Copper-dependent regulation of metal transporter expression in human intestinal epithelial cells

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Copper is an essential dietary trace metal required for a number of physiological and biochemical processes, but is highly toxic in excess. Copper can exist in either the reduced (Cu^{+}) or oxidised (Cu^{2+}) state and like iron can act as an important cofactor in a number of fundamental redox reactions. Despite its obvious importance to human metabolism, relatively little is known about the mechanisms involved in the intestinal absorption of copper. It has been suggested that the divalent metal transporter DMT1 might act as a common uptake pathway for a number of metal species (Gunshin *et al.* 1997). However, work in our laboratory has demonstrated that DMT1 primarily acts as a Fe^{2+} transporter with a much reduced affinity for other metals (Tandy *et al.* 2000). An alternative copper uptake pathway (CTR1) has been identified, which when expressed in HEK293 cells functions as a copper transporter (Lee *et al.* 2002). The purpose of our present work was to investigate the effects of copper on the expression of these two transporters in human intestinal Caco-2 cells.

Caco-2 cells were cultured for 21 days (the final 24 h in the presence or absence of 100 μM Cu^{2+}) in 25 cm^2 flasks and were used to isolate total RNA, which was subjected to RT-PCR for CTR1, DMT1 and β-actin (control). In parallel flasks, cellular protein was isolated for Western blotting.

CTR1 and DMT1 (non-IRE isoform) mRNA were unchanged by copper treatment, whereas DMT1 (+IRE) mRNA was decreased (control, 0.81 ± 0.10 a.u.; +Cu, 0.40 ± 0.11 a.u.; means ± S.E.M.; $n = 5$; $P < 0.02$, Student's unpaired t test). DMT1 protein levels were also decreased by copper treatment (control, 111.5 ± 6.4 a.u.; +Cu, 52.9 ± 10.8; $n = 4$; $P < 0.01$). Regulation of DMT1 (+IRE) mRNA by cellular iron status is thought to occur via differential interaction between an iron responsive element (IRE) in the 3′ untranslated region and cytoplasmic iron regulatory proteins (IRP). Recent evidence suggests that copper may also be able to regulate IRP binding to IRE by replacing iron in the iron-sulphur cluster present in IRP1 (Oshiro *et al.* 2002).

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Characterisation of the intestinal peptide transporter of the Antarctic haemoglobinless teleost *Chionodraco hamatus*

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Previous studies have demonstrated that transport systems for nutrients and ions occurring at the intestine of the Antarctic teleosts (Maffia *et al.* 1996, 2000) exhibit specific adaptations to low temperatures. In this work we extended our research on cold-adapted transporters to a proton oligopeptide transporter located at the intestine of the Antarctic haemoglobinless teleost *C. hamatus*. All fish were humanely killed. The presence in the icefish intestine of a low-affinity type peptide transport belonging to the proton oligopeptide transporter (POT) superfamily (Meredith & Boyd, 2000) was assessed by reverse transcription-polymerase chain reaction (RT-PCR) using human PepT-1-specific primers (Fig. 1).

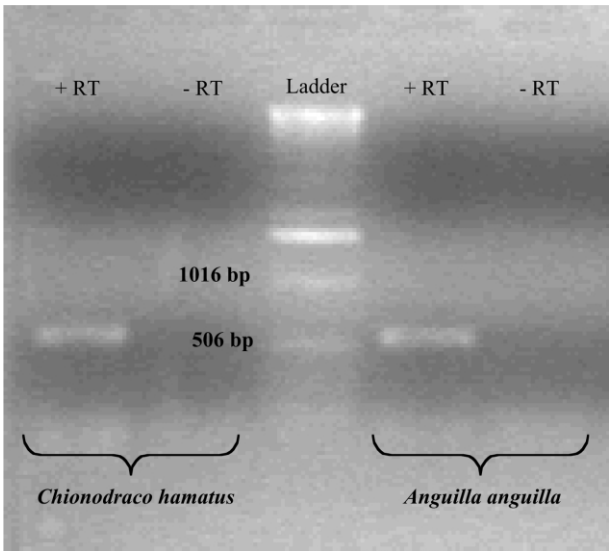

Figure 1. Expression of PepT1-related mRNA in *C. hamatus* and *A. anguilla* intestine by RT-PCR.

H^{+}/dipeptide cotransport activity was measured in the intestinal brush-border membrane vesicles (BBMV) by monitoring acridine orange fluorescence changes (Verri *et al.* 2000). In isolated BBMV, the icefish transporter displays a substrate specificity similar to that found in eel peptide transporter and a maximal transport activity falling in a narrow range of temperatures between −2 and 5 °C. Furthermore, the functional expression of peptide transporter was performed in a heterologous system by microinjection in *Xenopus laevis* oocytes of poly(A)+ RNA extracted from the intestinal mucosa of *C. hamatus*. In oocytes injected with poly(A)+ RNA, D-Phe-Ala uptake was 3-fold higher than that measured in water-injected oocytes. The expression was dose dependent, reaching maximum activity at 40 ng of poly(A)+ RNA and time dependent, reaching maximum activity on day 3 after injection. Poly(A)+ RNA-induced transport was inhibited by 10 mM Gly-Gln. Uptake performed at different temperatures (0–25 °C) was maximal at 25 °C. These data suggest the presence of a low-affinity type H^{+}/peptide transporter on the brush-border membrane of

C. hamatus intestine. When the intestinal peptide transporter of the Antarctic fish is expressed in the heterologous lipid microenvironment represented by *X. laevis* oocyte plasma membrane, its characteristic temperature dependence −2/5 °C) is modified (Fig. 2), thus suggesting a major role of the lipid component in the transport process. However, it cannot be excluded that cold adaptation might involve structural modifications of the transporter.

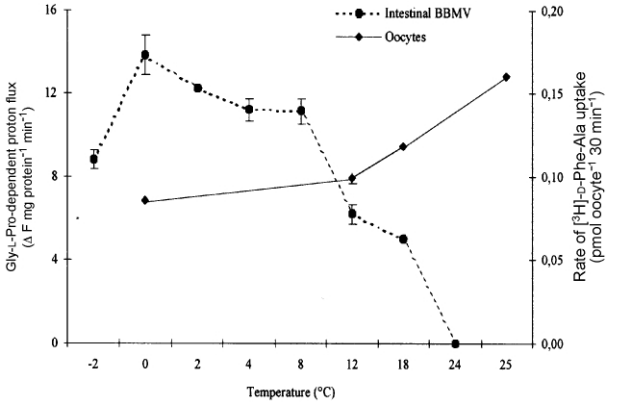

Figure 2. Temperature dependence of the H^+/dipeptide cotransport activity in the intestine of the Antarctic teleost *C. hamatus*.

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All procedures accord with current National guidelines.

Gastrin stimulates Reg expression in a human gastric epithelial cell line via RhoA GTPase

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The expression of peptides of the pancreatitis-associated protein/pancreatic stone protein family appears to be increased in response to tissue injury. One member of this group, Reg1α, is expressed in gastric epithelial cells, and may be a gastric growth factor (Fukui *et al.* 1998). The expression of Reg1α in stomach is controlled by the gastric hormone gastrin (Higham *et al.* 1999). We have examined the transcriptional mechanisms controlling Reg1α gene expression.

A sequence of 2.1 kb of the promoter region of the rat Reg gene was cloned by PCR and sequenced. Deletional mutants were cloned into a luciferase reporter vector, and luciferase expression was determined in AGS-GR cells (10^5 cells in 6-well dishes) which express the gastrin/cholecystokinin-B receptor. Gastrin (1 nM, 8 h) stimulated luciferase expression of the smallest deletional mutant (104 bp upstream of the transcriptional start site) 5.2 ± 0.4-fold (mean ± S.E.M., $n = 12$); there was no significant difference in the relative increase in luciferase activity of the 104 bp and the 2.1 kb promoter sequences in response to gastrin. Mutations within the 104 bp sequence suggested a putative Sp1 site was important for basal and gastrin-stimulated expression. The effect of gastrin on the 104 bp sequence was almost abolished by *Clostridium botulinum* C3-exoenzyme (24 h, 800 ng ml^{-1}), which inhibits the small GTPase, RhoA. Consistent with this observation, when cells were co-transfected with a dominant negative RhoA construct (N19RhoA; 0.5 μg) and the 104 bp Reg-luciferase expression vector (0.5 μg), the response to gastrin was significantly inhibited (1.6 ± 0.2-fold, $P < 0.05$, unpaired t test). Moreover, co-transfection of cells with a constitutively active form of RhoA (L63RhoA) significantly increased expression of the Reg-luciferase vector (5.7 ± 0.4-fold, $P < 0.05$); in contrast, constitutively active forms of the related GTPases, Rac1 and cdc42 had little or no stimulatory effect on Reg1α expression.

The data suggest that expression of the Reg gene is regulated by gastrin and that activation of RhoA is part of the transduction mechanism.

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Immunohistochemical localization of the $P2Y_4$ receptor in human bowel

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It is well established that purines influence gastrointestinal motility, secretion and absorption by acting on smooth muscle and the epithelium; moreover they are involved in synaptic transmission in the myenteric and submucosal ganglia (Burnstock, 2001). However, the role of pyrimidines in gastrointestinal functioning is less well established. Three metabotropic receptors of the P2Y class are activated by pyrimidines, $P2Y_2$ and $P2Y_4$ by uridine triphosphate and $P2Y_6$ by uridine diphosphate. Here we have examined the immunolocalisation of the UTP-specific receptor, $P2Y_4$, in human full thickness bowel.

Archival, paraffin-embedded bowel tissue (2 colon, 3 ileum) was obtained from the Department of Pathology, Glasgow Royal Infirmary and sectioned at 5 μm. Immunohistochemistry protocols were applied and, following microwave antigen retrieval, the $P2Y_4$ receptor was localized using the avidin-biotin complex (ABC) method. Rabbit monoclonal primary antibody (Alomone) was applied at 1:125 for 16 h at 4°C and immunoreactive sites visualized by sequential application of horse biotinylated secondary antibody, ABC complex and diaminobenzidine (Vector Laboratories). Sections were counterstained with haemotoxylin, dehydrated, cleared and mounted. Horse pre-immune serum was used as negative control.

In all specimens, strong immunoreactivity (IR) was observed in neurones in the myenteric, inner and outer submucosal ganglia. The longitudinal and circular muscle layers were negative for $P2Y_4$-like IR. Strong IR was also observed in the endothelium of the blood vessels traversing the submucosal layer. In ileum the epithelial cells were negative or showed only very faint IR. However, throughout the epithelium, specific strong IR was observed in a subpopulation of epithelial cells. These positively stained cells were found toward the base of crypts, although not exclusively, and the majority occurred singly or in pairs. The positively staining flame-like cells demonstrated a granular appearance, either apically, basolaterally or both. No evidence of

this positively staining epithelial subpopulation was observed in the colon.

Here, we report that $P2Y_4$ receptor IR was found in all patients in subpopulations of ganglion cells of the myenteric and submucosal plexuses, suggesting that synaptic transmission or modulation may be mediated by pyrimidines. The subpopulation of epithelial cells observed remains to be identified but may represent either Paneth or enteroendocrine cells. Since the majority of cells occur singularly or in pairs and are not exclusively restricted to the base of crypts they are likely to be enteroendocrine in origin.

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All procedures accord with current local guidelines.

Alterations of epithelial barrier properties of rat ileum following X-irradiation

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Exposure of the digestive tract to ionising radiation results in both morphological and functional alterations of the small intestine with the terminal ileum being a particular site of injury. The aim of this work was to characterize, in parallel, epithelial barrier properties of rat ileum as determined by the passage of different sized molecules and spatial localization junctional proteins following increasing radiation dose.

Animals were exposed to a single hemi-body irradiation (6–12 Gy) under gaseous anaesthesia (isoflurane 2.5 %, 0.4 l min^{-1}) and studied 3 days after exposure. After euthanasia (overdose of sodium pentobarbitone), samples of terminal ileum were placed in Ussing chambers for measurements of ^{14}C-mannitol, FITC-Dextran-4 kDa and ^{3}H-Dextran-70 kDa fluxes, short-circuit current (I_{sc}) and transepithelial resistance (R_T). Ileal samples were treated either for immunohistochemical analyses of junctional proteins (ZO-1, β-catenin) and pan-cytokeratin using confocal microscopy or for standard histological analyses with haematoxylin-eosin-saffron staining. All experiments were conducted according to the French regulations for animal experimentation (Ministry of the Agriculture Act No. 87848, October 19, 1987).

Following a 6 Gy irradiation I_{sc} was increased from 26 ± 5 μA cm^{-2} (controls $N = 22$) to 64 ± 16 μA cm^{-2} (irradiated $N = 5$) (means ± S.E.M., $P < 0.01$, one-way ANOVA). R_T was increased from 35 ± 3 Ω cm^2 (controls $N = 22$) to 50 ± 3 Ω cm^2 (irradiated $N = 5$, $P < 0.05$), but permeability to mannitol and dextran was unchanged. Analysis of pan-cytokeratin and β-catenin staining showed a slight increase of cell size with nevertheless a well-structured network in both villi and crypts. Standard histology confirmed these results.

At 3 days after 8 Gy R_T was decreased by 50 % concomitant with increased permeability to mannitol (×3), Dextran-4 kDa (×3) and Dextran-70 kDa (×2). A reduced and modified pattern of β-catenin staining was observed, suggesting a relocalization of this protein. The effect of irradiation on ZO-1 was more marked with very little staining being detected in either villi or crypts. An amplification of these effects occurred with increasing radiation dose where for ⩾ 10 Gy R_T was quasi-null and mannitol and 4.4 kDa dextran permeability was markedly increased (×7). Major histological alterations with epithelial discontinuity were also observed.

In conclusion, these results demonstrate radiation dose-dependent differences in the response of rat small intestinal epithelium: at a lower dose (6 Gy) increased R_T was observed, whereas an opposite effect (decreased R_T, enhanced permeability) was obtained at higher doses (⩾ 8 Gy). The latter were associated with a disorganization of the small intestinal epithelium.

All procedures accord with current National guidelines.

Putative implication of Ca^{2+} and NO-mediated pathways in radiation-induced alteration of VIP-stimulated secretory response in rat distal colon

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Previous data have shown a radiation-induced hypo-responsiveness of vasoactive intestinal peptide (VIP)-stimulated short-circuit current responses (I_{sc}) in rat distal colon. This finding was associated with reduced cAMP accumulation in isolated crypts and decreased adenylyl cyclase (AC) activity. Such alterations may involve inhibition of the cAMP pathway by $[Ca^{2+}]_i$, cGMP or altered expression of AC isoforms. The present study has addressed such possible mechanisms using co-stimulation studies in addition to determination of the frequency and localization of the AC isoforms present in colonic mucosa.

Animals were exposed to a single abdominal irradiation (9 Gy) under gaseous anaesthesia (isoflurane 2.5 %, 0.4 l min^{-1}) and studied 4 days after exposure. After euthanasia (overdose of sodium pentobarbitone), samples of distal colon were used for (a) I_{sc} determinations in Ussing chambers and (b) localization in full-thickness tissue of eight AC isoforms by immuno-histochemistry. All experiments were conducted according to the French regulations for animal experimentation (Ministry of the Agriculture Act No. 87848, October 19, 1987).

In control animals, VIP (1 μM) and carbachol (50 μM) in combination increased ΔI_{sc} responses to 143 ± 32 μA cm^{-2} (mean ± S.E.M., $n = 5$), which was higher than VIP alone (94 ± 15 μA cm^{-2}), but not significantly different from carbachol alone (161 ± 15 μA cm^{-2}). Addition of sodium nitroprusside (SNP, 100 μM) slightly reduced the VIP response (76 ± 25 μA cm^{-2}). Irradiation reduced VIP-stimulated I_{sc} responses but carbachol- or VIP + carbachol-stimulated responses were unchanged. VIP and SNP co-stimulated ΔI_{sc} responses, however, were reduced by 41 % ($P \leqslant 0.05$, Student's unpaired t test, $n = 5$). Addition of an iNOS inhibitor (L-NIL) did not markedly restore this hyposensitivity.

For the first time, localisation of most of the AC isoforms has been demonstrated in rat distal colon. Only isoforms III (upregulated by Ca^{2+}), IV and V/VI (downregulated by Ca^{2+} and NO) were present in crypts. Isoform staining was greatest at the surface and declined toward the crypt base. After irradiation, only the upper part of the crypt remained stained without changes of the predominant isoforms.

In conclusion, these data show that alteration of VIP-induced I_{sc} responses by $[Ca^{2+}]_i$-mediated pathways is unlikely in both control and irradiated groups. However, NO appears to exacerbate the radiation-induced hyporesponsiveness to VIP, which is in agreement with the presence of isoform V/VI in

crypts. Thus alteration of the cAMP pathway may be, in part, due to an inhibitory effect of NO perhaps via cGMP. Furthermore, reduced numbers of AC units are in accordance with the lower VIP responses seen 4 days after irradiation.

All procedures accord with current National guidelines.

Amidated gastrin induces proliferation via the cholecystokinin type 2 (CCK_2) receptor in Barrett's oesophagus

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Barrett's oesophagus is a premalignant metaplastic mucosa that is highly proliferative and characterised by mucosal instability (Hong *et al.* 1995). In the last two decades there has been a dramatic increase in the incidence of Barrett's oesophagus and associated oesophageal adenocarcinoma in western populations (Blot *et al.* 1991). Factors associated with its development and malignant progression are poorly understood. Gastrin has a complex role in the regulation of epithelial cell proliferation and differentiation of gastrointestinal mucosa, primarily mediated through the classical COOH-terminally amidated peptides (Dockray, 1999). Gastrin has also been implicated as a mitogen for a number of GI and extra-GI tumours (Rehfeld *et al.* 1989; Nemeth *et al.* 1993). The aim of this study was to determine whether gastrin could influence cellular events in Barrett's oesophagus.

Four patient groups were identified: control, reflux disease, Barrett's metaplasia and adenocarcinoma. Endoscopic biopsies were collected from 3–5 cm above the gastro-oesophageal junction (GOJ) and 12–15 cm above the GOJ or 3 cm above the upper limit of the Barrett's mucosa. The receptor was identified by RT-PCR and quantified by Northern analysis. [^{125}I]-G17-autoradiography was used to localise the CCK_2 receptor in frozen sections of Barrett's mucosa. [^{3}H]-thymidine and BrdU incorporation were used to determine proliferation in response to G17 in Barrett's mucosal biopsies incubated *ex vivo*. The Salford and Trafford, and the Mersey Research Ethics Committees granted ethical approval for the study. All patients provided written informed consent.

Reverse transcription polymerase chain reaction (RT-PCR) identified expression of the CCK_2 receptor in the lower and mid-oesophagus in 3 of 8 controls (37%), 5 of 7 patients with oesophagitis (71%), 10 of 10 patients with Barrett's metaplasia (100%) and 7 of 12 patients with oesophageal adenocarcinomas (58%). By Northern analysis a 2.1 kb mRNA, compatible with the CCK_2 receptor, was identified in PCR-positive samples. [^{125}I]-G17 bound to columnar epithelial cells within the glandular and lower crypt regions of Barrett's mucosa. In experiments examining proliferation of Barrett's mucosa incubated *ex vivo*, 10 nM G17 induced a 2-fold ($n = 7$, $P = 0.0257$, unpaired t test) increase in [^{3}H]-thymidine incorporation at 24 h. This response was abolished by the addition of 100 nM of the specific CCK_2 receptor antagonist L-740,093.

In summary, expression of the CCK_2 receptor appears to be related to the degree of mucosal inflammation, with mRNA for the receptor present in a greater number of patients with Barrett's/reflux oesophagitis compared with controls. CCK_2 receptor mRNA is ubiquitously expressed in Barrett's mucosa with the receptor located on epithelial cells within the proliferative zone of the tissue. Amidated gastrin can be shown to induce proliferation in Barrett's mucosa via the CCK_2 receptor.

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All procedures accord with current local guidelines and the Declaration of Helsinki.

Analysis of paracrine signals activating the mitogen activated protein (MAP) kinase pathway in heterogeneous cell populations

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Stimulation of G-protein-coupled receptors (GPCR) linked to $G\alpha_{q/11}$ may activate the MAP kinase pathway either directly, i.e. by intracellular mechanisms, or indirectly by increased shedding of growth factors of the epidermal growth factor (EGF) family, which then act as paracrine or autocrine mediators (Prenzel *et al.* 1999). The potential presence of the two mechanisms in different members of heterogeneous cell populations clearly complicates the interpretation of responses to GPCR stimulation. We have distinguished these mechanisms using flow cytometry of heterogeneous cell populations defined by expression of green fluorescent protein (GFP).

The human gastric epithelial cell line, AGS, stably transfected with the gastrin-CCK_B receptor (AGS-G_R cells) was co-cultured with ASG cells that had been stably transfected with GFP and which do not express the gastrin-CCK_B receptor (Varro *et al.* 2002). Activation of the MAP kinase pathway was studied by flow cytometry using antibodies to phospho p42/44 MAP kinase, or total MAP kinase, and detection by AlexaFluor647-conjugated second antibody (Chow *et al.* 2001), measuring fluorescence in each cell type by gating on GFP fluorescence.

There were similar increases in phospho-p42/44 MAP kinase in AGS-G_R (2.4 ± 0.5-fold, mean ± S.E.M., $n = 5$) and AGS-GFP (2.3 ± 0.4) cells in response to the EGF receptor ligand, transforming growth factor (TGF)-α (100 ng ml^{-1}, 30 min) and a smaller (1.6 ± 0.1 and 1.8 ± 0.2, respectively) increase was seen with acidic fibroblast growth factor (FGF, 25 ng ml^{-1}). In co-cultures, gastrin (1 nM, 30 min) increased phospho-p42/44MAP kinase in both ASG-G_R (3.1 ± 0.4-fold) and AGS-GFP cells (1.5 ± 0.1-fold). The responses in AGS-GRP cells to gastrin were fully reversed by AG1478 (3 μM), which inhibits EGF-receptor tyrosine kinase activity ($P < 0.05$, unpaired t test). In contrast, in the same co-cultures, AG1478 did not significantly inhibit the AGS-G_R cell responses to gastrin. Interestingly, neither the inhibitor of erb-B-2 receptor tyrosine kinase activity, AG825 (5 μM) nor the inhibitor of FGF-receptor tyrosine kinase SU5402 (25 μM) had any effect on gastrin-induced p42/44MAP kinase phosphorylation in either cell type.

We conclude that flow cytometry may be used to distinguish activation of signalling pathways in mixed populations of cells. Gastrin stimulation of MAP kinase activation can be partly direct, i.e. intracellular, and partly due to release of EGF-receptor ligands.

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Distribution of mitochondria in mouse pancreatic acinar cells

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It is known that the density and distribution of mitochondria in a cell reflects its metabolic activity and function. At present, there is no general consensus on the preferential distribution of distinct subpopulations of mitochondria in pancreatic acinar cells (Tinel *et al.* 1999; Gonzalez *et al.* 2000). In this study the distribution of mitochondria in isolated pancreatic acinar cells and acinar cells of intact pancreas was investigated using confocal microscopy and transmission electron microscopy (TEM).

Pancreatic acinar cells were obtained from the isolated pancreas of CD-1 male mice killed by rapid cervical dislocation as described previously (Osipchuk *et al.* 1990). In isolated cells loaded with MitoTracker Red (fluorescent mitochondrial probe), mitochondria were predominantly situated in the peri-granular, subplasmalemmal and peri-nuclear regions. Subsequent applications of TEM fixatives caused partial leakage of the probe out of mitochondria, but overall the distribution of MitoTracker Red was unchanged, suggesting that the localization of mitochondria is not affected by the fixation procedure. TEM was performed on isolated acinar cells and on acinar cells of the intact pancreas. The distribution of mitochondria was quantified by calculating the percentage of cross-sectional area that mitochondria occupied in different regions: peri-granular, peri-nuclear and subplasmalemmal. In isolated acinar cells the highest density of mitochondria was in the peri-granular region, $25.69 \pm 1.58\,\%$ (mean ± S.E.M.), then the subplasmalemmal region, $12.61 \pm 0.77\,\%$, and the peri-nuclear region, $9.07 \pm 0.97\,\%$ ($n = 26$); each value was found to be significantly different ($P < 0.01$) from the non-specific and whole cell values using Student's paired t test. Similar results were obtained from acinar cells of intact pancreas: peri-granular, $22.9 \pm 1.95\,\%$, subplasmalemmal, $12.45 \pm 0.78\,\%$ and peri-nuclear regions, $9.05 \pm 0.94\,\%$ ($n = 26$); these results were also significantly different from the non-specific and whole cell values ($P < 0.01$). Mitochondria were found only in cell areas where endoplasmic reticulum (ER) was present and a close localization between the ER membrane and the mitochondrial membrane could be seen.

We conclude that in pancreatic acinar cells mitochondria are preferentially distributed to peri-granular, subplasmalemmal and peri-nuclear regions in very close association with the ER and this distribution is not affected by isolation or fixation procedures.

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All procedures accord with current UK legislation.

Non-steroidal anti-inflammatory drugs inhibit *Helicobacter pylori* VacA toxin-induced vacuolation of human gastric epithelial cells in culture

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The relationship between non-steroidal anti-inflammatory drugs (NSAIDs) and *Helicobacter pylori* (*H. pylori*) in causing gastric mucosal injury is still under debate. VacA toxin is an important *H. pylori* virulence factor that causes cytoplasmic vacuolation in cultured cells (Ricci *et al.* 2000). VacA may act as a channel-forming toxin: endocytosed VacA channels could stimulate the turnover of endosomal V-ATPase by increasing the permeability of the endosomal membrane to anions. This would lead to the accumulation of osmotically active species causing an osmotic imbalance of late endosomes with subsequent vacuole formation. This study was designed to investigate whether and how NSAIDs interfere with VacA-induced vacuolation of human gastric epithelial cells in culture.

MKN 28 cells were incubated for 16 h at 37 °C with different concentrations of NH_4Cl in the absence or presence of VacA and of different NSAIDs (0.1–1 mM indomethacin; 0.1–1 mM aspirin; 0.01–1 mM NS-398, a selective cyclooxygenase-2 inhibitor), prostaglandin E_2 (PgE_2; 10^{-7}–10^{-5} M) or arachidonic acid (AA; 0.01–0.05 mM). To study NSAID action on already developed cell vacuolation, cells were incubated for 16 h with 4 mM NH_4Cl or 4 mM NH_4Cl plus VacA and then for an additional 8 h in the absence or presence of different NSAIDs. Cell vacuolation was quantified by neutral red uptake assay (Ricci *et al.* 2000). We also investigated NSAID action on cell binding and internalization of VacA, using a protease-protection assay and SDS-PAGE followed by Western blotting. Moreover, we compared NSAID action with that of 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), an NSAID itself known as a specific inhibitor of VacA action via its chloride channel blocking activity and exhibiting structural similarities with NSAIDs we used. Results were expressed as the mean ± S.E.M. of three independent experiments. The statistical significance of the differences was evaluated by ANOVA and Newman-Keuls Q-test.

We found that (1) NSAIDs dose-dependently and significantly ($P < 0.05$) both prevented and reverted VacA-induced vacuolation of MKN 28 cells, causing a 55–70 % reduction in neutral red uptake with the highest dose used (e.g. VacA: 2.05 ± 0.10; VacA + 1 mM indomethacin: 0.61 ± 0.04 $\mu g/\mu g$ cell protein; (2) neither PgE_2 nor AA affected NSAID-dependent inhibition of VacA vacuolating action; (3) none of NSAIDs used impaired cell binding or internalization of VacA; (4) NSAIDs and NPPB, in a very similar manner, significantly ($P < 0.05$) inhibited ammonia-dependent cell vacuolation and reduced the VacA potentiating effect on ammonia action.

Our findings may suggest that NSAIDs counteract VacA cytotoxicity through inhibition of VacA channel activity as well as of endogenous anionic channels required for vacuole genesis.

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Carbachol-stimulated HCO_3^- secretion in rat distal colon is sensitive to the CFTR channel blocker diphenylamine-2-carboxylic acid (DPC)

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The cystic fibrosis transmembrane conductance regulator (CFTR) can conduct both Cl^- and HCO_3^- ions although there is a preference for Cl^- (Kunzelmann *et al.* 1991). In rat colon, direct manipulation of intracellular cAMP can stimulate HCO_3^- secretion through a pathway dependent on CFTR (Cuthbert *et al.* 1999). Here we have examined the effects of carbachol on HCO_3^- secretion through CFTR in rat distal colon.

The terminal segment of colon (5 cm) was removed from adult male Wistar rats that had been humanely killed by cervical dislocation. After washing in Krebs solution (mM: NaCl 119, KCl 4.7, $NaHCO_3$ 24.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11.1) the smooth muscle was removed and the mucosal sheet mounted in an Ussing chamber (area 0.5 cm^2) where apical and basolateral baths contained Krebs solution at 37 °C, gassed with 95 % O_2 and 5 % CO_2. Ion replacement experiments were performed using low-Cl^- Krebs solution prepared by iso-osmotically replacing NaCl with sodium gluconate. In addition, Cl^- and HCO_3^- were replaced using the following solution (mM: sodium gluconate 130, potassium gluconate 5, $MgSO_4$ 1.2, calcium gluconate 5.8, Hepes 10, glucose 10, pH 7.4 with NaOH). All values represent the change in I_{SC} (ΔI_{SC}) from baseline (mean ± S.E.M.), and Student's unpaired t test was used for statistical analysis ($P < 0.05$ was considered significant).

Spontaneous short-circuit current (I_{SC}) was 17.0 ± 2.0 μA cm^{-2} ($n = 60$) with a resistance of 105 ± 4 Ω cm^{-2} and an open circuit transepithelial potential difference of −2.0 ± 0.2 mV. Basolateral carbachol (100 μM, $n = 14$) stimulated a triphasic response consisting of an initial increase in I_{SC} (phase 1, 12.4 ± 2.0 μA cm^{-2}) followed by a decrease (phase 2, 6.3 ± 2.4 μA cm^{-2}) then a large increase (phase 3, 55.9 ± 8.3 μA cm^{-2}) that slowly decayed to a stable plateau. Apical amiloride (100 μM, $n = 19$) did not alter the carbachol-stimulated response. Basolaterally applied bumetanide (100 μM, $n = 10$) significantly attenuated all three phases of the carbachol-stimulated ΔI_{SC} (phase 1, 4.6 ± 2.0 μA cm^{-2}, $P < 0.02$; phase 2, 0.9 ± 0.7 μA cm^{-2}, $P < 0.05$; phase 3, 7.4 ± 0.9 μA cm^{-2}, $P < 0.001$). The carbachol-evoked ΔI_{SC} was also significantly reduced compared with control, but not abolished, in low-Cl^- Krebs solution ($P < 0.02$ for all three phases, $n = 10$). In Cl^-- and HCO_3^--free conditions, however, the carbachol-evoked ΔI_{SC} was abolished ($n = 3$). In low-Cl^- conditions where there is still a residual HCO_3^- secretion in response to carbachol, DPC (100 μM apically and basolaterally, $n = 6$) was found to significantly reduce phases 1 and 3 of the ΔI_{SC} (phase 1, 0.5 ± 0.1 μA cm^{-2}, $P < 0.01$; phase 3, 5.0 ± 3.2 μA cm^{-2}, $P < 0.05$).

These results suggest that carbachol-stimulated ΔI_{SC} is due to both Cl^- and HCO_3^- secretion, although Cl^- secretion is predominant, similar to results in porcine bronchial epithelia with acetylcholine (Trout *et al.* 1998). Blocking the Cl^- component of ΔI_{SC}, using bumetanide or low-Cl^-, reveals the HCO_3^- current, which can be significantly attenuated by the CFTR channel blocker DPC, suggesting that HCO_3^- is carried through CFTR in rat distal colon.

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All procedures accord with current UK legislation.

Immunocytochemical localisation of neuronal nitric oxide synthase in normal and inflamed (ulcerative colitis, Crohn's disease) human colon

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Nitric oxide (NO) is an important neuronal signalling molecule (Bult *et al.* 1990) which mediates smooth muscle relaxation in the gastrointestinal tract and has been implicated in gastrointestinal motility disorders (Vanderwinden *et al.* 1992). The motility abnormalities in inflammatory bowel disease (IBD) may also, in part, be due to alterations in nitrergic innervation of the inflamed colon. In this study we examined the distribution and density of neuronal nitric oxide synthase (nNOS) in the colon of healthy (normal) controls and in IBD.

Archival, formalin-fixed, colonic specimens from normal controls (non-stenotic carcinoma, $n = 6$), Crohn's disease (CD, $n = 6$) and ulcerative colitis (UC, $n = 6$) were sectioned at 6 μm. ABC-Immunoperoxidase staining was performed with a polyclonal antibody directed against nNOS (Chemicon, UK). North Glasgow Universities NHS Trust granted ethical permission for this study. Immunoreactivity was assessed in the circular and longitudinal muscle, and in the myenteric plexus using an arbitrary grading system of 0–4, representing absent, sparse, moderate, abundant and very abundant nNOS immunoreactivity.

nNOS-like immunoreactivity (IR) occurred in both normal and diseased bowel. The most striking area of immunoreactivity was in the myenteric plexus where strong staining of ganglion cells occurred. The proportion of specimens assigned a grade of abundant in the myenteric plexus was decreased in UC (3/6) and in CD (2/6) compared with controls (6/6). In the submucosal plexus, IR was dense in the cytoplasm of ganglion cells and connecting fibres. In the muscularis externa, the grading of abundant nNOS-like immunoreactive nerve fibres was greater in the circular than longitudinal muscle in all specimens. In both the circular (controls (5/6), UC (2/6), CD (1/6)) and longitudinal (controls (2/6), UC (0/6), CD (0/6)) muscle, the proportion of specimens graded as abundant were decreased in IBD. A small number of epithelial cells displayed nNOS IR; these cells (at most two per crypt) were located at the lower half of the crypt and were identified in all normal colon specimens. The proportion of specimens in which these nNOS immunoreactive cells were identified decreased in IBD where 4/6 UC specimens and 0/6 CD specimens displayed immunoreactivity.

The alterations of nNOS-like immunoreactivity observed in this study may represent cellular changes which contribute to defects in the control of colonic motility in IBD.

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All procedures accord with current local guidelines.

The anti-inflammatory effect of leptin on experimental colitis in rats is abolished under stressful conditions

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Leptin, which is a newly recognised 'anorexic' hormone produced mainly by adipose tissue, regulates food intake and energy balance. Plasma leptin concentrations increase acutely during infection and inflammation (Faggioni *et al.* 2001), suggesting a modulatory role of leptin in the hypothalamo-pituitary-adrenal (HPA) stress axis as an acute phase reactant (Maruna *et al.* 2001). It is well known that HPA axis and glucocorticoids have a proliferative activity on the immune system, while stress-induced overactivity of the HPA axis suppresses the immune response. However, it is not clear yet whether leptin has a pro-inflammatory (Barbier *et al.* 2001) or an anti-inflammatory (Brzozowski *et al.* 2001) activity in the pathogenesis of intestinal inflammation. Therefore, the present study was designed to investigate the effect of leptin on acute colonic inflammation and this effect was compared with that of acute stress, which is known to act via the HPA axis.

Experimental procedures were approved by the Marmara University Animal Care and Use Committee. After an overnight fast, Sprague-Dawley rats of both sexes (200–250 g; $n = 59$) were administered intrarectally (I.R.) under light ether anaesthesia with 4% (1 ml) acetic acid (colitis) (Sekizuka *et al.* 1988) or saline (control). Some rats were subjected to water avoidance stress for 30 min at the sixth hour following the administration of acetic acid or saline. Leptin (10 μg kg^{-1}; I.P.) or saline was injected immediately before and 6 h after the I.R. administrations. Rats were humanely decapitated at 24 h and the distal 8 cm of the colon were removed for macroscopic scoring and the determination of tissue wet weight index (WWI) and tissue myeloperoxidase activity (MPO; an indicator of tissue neutrophil infiltration). Data are means ± S.E.M. from 7–9 rats in each group and were analysed using ANOVA.

Acetic acid-induced colitis increased the macroscopic damage score (4.8 ± 0.6, $P < 0.001$), compared with control group (0.06 ± 0.06), as well as WWI ($P < 0.001$) and MPO activity ($P < 0.05$). Water avoidance stress in the control group had no significant effect, while its application in the colitis group reduced both macroscopic score (2.4 ± 0.3, $P < 0.01$) and WWI ($P < 0.01$). Similarly, in the leptin-treated colitis group, macroscopic score (2.8 ± 0.4, $P < 0.05$) and WWI ($P < 0.01$) were also reduced, but leptin *per se* had no effect on control animals (0.06 ± 0.04). When leptin treatment and stress were applied together in the colitis group, the reductions in the macroscopic score and WWI accomplished by either leptin or stress, were abolished. However, increased MPO activity in the colitis group was not affected by stress or leptin.

Our results indicate that leptin has an anti-inflammatory effect on acetic acid-induced colitis and this effect appears to be independent of neutrophils. Based on our present results, it may be speculated that leptin, acting via the HPA axis, has an immunomodulatory and an anti-inflammatory effect. However, when the HPA axis is over-stimulated by leptin during stressful conditions, immune dysfunction is likely. This supports the fact that the anorexia during acute infection, which is beneficial in the beginning, turns out to be deleterious in chronic infections and neoplastic diseases.

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All procedures accord with current local guidelines.

Deletion of the P-glycoprotein (mdr1a) gene unmasks regional variations in transcellular permeability in isolated mouse intestine *in vitro*

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P-glycoprotein (PGP) and related efflux transporters reduce the intestinal absorption of therapeutic agents but may also have a key protective role in preventing the entry of potentially toxic xenobiotics from the gut lumen. To date, we have little direct information on the impact of PGP on the permeability of the epithelial barrier and, in particular, the functional distribution of PGP effects along the gut, which may give further insight into its physiological importance. To address this we have investigated the regional permeability of PGP substrates and permeability probes using intestinal tissues from mdr1a (−/−) mice, which do not express functional PGP in the intestine, and wild-type mdr1a (+/+) mice.

Unstripped distal jejunum (DJ), ileum (IL), proximal (PC) and distal colon (DC) was removed from humanely killed male FVB (mdr1a (+/+) or mdr1a (−/−)) mice, and mounted in modified Ussing chambers, using methods similar to those of Stephens *et al.* (2002). Apical to basolateral (A-B) apparent permeability (P_{app}) in each region was measured for the PGP substrates digoxin (DIG, 40 μM) and paclitaxel (TAX, 20 μM). Propranolol (PRO, 100 μM) and mannitol (MAN, 100 μM) were also used as markers of passive transcellular and paracellular permeability, respectively. All compounds were labelled with either [^{3}H] or [^{14}C] (0.2 μCi ml^{-1}).

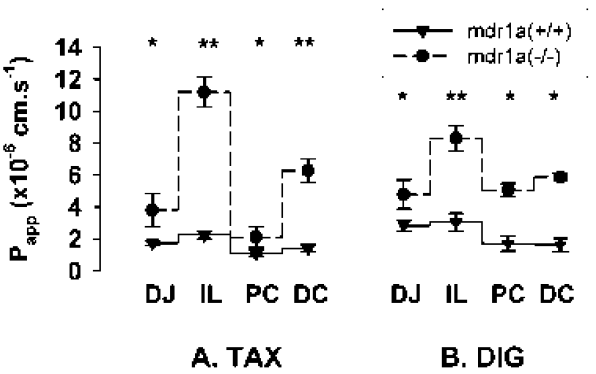

Figure 1. Regional variation in TAX and DIG A-B P_{app} in isolated intestine from mdr1a (+/+) and mdr1a (−/−) mice. Values are mean A-B P_{app} ± S.E.M., $n = 4$ for each data point. *$P < 0.05$ cf. ileum; **$P < 0.05$ cf. proximal colon (mdr1a (−/−) only, ANOVA).

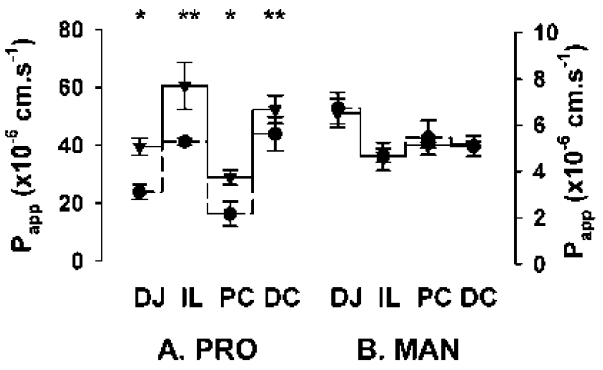

Figure 2. Regional variation in PRO and MAN A-B P_{app} in isolated intestine from mdr1a (+/+) and mdr1a (−/−) mice. Values are mean P_{app} ± S.E.M., $n = 4$ for each data point. *$P < 0.05$ cf. ileum; **$P < 0.05$ cf. proximal colon (both strains, ANOVA).

In mdr1a (+/+) intestine, the permeability of TAX ($n = 4$) and DIG ($n = 4$) was restricted, which was manifested as low absorption that varied little from jejunum to distal colon (Fig. 1). In contrast, active PGP-mediated efflux of TAX and DIG was abolished in mdr1a (−/−) tissues and this was accompanied by marked region-dependent increases in passive drug permeability (Fig. 1). Permeability of the passive transcellular marker PRO, whose transport is unaffected by PGP, showed similar regional variations in both mdr1a (+/+) and mdr1a (−/−) tissues (Fig. 2), indicative of inherent differences in transcellular permeability that are masked by differential expression of PGP. Permeability to the paracellular marker MAN was identical in both mouse strains and unlike PRO showed no regional variation. These data suggest that differential PGP expression along the gut maintains xenobiotic absorption at a low level and, in doing so, minimises regional variations in transcellular permeability.

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Glucagon-like peptide 2 (GLP-2) abrogates the effect of total parenteral nutrition (TPN) on intestinal amino acid and peptide transporters in rats

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Previously we have demonstrated that TPN alters amino acid and peptide transporter mRNA levels in rat intestine (Howard *et al.* 2001). We looked at eight different transporters and identified three subgroups: those which showed (i) increased duodenal but not ileal levels during TPN (ATA2, ASCT1 and GLYT1); (ii) increased levels in ileum only (ASCT2, NBAT, EAAC1, PepT1); and (iii) no effect of TPN (CAT1). We have now investigated how co-infusion of the intestino-trophic factor GLP-2 influences these responses.

Rats were established on TPN given into the jugular vein (Hypnorm, 0.1 ml, I.M.) and received no oral intake for 7 days. A second group were given TPN supplemented with GLP-2 (100 μg per rat per day). Orally fed control animals were given saline infusions and had free access to oral diets. After 7 days animals were humanely killed and cDNA was prepared from duodenal and ileal mucosa. mRNA levels, expressed in arbitrary units relative to 18S rRNA, were measured by semi-quantitative polymerase chain reaction.

In both duodenum and ileum GLP-2 abrogated any effects of TPN, maintaining mRNA levels at orally fed control values. For example, compared with orally fed animals, duodenal content of the neutral amino acid transporter ASCT1 mRNA was increased 1.9-fold by TPN but remained unchanged with TPN + GLP-2 co-infusion (orally fed, 1.00 ± 0.09; TPN, 1.87 ± 0.05, $P < 0.01$; TPN + GLP-2, 0.92 ± 0.1, $P > 0.05$, means ± S.E.M., $n = 6$, unpaired t test). Neither TPN nor TPN + GLP-2 had an effect on ileal ASCT1 mRNA level (orally fed, 1.00 ± 0.03; TPN, 0.92 ± 0.04; TPN + GLP-2, 0.85 ± 0.12; $P > 0.05$). Comparable results were found for ATA2, ASCT2, NBAT and PepT1. However, both GLYT1 and EAAC1 ileal mRNA levels were significantly reduced by TPN + GLP-2 co-infusion when compared with orally fed animals (GLYT1: orally fed, 1.00 ± 0.03, TPN + GLP-2, 0.52 ± 0.07, $P < 0.01$; EAAC1: orally fed, 1.00 ± 0.06, TPN + GLP-2, 0.62 ± 0.12, $P < 0.05$).

Co-infusion of GLP-2 during TPN prevents TPN-mediated increases in intestinal amino acid and peptide transporter mRNA levels. This suggests that GLP-2 is involved in maintaining normal intestinal function, and this may be compromised in the absence of enteral nutrition.

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All procedures accord with current UK legislation.

Expression of orexin-1 receptors by rat vagal afferent neurones

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Stimulation of vagal afferent neurones serving the gut inhibits food intake and activates autonomic reflexes regulating digestion. Some of these effects are mediated by the intestinal hormone cholecystokinin (CCK) which acts on CCK-A receptors expressed by vagal afferent neurones (Burdyga *et al.* 2001). The neuropeptides orexin A and B are produced in the hypothalamus and gut, and act at orexin-1 or -2 receptors (OX-R1, -R2) to stimulate food intake (Kirchgessner, 2002). We have examined OX-R1 expression by vagal afferent neurones.

The expression of OX-R1 in rat nodose ganglia was examined by RT-PCR, cloning and sequencing, and by immunocytochemistry, using methods similar to those described recently (Burdyga *et al.* 2001). The effect of orexin A on the discharge of afferent nerve fibres from the jejunum was studied in rats anaesthetised with pentobarbitone (60 mg kg^{-1}, I.P.) and then humanely killed (Lal *et al.* 2001).

Primers specific for rat OX-R1 revealed a band of the predicted size in rat nodose ganglia following RT-PCR. Cloning and sequencing of the product confirmed its identity. Primers specific for the orexin precursor and OX-R2 did not yield products in RT-PCR of the same starting material. OX-R1-, but not OX-2R-like immunoreactivity was identified in a subset of neurones where the localisation was predominantly vesicular. Approximately 80 % of these neurones also expressed the CCK-A receptor.

Administration of orexin A (up to 30 nmol, I.A.) did not change the discharge of intestinal afferent nerve fibres in anaesthetised rats. In contrast, CCK8 (100 pmol, I.V.) stimulated the discharge of these nerve fibres. Administration of orexin A (1 nmol, I.V.) immediately prior to CCK8 significantly inhibited responses (peak increase in discharge after vehicle and CCK, 114 ± 11 % compared with CCK alone; *vs.* 72 ± 5 % after orexin A and CCK; mean ± S.E.M., $n = 5$–8, $P < 0.05$, Mann-Whitney rank sum test).

We conclude that vagal afferent neurones expressing the CCK-A receptor may also express the OX-R1, and that stimulation of the latter inhibits responses to CCK. Stimulation of food intake by orexin might therefore be mediated, partly, by suppressing CCK-evoked satiety signals from the gut.

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All procedures accord with current UK legislation.

Enterochromaffin-like (ECL) cell hyperplasia in transgenic mice with circulating progastrin but not amidated gastrin

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Multiple biologically active peptides are generated from the precursor of the gastric hormone gastrin (Dockray *et al.* 2001). In particular, the precursor peptide, progastrin, has proliferative effects that are distinct from those of the well-known acid-stimulating, COOH-terminally amidated gastrins (Wang *et al.* 1996).

In order to study the effects of progastrin *in vivo*, independently of the other gastrins, we crossed mice over-expressing a human progastrin transgene, hGas mice (Wang *et al.* 1996), with mice in which the wild-type gastrin gene had been deleted by homologous recombination, Gas-Ko mice (Koh *et al.* 1997). Offspring were back-crossed into Gas-Ko mice for at least three generations, selecting for animals that were −/− for the wild-type gene, and +/− for the progastrin transgene, i.e. hGas × Gas-Ko mice. Animals were humanely killed where appropriate.

Plasma concentrations of amidated gastrin determined by radioimmunoassay were below the limit of detection in hGas × Gas-Ko mice and control litter mates (hGas −/−) (< 10 pM). In contrast, plasma progastrin concentrations were 560 ± 150 pM (mean ± S.E.M., $n = 27$) in hGas × Gas-Ko mice compared with undetectable (< 100 pM) in control litter mates. Immunocytochemistry using antibodies to chromogranin A (CgA) revealed an approximately 3-fold increase in positive cells in hGas × Gas-Ko mice (19 ± 4 cells per mm horizontal to the gland axis, $n = 3$) compared with control litter mates (7 ± 3; $P < 0.05$, unpaired t test). In EM-immunogold studies, these antibodies labelled secretory vesicles in cells with the morphology of ECL cells. In contrast, the abundance of two other endocrine cell populations, D- and X-cells (marked by somatostatin and ghrelin, respectively), was not different from controls. Intragastric pH in both hGas × Gas-Ko mice (4.9 ± 0.2, $n = 6$) and their control litter mates (4.3 ± 0.1), was significantly higher than in wild-type mice (2.6 ± 0.2; $P < 0.05$).

Thus ECL cell hyperplasia can be associated with increased circulating progastrin in the absence of amidated gastrin. The cellular mechanisms and possible interactions between progastrin and other gastrins remain to be elucidated.

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All procedures accord with current UK legislation.

EGF enhanced transcription of the amino acid transporter hATB^0 in COS-7 cells

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System B^0 is a sodium-dependent, neutral amino acid transporter with broad specificity. The cloned transporter hATB^0 generates system B^0-like activity (Kekuda *et al.* 1996) and is regulated by epidermal growth factor (EGF) (Torres-Zamorano *et al.* 1997). We describe here the regulation of hATB^0 by EGF in COS-7 cells.

Cells were grown for 4 days in control medium, with or without 100 ng ml^{-1} EGF for the last 7 h of growth. Total RNA was extracted and hATB^0 mRNA levels were measured by semi-quantitative RT-PCR, normalised against 18S rRNA levels. Compared with control, hATB^0 mRNA was increased upon EGF supplementation by 1.63 ± 0.23f-fold (mean ± S.E.M., $n = 8$, $P < 0.05$, Student's paired t test).

The promoter region of hATB^0 was sequenced. Use of the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information, revealed a 100 % match at the 5′ end of the hATB^0 cDNA (accession number U53347) with a CpG island clone (Cross *et al.* 1994). The clone was sequenced, which identified 170 bp of the promoter region of the hATB^0 gene. BLAST searches using this promoter sequence identified a further 5 kb of the 5′-flanking region. Putative *cis*-acting binding sites were identified by use of the transcription factor matrix search programme MatInspector (Quandt *et al.* 1995). The promoter contains several GC-rich regions that bind the ubiquitous transcription factor Sp1, which has been shown to be involved in EGF-induced promoter activity.

A DNA fragment containing the region from −932 to +42 of the hATB^0 gene, which includes six putative *cis*-acting Sp1 sites, was generated by PCR and inserted 5′ to the coding region of the luciferase gene of the vector pGL3-basic (Promega), to create a reporter construct. Cells were transfected with reporter construct DNA by calcium phosphate co-precipitation and grown for 48 h in control medium, with or without 100 ng ml^{-1} EGF for the last 7 h of growth. Cells were then harvested and assayed for luciferase activity. Compared with control, hATB^0 promoter activity was increased by 1.37 ± 0.05-fold ($n = 5$, $P<0.05$) in the presence of EGF.

We conclude that the EGF-induced increase in $hATB^0$ mRNA in COS 7 cells results from increased transcription and suggest that this effect is mediated through *cis*-acting Sp1 sites.

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