3). The tissue histamine concentration was greatly reduced (−70%), while the HDC activity seemed unaffected (Figure 4).

Repeating the infusion of 10 μmol l^{-1} endothelin or 1 mmol l^{-1} adrenaline 2 h later induced a histamine response that was only a fraction of the first response (Figure 5). With longer time intervals between the challenges (longest interval 48 h), the response was found to recover. The response to adrenaline had recovered to reach approximately 50% of the original response after 24 h.

Immunocytochemical and electron-microscopic analysis of the effect of endothelin on the ECL cells In an attempt to identify the source of histamine released in response to endothelin, we examined its effect on the histamine-storing cells in the rat stomach by histamine immunocytochemistry and electron microscopy. Histamine-immunoreactive ECL cells were notably few in the mucosa adjacent to the microdialysis probe, and the immunofluorescence intensity of those cells that remained visible was greatly reduced compared to ECL cells on the contralateral side of the stomach (Figure 6). The mast cells were not affected (not shown). Electron microscopic analysis of the mucosa close to the probe revealed that the ultrastructure of endothelin-exposed ECL cells differed from that of ECL cells on the contralateral side of the stomach in that they displayed a greatly reduced number of secretory vesicles (−70%) (Table 1, Figure 7); the numbers of granules and microvesicles were unchanged.

Gastric histamine response to endothelin and adrenaline after treatment with α-FMH or omeprazole In order to pursue the problem of the cellular source of histamine that is mobilized by endothelin and adrenaline, we made use of two drugs with known effects on ECL cells, namely the HDC inhibitor α-FMH and omeprazole.

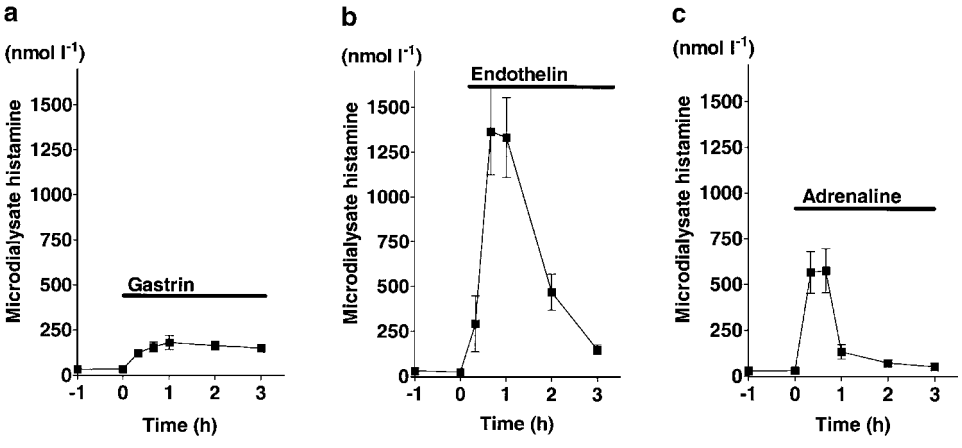

Figure 2 Time course of gastric histamine mobilization in response to gastrin (0.1 mmol l^{-1} (a)), endothelin (30 μmol l^{-1} (b)) and adrenaline (1 mmol l^{-1} (c)). Stimulation started at time 0 and lasted for 3 h (indicated by the horizontal line). The compounds were administered *via* microdialysis probes in the gastric submucosa. Mean ± s.e.m., $n = 6–9$.

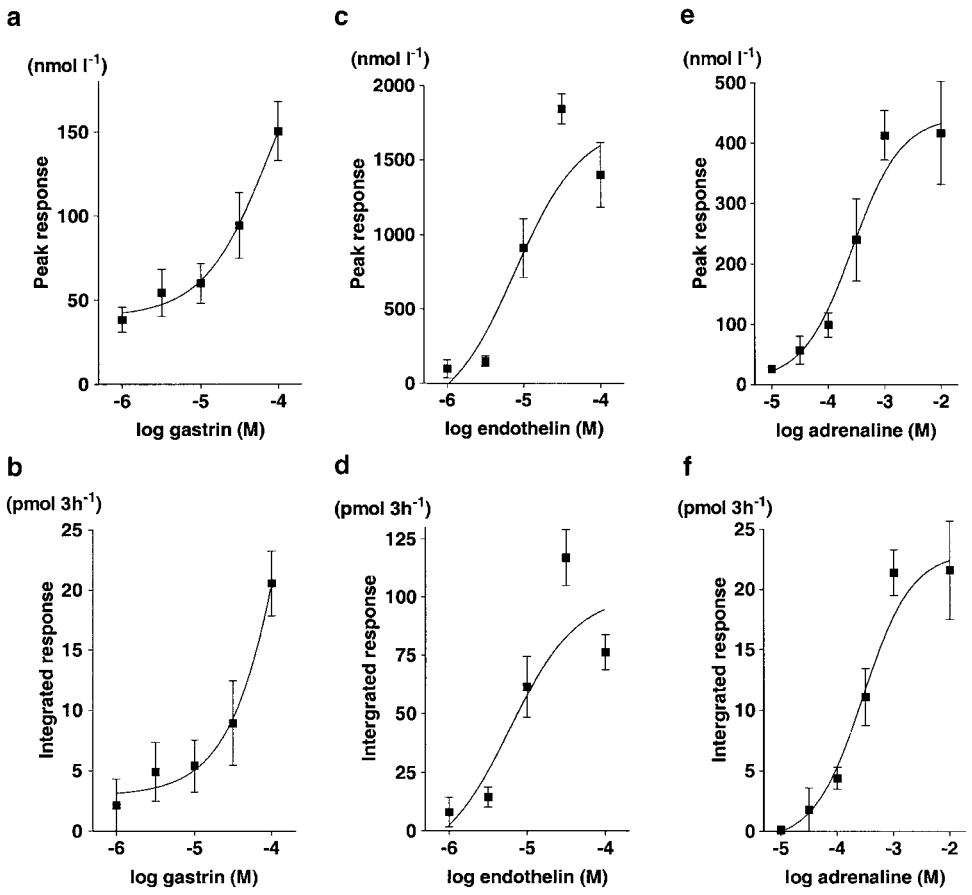

Figure 3 Concentration–response curves for the histamine response to gastrin (a, b), endothelin (c, d) and adrenaline (e, f). The results are from experiments such as those shown in Figure 2, and are expressed as either peak response (a, c, e) or integrated response (b, d, f) (see Methods). The compounds were administered *via* microdialysis probes in the gastric submucosa. The results shown include data from an earlier study (Norlén *et al*., 2001). Mean ± s.e.m., $n = 6–9$.

Continuous subcutaneous infusion of α-FMH reduced the concentration of histamine in the oxyntic mucosa of freely fed rats by 80% (from 54 ± 5 to $11 \pm 2\,\mu g\,g^{-1}$), while the HDC activity (as expected) was almost nonmeasurable (not shown). The basal microdialysate histamine concentration was reduced by 52% ($P < 0.001$), and the endothelin-induced histamine release by 75% ($P < 0.001$) (Figure 8).

Treatment with omeprazole for 1 week before (and during) microdialysis raised the serum gastrin concentration (from 40 ± 5 to $206 \pm 22\,pmol\,l^{-1}$) and the basal microdialysate histamine concentration (from 50 ± 6 to $340 \pm 49\,nmol\,l^{-1}$) (see also Konagaya *et al*., 2001). Under these circumstances, local administration of $0.1\,mmol\,l^{-1}$ gastrin failed to increase the microdialysate histamine concentration (probably because of the prevailing hypergastrinemia). Nonetheless, local administration of endothelin ($10\,\mu mol\,l^{-1}$) or adrenaline ($1\,mmol\,l^{-1}$) caused a quite spectacular release of histamine, in fact twice that of rats not receiving omeprazole (Figure 9). Endothelin and adrenaline lowered the local HDC activity of the omeprazole-treated rats from 184.5 ± 16.4 to 81.6 ± 9.3 and $106.5 \pm 11.6\,pmol\ ^{14}CO_2\,mg^{-1}\,h^{-1}$, respectively ($P < 0.001$) (not shown in the figure).

Effect of somatostatin and misoprostol on gastrin- and endothelin-induced histamine mobilization The histamine-mobilizing effect of intravenous infusion of gastrin was greatly suppressed by somatostatin and misoprostol, whereas the histamine-mobilizing effect of submucosal administration of endothelin was unaffected (Figure 10).

Effect of endothelin and adrenaline on the mobilization of histamine from ECL cells in primary culture

Studies of isolated ECL cells in primary culture showed that gastrin stimulated histamine mobilization four-fold, while endothelin had no effect. Adrenaline had a modest stimulatory effect compared to gastrin (Figure 11).

Discussion

The present study is concerned with the effects of local submucosal administration of endothelin and adrenaline (*via* microdialysis probes) to the rat stomach. The effects include local gastric mucosal damage (along the probe) and a massive but short-lasting mobilization of gastric mucosal histamine. One of the aims of the study was to explain the lesions and to examine the possibility of a link between the mucosal damage and the histamine release. The other aim was to identify the

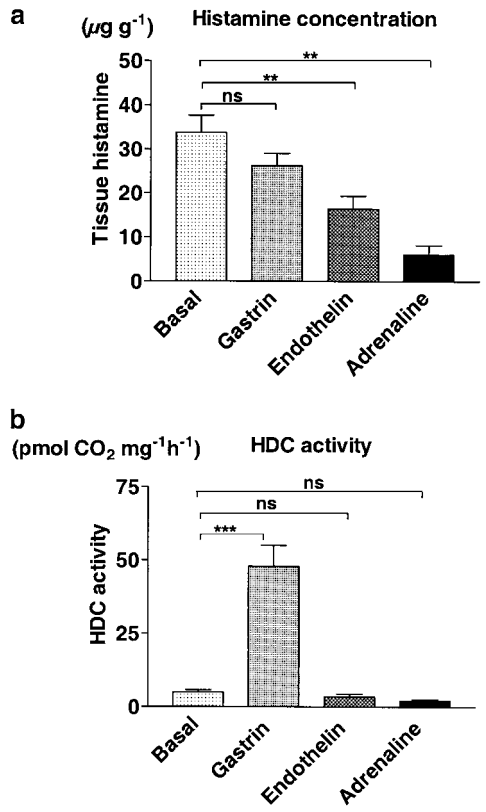

Figure 4 Histamine concentration (a) and HDC activity (b) in small whole-wall specimens collected along the microdialysis probes in the stomachs of fasted rats challenged for 3 h with 0.9% NaCl (basal), gastrin (0.1 mmol l^{-1}), endothelin (10 μmol l^{-1}) or adrenaline (1 mmol l^{-1}) administered *via* the probes. Mean ± s.e.m., $n = 6-8$. (**) $P<0.01$, (***) $P<0.005$.

cellular source of the mobilized histamine and to explore the mechanisms behind the histamine release.

Mechanisms behind the gastric mucosal injury

Unlike gastrin, both endothelin and adrenaline induced gastric mucosal damage. This occurred independently of histamine because of the following reasons: (1) local administration of histamine itself into the gastric submucosa failed to cause mucosal damage. Histamine was administered in concentrations that can be expected to exceed the histamine concentrations achieved upon local infusion of endothelin and adrenaline. (2) Local administration of histamine H_1- and H_2-receptor antagonists failed to prevent the endothelin-induced mucosal damage. Both endothelin and adrenaline are powerful vasoconstrictors, and it seems conceivable that the mucosal damage was caused by ischemia (see Wallace *et al*., 1989; Michida *et al*., 1994; Watanabe *et al*., 2000). α-Adrenergic blockade (prazosine) prevented the adrenaline-evoked mucosal damage (Norlén, unpublished observation), in support of the view that vasoconstriction (ischemia?) is the cause of the injury. It is well known that subjecting rats to gastric ischemia will induce severe mucosal damage, and that the effect of the ischemia can be prevented by acid blockade (Kitano *et al*., 1997). As could be expected therefore, inhibition of acid secretion by systemic administration of an H_2-receptor antagonist (ranitidine) or a proton pump inhibitor (omeprazole) prevented the endothelin-evoked mucosal injury (without preventing the mobilization of histamine). The most plausible explanation of the mucosal injury is that endothelin and adrenaline cause ischemia that undermines the resistance of the mucosal barrier to the tissue-damaging effect of acid back diffusion. The significance of the acid is illustrated by the fact that neither endothelin nor adrenaline induced mucosal damage upon blockade of acid secretion. The ability of endothelin and adrenaline to mobilize histamine does not contribute to the mucosal injury.

Hence, endothelin and adrenaline probably damage the mucosa through local ischemia, thereby disrupting the protective barrier of the mucosa and exposing it to tissue-damaging acid back diffusion. Without acid, there is no mucosal damage.

Mechanisms behind the release of gastric histamine

Cellular source of mobilized histamine ECL cells are numerous, while mast cells are few in the oxyntic mucosa of the rat stomach (Håkanson *et al*., 1986). The latter cells are found mainly at the mucosal surface and in the submucosa; they harbor about 20% of the histamine in the rat stomach (Andersson *et al*., 1992). The mobilization of gastric histamine in response to endothelin and adrenaline was associated with a reduced histamine concentration in the mucosa close to the probe. Light microscopy showed that endothelin reduced the intensity of histamine immunostaining in ECL cells in the vicinity of the probe. Mast cells seemed not to be affected. Electron microscopy, moreover, revealed a reduced number of histamine-storing organelles, that is, secretory vesicles, in the ECL cells.

α-FMH is known to deplete the ECL cells of histamine (by irreversible inhibition of HDC), while leaving mast cells unperturbed (Andersson *et al*., 1992). The results of the present study confirm that α-FMH reduces the mucosal histamine concentration by depleting the ECL cells of their histamine. Not unexpectedly, α-FMH greatly reduced the amount of histamine that could be mobilized by endothelin, in line with the view that ECL cells are the prime source of mobilized histamine. In fact, however, some histamine could be mobilized by endothelin even after α-FMH pretreatment. It is an open question whether it comes from mast cells or represents mobilization of residual histamine in the ECL cells.

The proton pump inhibitor omeprazole inhibits acid secretion, which results in hypergastrinemia (Larsson *et al*., 1986). Omeprazole-induced hypergastrinemia accelerates ECL-cell histamine formation and secretion (Konagaya *et al*., 2001), and the amount of histamine that can be mobilized will increase accordingly. Indeed, endothelin and adrenaline mobilized much larger amounts of histamine in omeprazole-treated rats than in control rats. This might be expected if the ECL cells are the prime source of histamine. Clearly, endothelin does not depend on acid back diffusion to mobilize histamine from the ECL cells.

The results of histamine immunocytochemistry and the analysis of ECL-cell ultrastructure together with the results of experiments using α-FMH and omeprazole support the conclusion that endothelin and adrenaline mobilize histamine from ECL cells.

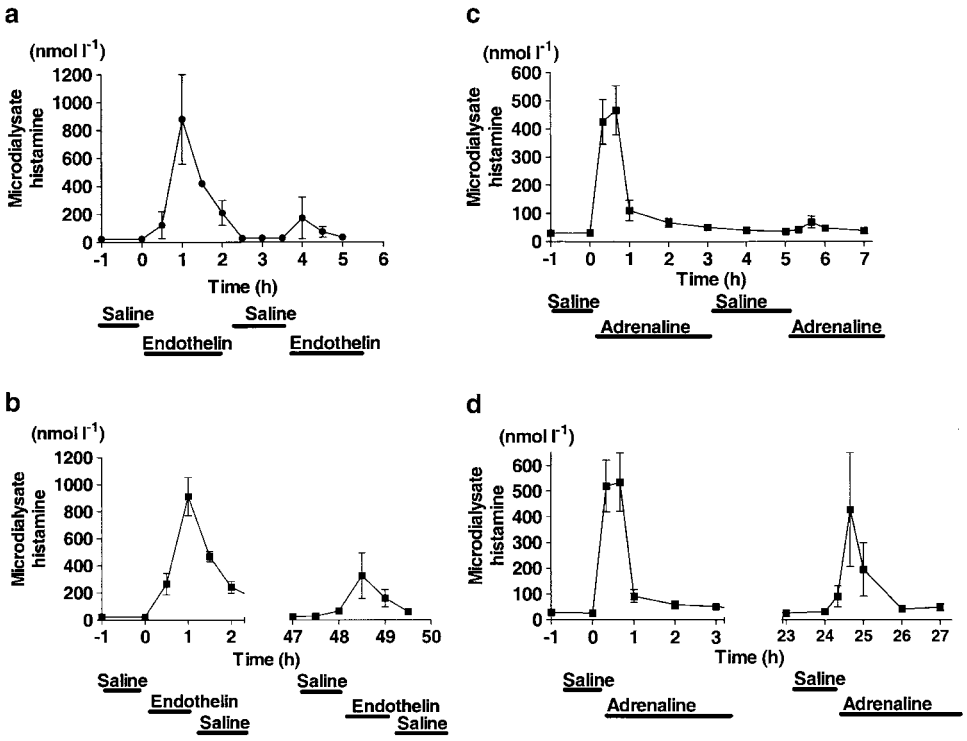

Figure 5 Microdialysate histamine concentration in response to repeated submucosal microinfusion of endothelin (10 μmol l^{-1}) 3 (a) or 48 (b) h after the first administration. In another series of experiments, adrenaline (1 mmol l^{-1}) was given twice, 5 (c) or 24 (d) h after the first administration. After 24 h, the response was greater than that after 5 h ($P<0.05$). The agents were administered *via* the microdialysis probes as indicated. Mean $\pm$ s.e.m., $n=4-5$.

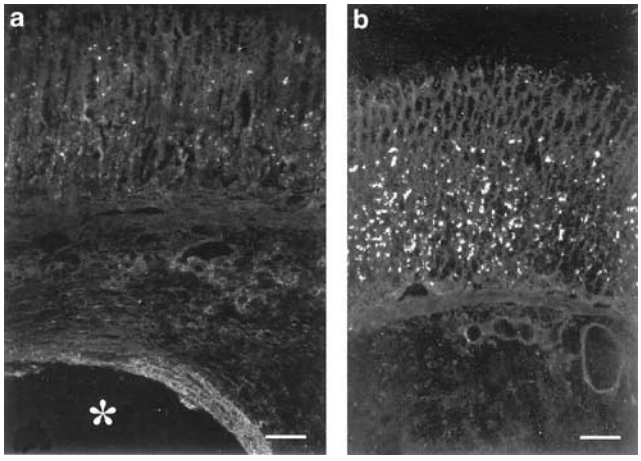

Figure 6 Histamine immunofluorescence in ECL cells in the gastric mucosa of rats exposed to endothelin (10 μmol l^{-1} for 3 h) *via* a microdialysis probe. Tissue specimens were collected close to the probe (a), and from the contralateral side of the stomach (b). Immunofluorescent ECL cells were greatly reduced in number near the probe compared to the contralateral side of the stomach. The probe is indicated by asterisk. Bar = 100 μm.

Mechanisms that determine the magnitude and duration of the histamine response The pattern of histamine mobilization differed greatly between gastrin on the one hand and endothelin and adrenaline on the other (see also Norlén *et al*., 2001). The sustained histamine response to gastrin probably reflects the fact that only small amounts of histamine are being secreted (through exocytosis) from the ECL cells per unit time (Lindström *et al*., 1997; 2001a, b), and that histamine lost is being continuously replaced by newly synthesized histamine as a result of accelerated formation (Håkanson *et al*., 1994). The small amount of histamine that is released by gastrin is thought to reflect the size of the immediately releasable pool of secretory products ($\sim$1% of the histamine pool), which in turn corresponds to the number of secretory vesicles that are docked onto the cell membrane and primed for secretion (Zhao *et al*., 1999; Lindström *et al*., 2001a, b).

In the short term, endothelin mobilized more histamine than did gastrin. We suggest that endothelin (and adrenaline) manages to mobilize not only the immediately releasable histamine pool (docked and primed secretory vesicles), but also the more slowly releasable pool (secretory vesicles in the docking zone) and parts of the reserve pool (secretory organelles in transit from the Golgi area to the release site). Moreover, microvesicles were unchanged in number. Earlier reports have suggested that endocytotic microvesicles increase in number in situations characterized by accelerated exocytosis (Zhao *et al*., 1999) (reflecting the coupling between exocytosis and endocytosis). This was not the case in endothelin-exposed ECL cells. The present ultrastructural and chemical findings showed that a near-maximally effective dose of endothelin released about 70% of the secretory vesicles (and a corresponding portion of the histamine store) of the ECL cells. Hence, the massive histamine mobilization was accompanied by the loss of most of the histamine-storing secretory organelles. Accelerated exocytosis is unlikely to explain these findings.

Table 1 Quantitative electron microscopy of ECL cells exposed to endothelin *versus* control ECL cells

Treatment	*No. of cells examined (rats)*	*Granules*		*Secretory vesicles*		*Microvesicles*	
		No./profile	*Vol. (%)*	*No./profile*	*Vol. (%)*	*No./profile*	*Vol. (%)*
Control	31 (3)	0.6±0.3	0.2±0.1	34.8±4.5	14.1±1.7	16.9±1.8	1.7±0.2
Endothelin	41 (3)	0.9±0.1	0.3±0.1	8.6*±1.2	7.4*±1.0	11.5±1.3	2.6±0.3

Comparison between endothelin-exposed cells and control cells. *$P<0.05$. Endothelin (0.1 mmol l^{-1}) was given by submucosal microinfusion (*via* microdialysis probes) for 3 h. Endothelin-exposed ECL cells are close to the probe. Control ECL cells are from the contralateral side of the same rat.

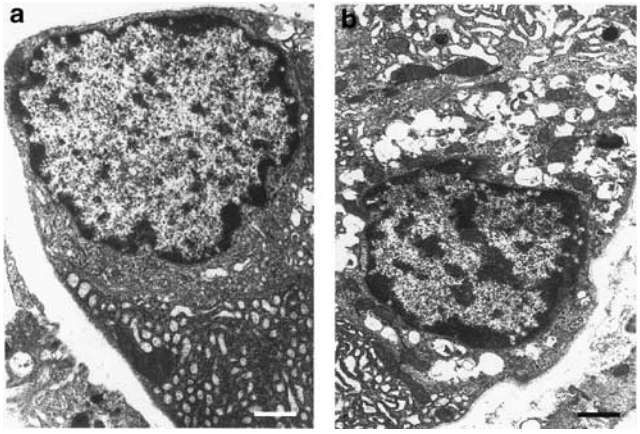

Figure 7 Electron micrographs of ECL cells in the gastric mucosa of rats exposed to submucosal microinfusion of endothelin (10 μmol l^{-1} for 3 h) *via* a microdialysis probe. An ECL cell close to the probe (a) and a control cell (b) from the contralateral side of the stomach. Typical secretory vesicles (examples indicated by arrows) were greatly reduced in number in ECL cells near the probe compared to the contralateral side of the stomach. Bar = 200 μm.

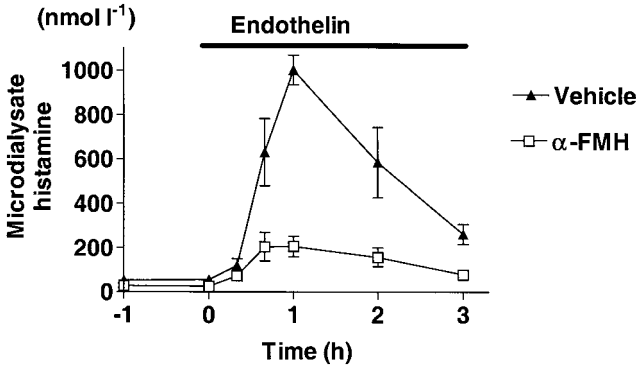

Figure 8 Endothelin-evoked histamine mobilization after continuous subcutaneous infusion of α-FMH (3 nmol kg^{-1} h^{-1}) for 20 h prior to microdialysis. Comparison with vehicle-treated rats. Endothelin was administered *via* microdialysis probes for 3 h, as indicated by the horizontal line. Pretreatment with α-FMH greatly suppressed the histamine response to endothelin. Mean±s.e.m., $n=4$–5.

The transient nature of the histamine response to endothelin and adrenaline may be a consequence of (1) prompt exhaustion of all releasable histamine pools, (2) failure of endothelin/adrenaline to activate HDC to replace lost histamine, and/or (3) mobilization of local agents, such as

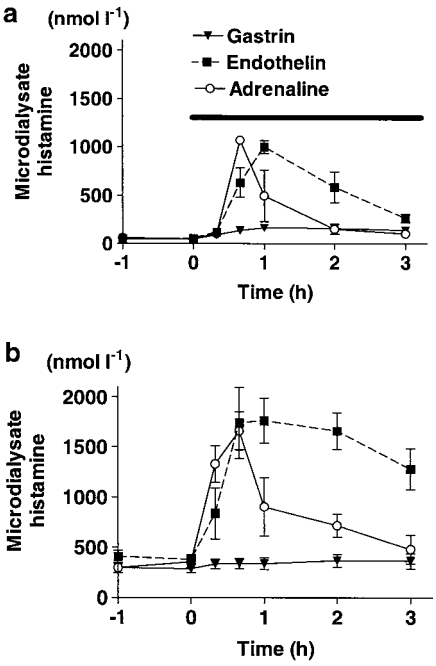

Figure 9 Time course of the histamine response to gastrin (0.1 mmol l^{-1}), endothelin (10 μmol l^{-1}) and adrenaline (1 mmol l^{-1}) in freely fed (a) and omeprazole-treated rats (b). The substances were administered *via* microdialysis probes, as indicated by the horizontal line. Note the enhanced histamine response to endothelin and adrenaline following pretreatment with omeprazole. Mean± s.e.m., $n=4$–8.

somatostatin or prostaglandins, capable of inhibiting exocytotic histamine release (Lindström & Håkanson, 1998; Norlén *et al*., 2001).

Our results are in line with the view that exhaustion of the histamine stores in the ECL cells explains why the histamine release is short-lasting. Histamine may be exhausted because the mechanisms responsible for the formation and storage of the amine are impaired. Indeed, endothelin and adrenaline do not activate HDC, which will delay the resynthesis of histamine. Whether histamine storage is impaired as well remains to be studied.

It cannot be excluded that the mobilization of histamine is also transient, because endothelin releases agents that inhibit histamine release, such as somatostatin and prostaglandins (Lindström & Håkanson, 1998; Norlén *et al*., 2001). However, this explanation seems unlikely because both somatostatin and the prostanoid misoprostol were without effect on the histamine response to endothelin.

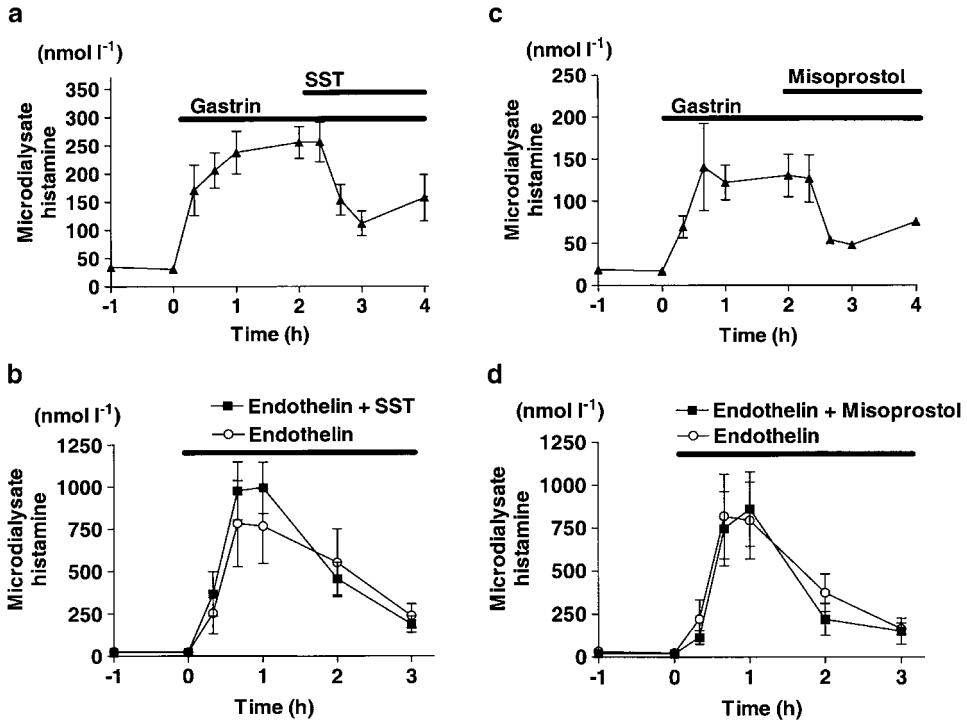

Figure 10 Effect of somatostatin (SST) (a, b) or misoprostol (c, d) on gastrin-induced histamine mobilization (a, c) and on endothelin-induced histamine mobilization (b, d). Gastrin (3 nmol $kg^{-1} h^{-1}$) was given by intravenous infusion for 4 h as indicated. SST (0.1 mmol l^{-1}), misoprostol (0.1 mmol l^{-1}) and endothelin (10 μmol l^{-1}) were administered *via* microdialysis probes as indicated. Mean $\pm$ s.e.m., $n = 3-7$.

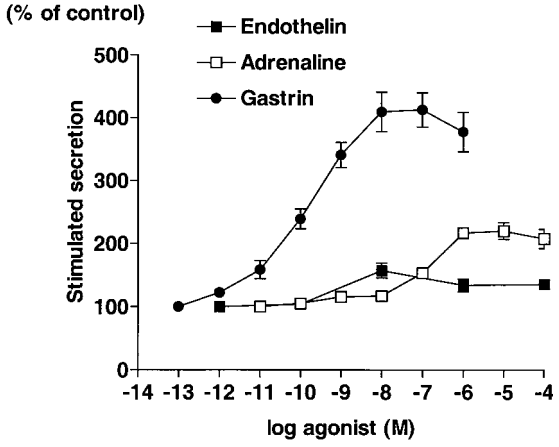

Figure 11 Effect of increasing concentrations of gastrin, endothelin and adrenaline on histamine secretion from isolated ECL cells in primary culture. Gastrin has a powerful effect, adrenaline is moderately active, and endothelin is without any effect. The control is baseline before adding stimuli. Mean $\pm$ s.e.m., $n = 5$ (n is the number of independent cell preparations).

The results favor the view that mobilization of ECL-cell histamine by endothelin and adrenaline is short-lasting, because the histamine stores are rapidly exhausted and because histamine resynthesis is slow.

The nature of the histamine-mobilizing signal elicited by endothelin and adrenaline Endothelin is inactive, and adrenaline only moderately active (compared to gastrin) in releasing histamine from ECL cells in primary culture (see Lindström *et al*., 1997). This contrasts with the quite spectacular effects of endothelin and adrenaline *in vivo*, indicating that the two vasoconstrictors affect ECL cells *in situ* in an indirect manner. α-Adrenergic blockade (prazosine) abolished the response to adrenaline (Norlén, unpublished observation), favoring the view that vasoconstriction (and possibly ischemia) is the causative factor.

Although the histamine responses to endothelin and adrenaline were short-lasting, the two agents had in fact quite long-lasting effects on the ECL cells, revealed by the fact that the cells became refractory to repeated stimulation. Indeed, the cells were found to need several days to recover from exposure to a single, near-maximal dose of either endothelin or adrenaline. The fact that the cells did recover indicates that the two agents did not damage them irrevocably.

Conceivably, vasoconstriction/ischemia is responsible for damaging the cells, and for the consequent histamine mobilization. It is noteworthy that acid blockade (following pretreatment with ranitidine or omeprazole) did not prevent endothelin from mobilizing histamine. Consequently, mobilization of histamine from ECL cells does not depend on acid back diffusion.

We propose that vasoconstriction-evoked ischemia is responsible for the massive endothelin/adrenaline-induced histamine mobilization from the ECL cells, and that acid back diffusion does not contribute to it. The histamine-mobilizing signals elicited by the two vasoconstrictors remain unidentified.

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