The histamine H₃ receptor agonist N^m-methylhistamine produced by *Helicobacter pylori* does not alter somatostatin release from cultured rabbit fundic D-cells

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Abstract

Background—The mechanisms underlying the suppression of somatostatin dependent reflexes in *Helicobacter pylori* infection are not fully determined. The *H. pylori* product N^m-methylhistamine and inflammatory mediators such as tumour necrosis factor- α (TNF- α) may be responsible for the alterations in somatostatin release.

Aims—To examine the effect of N^m-methylhistamine on somatostatin release from cultured somatostatin-secreting D-cells.

Methods—Rabbit fundic D-cells were obtained by collagenase-EDTA digestion and enriched by centrifugal elutriation and cultured for 40 hours. The effects of N^m-methylhistamine on somatostatin release soon after stimulation (two hours) and after more prolonged exposure (24 hours) were assessed.

Results—N^m-Methylhistamine (1 nM–1 μ M) had no effect on basal or carbachol or adrenaline stimulated release over two hours. Similarly with prolonged exposure no effect on somatostatin cell content or release was identified. In contrast, TNF- α (24 hours) led to a dose dependent fall in both somatostatin content and release.

Conclusions—N^m-Methylhistamine had no direct inhibitory effects on D-cells, but TNF- α both significantly reduced the cellular content and inhibited release. Inflammatory cytokines, rather than N^m-methylhistamine, are therefore likely to be responsible for directly inhibiting D-cell function in *H. pylori* infection.

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Keywords: D-cell; *Helicobacter pylori*; H₃ receptor; N^m-methylhistamine; somatostatin; tumour necrosis factor α

Disordered gastroduodenal secretory physiology is believed to be important in the pathogenesis of *Helicobacter pylori* induced duodenal disease. Hypersecretion of gastrin and acid promotes duodenal ulceration, and, although the exact mechanism remains open to question, clearance of the infection and the associated inflammation from the stomach is associated with resolution of the physiological changes.¹ Somatostatin is the major inhibitory influence on gastrin and acid secretion, and

considerable experimental evidence has either indirectly or directly suggested that impaired somatostatin dependent inhibition is central to understanding the pathophysiology of *H. pylori* infection.

The mechanisms underlying the reduction in somatostatin secretion are not yet fully understood, but it seems likely that factors produced in the *H. pylori* infected mucosa regulate somatostatin-secreting (D-cell) function. Factors likely to inhibit D-cell function may be directly produced by *H. pylori* or liberated by the inflammatory cells infiltrating the mucosa.¹ N^m-Methylhistamine is produced by *H. pylori*, and, as an agonist at H₃ receptors, may be capable of inhibiting somatostatin secretion,² while tumour necrosis factor- α (TNF- α), which can alter endocrine cell function in other systems,³ appears to be a good candidate from the multitude of inflammatory products. We have previously shown that prolonged exposure to TNF- α leads to impaired somatostatin release from canine D-cells.⁴

The hypothesis that N^m-methylhistamine may be responsible for inhibiting somatostatin dependent pathways stems from the report of the inverse relation between mucosal N^m-methylhistamine and somatostatin levels in *H. pylori* infection,² and some data suggesting that (R)-methylhistamine (another H₃ agonist) reduces somatostatin release from preparations of gastric antrum in vitro.⁵ However, other data have suggested that the H₃ receptor does not alter gastric somatostatin and gastrin.⁶

Therefore we examined the effects of both N^m-methylhistamine and TNF- α on isolated cultured rabbit D-cells, in order to gain further understanding of how *H. pylori* infection depresses somatostatin release.

Materials and Methods

CHEMICALS

N^m-Methylhistamine was purchased from Calbiochem (Nottingham, UK), ranitidine from Glaxo (Greenford, Middx, UK), and octreotide from Sandoz (Camberley, Surrey, UK). Ham's F12/Dulbecco's modified Eagle's culture medium (50:50, v/v), glutamine, Hanks balanced salt solution, and fetal calf serum were from Gibco (Paisley, Scotland, UK). Basement membrane Matrigel was from Universal Biologicals (London, UK). All other chemicals and reagents were from Sigma (Poole, Dorset, UK).

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Figure 1 Effect of *N*^m-methylhistamine on somatostatin release from cultured D-cells. Somatostatin release over two hours in the presence of increasing concentrations of *N*^m-methylhistamine was measured in the basal state or when cells were co-stimulated with cholecystokinin 10 nM (CCK) or adrenaline (EPI) and isobutylmethylxanthine (IBMX) both 100 μ M. Values are mean (SEM), *n* = 3.

Figure 2 Effect of carbachol (10 μ M) and octreotide (10 nM) on somatostatin release stimulated by cholecystokinin (CCK; 10 nM) and adrenaline with isobutylmethylxanthine (both 100 μ M; EPI). Values are mean (SEM), *n* = 3. **p* < 0.05 compared with the stimulated somatostatin release.

Figure 3 Effect of combining *H*₁ receptor antagonism (chlorpheniramine 10 μ M), *H*₂ receptor antagonism (ranitidine 10 μ M), and the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 100 μ M) on somatostatin release from D-cells stimulated with *N*^m-methylhistamine (100 nM) (*N*^mMH). Values are mean (SEM), *n* = 3.

D-CELL ENRICHMENT AND CULTURE

Rabbit fundic D-cells were obtained from eight New Zealand White rabbits (2–2.5 kg) by sequential digestion of fundic mucosa in collagenase and EDTA and followed by enrichment by counterflow elutriation using a Beckman JE 5.0 standard elutriator rotor as previously described.⁷ The D-cell enriched fraction was then cultured in Matrigel coated 24 well tissue culture plates in complete culture medium (Ham's F12/Dulbecco's modified Eagle's medium 50:50 nutrient mix containing 10 mM Hepes, pH 7.4, 0.22% NaHCO₃, 10% fetal calf serum, 1 mg/l hydrocortisone, 8 mg/l insulin, 100 mg/l streptomycin, 100 mg/l penicillin, 100 mg/l gentamicin) at a density of 1 million cells/well. Matrigel coated wells had been previously prepared by coating each well with 100 μ l Matrigel diluted 1:7 with sterile water, allowing it to set, and then equilibrating with culture medium as previously described.⁸ The D-cell fraction was initially cultured for 40 hours in an atmosphere of 5% CO₂/95% air. After this 40 hour period, either the effect of *N*^m-methylhistamine on somatostatin release over a two hour release period or the effect of treatment with *N*^m-methylhistamine for 24 hours on subsequent release was assessed. For the prolonged treatment experiments, the wells were washed three times with release buffer (Earl's balanced salt solution containing 0.1% bovine serum albumin and 10 mM Hepes, pH 7.4) to remove dead and non-adherent cells. Then 1 ml complete culture medium was added to each well and increasing concentrations of *N*^m-methylhistamine as appropriate. Cells were cultured for a further 24 hour period. TNF- α in increasing concentrations was used as a positive control.

HORMONE RELEASE EXPERIMENTS

Release studies, whether for short term or prolonged exposure, were performed in the same way. Wells were washed three times with release medium, and then release medium and standard stimulants (or inhibitors) with *N*^m-methylhistamine as appropriate (final volume 1 ml) were added. Cells were incubated for two hours at 37°C in an atmosphere of 95% air/5% CO₂. Cholecystokinin (CCK) and adrenaline with isobutylmethylxanthine were used as positive stimulants, and carbachol and octreotide as control inhibitors. After this two hour release period, the medium was aspirated, centrifuged to pellet any non-adherent cells, and the supernatant stored at -70°C until assayed for somatostatin. Cellular somatostatin was extracted by boiling the cells in distilled water.

SOMATOSTATIN DETERMINATION

Somatostatin concentrations were determined by radioimmunoassay using K2 anti-somatostatin serum (kindly provided by Professor S R Bloom and Dr M Ghatei, RPMS, London, UK) with human ¹²⁵I-somatostatin-14 as tracer and human somatostatin-14 as standard. Half maximal inhibition of binding occurred at 2 fmol/ml. Intra-assay and inter-assay variability were respectively 7 and 8%.⁷

Figure 4 Effect of 24 hours preincubation with tumour necrosis factor- α (TNF- α) (A) and N^m-methylhistamine (B) on the somatostatin content of cultured D-cells. Results are expressed as percentage of control cells on the same culture plate. Values are mean (SEM), $n = 5$. * $p < 0.05$ compared with control (no TNF- α).

Figure 5 Effect of 24 hours preincubation with tumour necrosis factor (TNF- α) (A) and N^m-methylhistamine (B) on the somatostatin release from cultured D-cells in response to cholecystokinin 10 nM. Results are expressed as percentage of control cells on the same culture plate. Values are mean (SEM), $n = 5$. * $p < 0.05$, ** $p < 0.01$ compared with controls (no TNF- α).

CELL NUMBER AND TOXICITY

A modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to assess the effect of prolonged incubation in TNF- α or N^m-methylhistamine on cell number and viability.⁹ After the 24 hour culture period, cells were cultured in complete culture medium containing 0.5 mg/ml MTT for three hours at 37°C. The medium was then removed, and 0.04 M HCl in propan-2-ol was added to extract the resulting formazan product from the cells. The resulting optical density at 550 nm was determined.

STATISTICAL ANALYSIS

Each condition was tested on each animal preparation in duplicate wells and the somatostatin release was compared with that obtained from control wells (no TNF- α or N^m-methylhistamine treatment) from the same 24 well plate. The mean values from one animal preparation were regarded as $n = 1$, and results are the from three to five separate animal preparations. Results are expressed as mean (SEM). Values were compared by Student's paired t test. The significance level was set at $p < 0.05$.

Results

After 42 hours culture, the mean somatostatin content was 4370 (344) fmol/well, and basal and CCK stimulated release were 1.3 and 7.1% of cell content respectively. In those cells cultured for the full 66 hour period in control conditions, somatostatin cell content was 3082 (818) fmol/well. In the latter situation, the mean basal and CCK stimulated somatostatin release were 1.1 and 4.8% of cell somatostatin content respectively.

In the initial release experiments, N^m-methylhistamine had no effect on basal somatostatin release. Similarly, when D-cells were stimulated with CCK (a calcium/protein kinase C/diacylglycerol dependent stimulant) or adrenaline with isobutylmethylxanthine (cAMP-dependent stimulation), the addition of N^m-methylhistamine had no effect, positive or negative, on stimulated somatostatin release (fig 1).

Control experiments were performed with the muscarinic agonist carbachol and the somatostatin analogue octreotide, which have both previously been shown to inhibit somatostatin release from cultured D-cells.^{10 11} As shown in fig 2, both agents significantly inhibited somatostatin release stimulated by CCK (by 70 and 33% respectively) and that stimulated by adrenaline (by 80 and 49% respectively).

In a further attempt to detect any specific H₃ receptor mediated effect, which may have been obscured by H₁ or H₂ dependent effects on the D-cells or the contaminating gastric mucosal cells, experiments were performed in the presence of the selective receptor antagonists chlorpheniramine (H₁) and ranitidine (H₂). Antagonism of either or both receptors had no effect on somatostatin release in the presence of N^m-methylhistamine (fig 3). Similar results were seen with CCK stimulated cells (data not shown). The addition of the phosphodiesterase inhibitor, isobutylmethylxanthine, did not alter the effect of N^m-methylhistamine (fig 3).

Pretreatment of cultured D-cells for 24 hours with TNF- α led to a dose dependent reduction in the cellular content of somatostatin (fig 4A). This effect was greatest at 10 and 100 ng/ml when the cell somatostatin content was reduced to 82 (5) and 79 (4)% of control respectively (both $p < 0.05$). However, pretreatment of D-cells with N^m-methylhistamine had no effect on the cell content of somatostatin (fig 4B).

Figure 6 Effect of *N*^m-methylhistamine and tumour necrosis factor- α (TNF- α) on cell numbers and viability. After 24 hours incubation in test agents, viable cell numbers were measured by MTT assay. Cell numbers were quantified by measuring the optical density at 550 nm of the extracted formazan product. Values are mean (SEM), $n = 3$.

The somatostatin release following stimulation with CCK was dose dependently reduced by pretreatment with TNF- α : significant reductions in somatostatin release were seen with 1, 10, and 100 ng/ml, when somatostatin release was reduced to 75 (12), 63 (5), and 53 (8) of the untreated control respectively (fig 5A). In contrast, pretreatment of D-cells with *N*^m-methylhistamine for 24 hours had no inhibitory effect on subsequent CCK stimulated somatostatin release (fig 5B).

Incubation in TNF- α or *N*^m-methylhistamine had no effect on cell numbers or viability, as measured by the MTT assay (fig 6).

Discussion

In this study we used an isolated cultured D-cell model to examine the potential pathophysiological effects of the *H. pylori* derived metabolite *N*^m-methylhistamine on the function of somatostatin secreting cells. *N*^m-Methylhistamine appeared to have no significant effect on the cells, and the conclusions that stem from this are that it is unlikely to be responsible for the suppression of somatostatin dependent reflexes in *H. pylori* infection and that H_3 receptors do not appear to directly alter the function of fundic D-cells. As this study has provided essentially negative results, it is important to address several factors that could have contributed to a false negative. It seems unlikely that either the species studied or the methodology contributed to such a result. Isolated rabbit fundic cells have been used previously to study regulation of histamine release by the H_3 receptor,¹² and elutriation and culture have previously been used to study the function and binding of the H_3 and other receptors.^{4, 7, 11, 13} Thus damage or loss of the receptor during processing seems unlikely.

This study was performed in fundic cells, and there may be some differences in physiology between antral and fundic D-cells.¹⁴ However, in *H. pylori* infection, the depression in somatostatin is seen in both areas of the gastric mucosa,^{2, 15} and if it is hypothesised that *N*^m-methylhistamine was responsible, it would follow that the effect would not be site specific. The study population is not pure D-cells; it contains contaminating mucus cells, some chief cells, and small numbers of ECL cells and immunocompetent cells. It is possible that confounding actions of *N*^m-methylhistamine on

non- H_3 receptors might have obscured any effect, but despite H_1 and/or H_2 antagonism, no effect of *N*^m-methylhistamine was shown. Similarly, the addition of isobutylmethylxanthine had no effect on *N*^m-methylhistamine stimulated D-cells. Agonists that activate cAMP (such as adrenaline) typically produce a rather weak somatostatin response in vitro,^{4, 16} and this can be amplified by phosphodiesterase inhibitors. Thus it again seems unlikely that *N*^m-methylhistamine is directly activating a stimulatory pathway by way of the H_3 receptor at the D-cell level. The cultured D-cells were responsive to control agonists (CCK and adrenaline) and inhibitors (carbachol and octreotide), which suggests that regulatory pathways were active in the study conditions.

As we have previously shown a biphasic effect with TNF- α in canine D-cells, with prolonged TNF- α treatment inhibiting somatostatin release,⁴ and having failed to detect any effect with two hours of treatment with *N*^m-methylhistamine, we next explored the effect of prolonged exposure of D-cells to *N*^m-methylhistamine. In this situation TNF- α acted as a control and useful confirmation of previous work. TNF- α caused a reduction in both cellular content and, more markedly, release of somatostatin. Again, no effect with *N*^m-methylhistamine was shown. After 24 hours, the bioactivity of the added *N*^m-methylhistamine, as assessed by a parietal cell bioassay,⁸ was about 40%. Thus excessive degradation cannot explain the lack of effect.

These data have important implications for our understanding of the normal physiological control of gastric secretory function and specifically of the pathogenesis of *H. pylori* related diseases. The pathogenic mechanisms underlying the enhanced release of gastrin in *H. pylori* infection remain the subject of some conjecture. It is possible that several factors, including ammonia,¹⁷ inflammation,¹⁷ and specific cytokines^{18, 19} directly stimulate antral G-cells to release gastrin, but there is considerable evidence that disordered regulation of somatostatin dependent pathways is important in the hypergastrinaemia and enhanced gastric acid secretion characteristic of *H. pylori* induced duodenal ulcer disease. Reduced mucosal levels of somatostatin immunoreactive peptide^{2, 20} and mRNA²¹ occur in *H. pylori* infected tissue, and decreased antral release of somatostatin was detected in duodenal ulcer patients before the appreciation of the central importance of *H. pylori*.²² More dynamic physiological studies showing impairment of inhibitory pathways, such as those normally induced by antral distension²³ or infusion of CCK,²⁴ in *H. pylori* associated duodenal ulcer disease, seem to confirm that the somatostatin secreting cell is central to understanding the altered physiology of *H. pylori* infection.

The mechanism for the impaired D-cell function is not clear. Reduced somatostatin peptide levels have been described in colonic inflammatory bowel disease,²⁵ and several inflammatory cytokines have been reported to alter endocrine cell functions in other experimental systems.³ The data presented in this

study confirm our previous observations in canine fundic D-cells that TNF- α reduces somatostatin content and more potently inhibits stimulated release.⁴ Despite using a wide range of N^{α} -methylhistamine concentrations, encompassing the dose-response range of the H_3 receptor,²⁶ we were unable to show any inhibitory action of *H. pylori* against D-cells. The exact role of the histamine H_3 receptor in the control of gastric secretory physiology remains intriguing but not fully defined. A variety of responses have been described in different experimental systems, and not all of these results are concordant. This may partly be due to the preparations used, as the modifying effects of local gastric acid, neurocrine or paracrine actions, systemic endocrine and long neural reflexes are different between the various in vitro models and in vivo studies. Specific H_3 agonists such as $(R)^{\alpha}$ -methylhistamine inhibit gastric acid secretion induced by indirectly acting secretagogues in dogs and cats,^{6, 27-30} an effect probably explained by the presence of inhibitory H_3 receptors on the fundic ECL cell,¹³ but have no effect on acid secretion in the rat.³¹ Variable results have been reported with H_3 agonists and antagonists on gastrin and somatostatin release. One study with cultured rat, canine, and human antral fragments showed that $(R)^{\alpha}$ -methylhistamine inhibited somatostatin release and enhanced gastrin release.⁵ A reduction in somatostatin release was reported in the isolated mouse stomach,³² but studies in rat fundic fragments have shown $(R)^{\alpha}$ -methylhistamine to either have no effect³¹ or reduce somatostatin release.³³ In contrast, in vivo studies have shown that parenteral administration of the H_3 agonist $(R)^{\alpha}$ -methylhistamine inhibits gastric acid secretion but has no effect on gastrin release induced by 2-deoxyglucose or food⁶ in cats, or on basal²⁸ or bombesin²⁷ or 2-deoxyglucose²⁸ stimulated gastrin release in dogs. Parenteral administration of histamine (the endogenous H_3 ligand) to dogs led to increased release of gastric somatostatin.³⁴ This effect was abolished by omeprazole, suggesting that the effect of histamine was indirect via the release of acid and not direct via histamine receptors on D-cells. Similar results were seen with the isolated perfused mouse stomach, where the stimulation of somatostatin secretion by histamine was abolished by cimetidine or neutralisation of gastric contents.³⁵ These data are consistent with the data presented in this study.

More recently, intragastric administration of N^{α} -methylhistamine was found to have a small stimulatory effect on basal acid secretion but without any effect on gastrin release.³⁶ Additional data have shown that thioperamide, which has often been used as the prototypical H_3 antagonist, may alter neurotransmitter release by H_3 independent means.³⁷ Thus results previously attributed purely to H_3 effects may need to be reinterpreted.

N^{α} -Methylhistamine is a non-specific agonist at histamine receptors, but is rather more potent than histamine itself at the H_3 receptor.²⁶ It has been detected in the gastric

juice and mucosa of *H. pylori* infected subjects. Levels correlated inversely with somatostatin concentrations, and after eradication of *H. pylori*, they were reduced to normal.² *H. pylori* itself appears to produce the appropriate methyltransferase enzyme to catalyse the formation of N^{α} -methylhistamine.² Thus it was attractive to speculate that bacterially derived *H. pylori* may lead to impaired D-cell function. Our data do not support the presence of a functional inhibitory H_3 receptor on the D-cells, and thus we must question whether the locally produced N^{α} -methylhistamine has any functional significance. In contrast, TNF- α had direct inhibitory effects on the D-cells, and it is possible that such products of inflammation are responsible for the deranged physiology. Mucosal somatostatin levels correlate inversely with the severity of inflammation in *H. pylori* infection.²⁰ The time course of resolution of the physiological abnormalities with anti-*H. pylori* treatment also favours inflammatory mediators rather than bacterially derived factors: the hypergastrinaemia persists in the early stages of antibacterial treatment despite severe depression of *H. pylori* and clearance of the polymorphonuclear cell infiltrate, and resolution of the physiology takes longer, as does clearance of the mononuclear cell infiltrate.³⁸

These data do not completely exclude a role for the H_3 receptor and N^{α} -methylhistamine in the regulation of D-cell function, although such an effect may be indirect. It is possible that an inhibitory action of the H_3 receptor is not mediated directly on the D-cell: inhibitory H_3 receptors are often located presynaptically,³⁹ and it is conceivable that the regulatory $(R)^{\alpha}$ -methylhistamine and thioperamide in antral pieces is mediated at the level of intrinsic neurons, possibly by controlling the release of calcitonin gene related peptide,^{40, 41} or by the regulation of other paracrine influences. Isolation and cloning of the putative H_3 receptor and more detailed studies with the next generation of selective agonists and antagonists⁶ will greatly enhance our understanding of this facet of gastric physiology and *H. pylori* pathophysiology.

This is the first study to examine the regulation of somatostatin secretion by histamine and related agents at the D-cell level. We have found no evidence that N^{α} -methylhistamine directly regulates D-cell functions. It appears on the present evidence that inflammatory products rather than N^{α} -methylhistamine are responsible for the somatostatin suppression in *H. pylori* infection.

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