Effect of nicotine on rectal mucus and mucosal eicosanoids

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Abstract

Because ulcerative colitis is largely a disease of non-smokers and nicotine may have a beneficial effect on the disease, the effect of nicotine on rectal mucosa in rabbits was examined. Nicotine was given subcutaneously by an Alzet mini-pump in doses of 0.5, 1.25, and 2 mg/kg/day for 14 days to three groups of eight animals and compared with eight controls. Mean (SD) serum nicotine concentrations (ng/ml) were 3.5 (1.1), 8.8 (2.3), and 16.2 (5.2) respectively in the treated groups. The thickness of adherent mucus on rectal mucosa in controls (median 36 μ m) was significantly reduced by low dose (22 μ m, $p=0.0011$), and increased by high dose nicotine (48 μ m, $p=0.035$). Incorporation of radioactive glucosamine into papain resistant glycoconjugates was unchanged, indicating that mucin synthesis was unaltered. Prostaglandins (PG) were reduced, in some cases significantly (6-keto $\text{PGF}_{1\alpha}$, $\text{PGF}_{2\alpha}$, and hydroxy-eicosatetraenoic acid), by nicotine, which showed an inverse dose dependence – with greatest inhibition in relation to the lowest dose. Nicotine, and possibly smoking, may affect colitis by an action on mucosal eicosanoids and on adherent surface mucus secretion in the rectum and large bowel.

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relationship between smoking and colitis may lead to therapeutic advances. We have therefore examined the effect of nicotine on colonic mucus and mucosal eicosanoids in an animal model.

Methods

ANIMALS

The rabbit model was chosen because the eicosanoid profile for colonic mucosa is similar to that in man, and preliminary studies in mice failed to produce adequate plasma nicotine concentrations. Thirty two male New Zealand white rabbits weighing between 2 and 2.5 kg were allocated randomly to four groups – a control group and three treatment groups with eight rats in each group. Nicotine hydrogen tartrate (BDH Ltd, Poole, England) was given over 14 days by subcutaneous infusion in doses equivalent to 0.5, 1.25, and 2 mg/kg/day of nicotine base, dissolved in saline, in the three treatment groups and saline was given to controls. An Alzet osmotic mini-pump (model 2ML2) with an infusion rate of 5 μ l/h was implanted under halothane anaesthesia and each animal was housed separately with food and water supplied freely. One ml of blood was taken from the ear vein initially and on days 6 and 14 for measurement of plasma nicotine.¹³ Each rabbit was weighed initially and at 14 days, when they were killed and the rectum, colon, and caecum removed. Measurements were performed without knowledge of the group to which animals belonged.

MEASUREMENT OF MUCUS THICKNESS

The visible layer of adherent mucus in the rectum was measured immediately using an inverted microscope on mucosal sections 1.6 mm in thickness as previously reported.¹⁴ The rectum was chosen for these measurements because it was the only site in the large bowel where flat mucosa could be gently dissected from the underlying tissue without distortion. In the remainder of the colon, there were large folds which made measurement unsatisfactory. Measurements from each piece of resected mucosa were made on three sections using an eyepiece graticule, and a minimum of 10 readings were taken on each section. Each animal was characterised by the mean of the readings made.

MEASUREMENT OF THE MUCIN SYNTHESIS RATE

Tissue samples from the rectum were suspended in a standard culture medium which included D [^3H]-glucosamine. The methodology used

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Ulcerative colitis is predominantly a disease of current non-smokers,¹ many of whom are ex-smokers who develop their colitis after stopping smoking.² Anecdotal reports suggest an improvement in colitis when some patients start smoking again.³⁻⁵ Nicotine may be the active agent responsible for this improvement and a pilot study of transdermal nicotine administration in patients with active disease seemed to show benefit.⁶

Mucus in the colon forms a continuous adherent layer on the epithelium, acting as a barrier between the mucosal epithelial cells and the luminal contents. Since ulcerative colitis is a mucosal disease, damage by luminal contents may play a role. Factors responsible for maintaining the mucus barrier may therefore be pertinent to the pathogenesis of colitis. These include synthesis and secretion of mucin,⁷ thickness of the adherent surface layer,^{8,9} and activity of luminal proteases which are responsible for digestion of the mucus gel barrier.¹⁰ Mucosal eicosanoids may also be relevant since, by analogy with the stomach, they probably stimulate mucin synthesis and secretion.^{11,12}

Elucidation of the mechanisms involved in the

was identical to that previously described for gall bladder mucosa.¹⁵ Mucosal explants were cultured for 24 hours at 37°C in an atmosphere of 95% O₂ and 5% CO₂ in a standard tissue culture incubator. Tissues underwent papain digestion for 72 hours to isolate carbohydrate-containing segments of the mucin, followed by exhaustive dialysis to remove low molecular weight material before measurement of 3H-glucosamine incorporation.¹⁶ This procedure has previously been shown to isolate papain resistant radioactive glycoconjugates, a very large proportion of which is mucin, free from other glycoconjugates and without significant loss of mucin carbohydrate.^{15, 16} Radioactivity incorporated into mucin glycoconjugate was expressed as dpm × 10³ 3H-glucosamine incorporated per gram wet weight of mucosa.

Preliminary studies were done to justify the use of wet weights of mucosa as representative of epithelial tissue mass. Twelve samples of fresh rectal mucosa, which varied in size between 2 and 42 mg, were blotted, weighed, and dried overnight at 40°C. They were then reweighed, homogenised, and further measurements made of both the protein¹⁸ and DNA¹⁹ content. The respective correlation coefficients relating wet weight of tissue to dry weight, protein, and DNA contents were 0.79, 0.95, and 0.98.

MEASUREMENT OF EICOSANOIDS

Tissue samples from the rectum and caecum (100 to 200 mg wet weight) were stored at -70°C for subsequent analysis of eicosanoids. On analysis, each sample was minced and homogenised in 1 ml of Krebs-Henseleit buffer pH 7.4 by means of an Ultra-Turrax homogeniser (Polytron, Kinematica, Switzerland) for 20 seconds on melting ice. Total protein content was determined by a micro-scale method using an ELISA reader at 600 nm (Instruchemie, Hilversum, The Netherlands). Each tissue sample was incubated with 0.125 µCi [1-14_c] - arachidonic acid (55 µCi/µmol, Amersham, UK) together with 2 µM calcium ionophore A23187 (Sigma) at 37°C for 15 minutes. Then 3H labelled compounds of prostaglandins 6-keto PGF_{1α}, PGF_{2α}, and PGE₂, thromboxane B₂, hydroxy-5, 8, 10-heptadecatreinoic acid (HHT), and 15-hydroxy eicosatetraenoic acid (15HETE) (Amersham, UK) were added as chromatographic standards and for the determination of the recovery. Samples were centrifuged for two minutes at 1600 g at 4°C. The supernatant was applied to a Sep Pak C₁₈ cartridge (Waters Ass, USA), diluted with methanol, and dried with a Savant Speed Vac concentrator. The pellet was dissolved in 250 µl of methanol and filtered through an Anotop 0.2 µm filter into an high performance liquid chromatography (HPLC) polypropylene microvial. Altogether 100 µl were injected onto two combined Nucleosil 5C₁₈ HPLC columns (3 × 20 mm, Chrompack, The Netherlands). HPLC was performed with a Hewlett-Packard 1084B liquid chromatograph with dual pumping system. Radioactivity was measured on-line with a Berthold LB506C monitor. The solvent system contained a gradient of 0.12% tri-fluoroacetic acid and 0.2%

triethylamine in water (pH 3.0) and acetonitril (Lichrosolv, Merck, Germany). The flow rate was 0.5 ml/min at 37°C. Picofluor (Packard Canberra, USA) was used as a premixed scintillator at a flow rate of 2.25 ml/min.²⁰⁻²²

FAECAL PROTEINASE ACTIVITY

Faecal samples from the rectum and caecum were stored at -70°C before processing. Samples were thawed to room temperature, suspended in 67 mmol/l sodium phosphate buffer, pH 7.5 containing 50 mmol/l sodium chloride, and centrifuged at 18 000 g for 45 minutes at 4°C. The supernatant was retained as a faecal extract. Proteolytic activity was measured by assay of new N-terminals formed on hydrolysis of peptide bonds.¹⁰ All samples were compared with zero incubation time controls. Proteolytic activity was expressed as mmol-N-terminals/min/g faeces.

HISTOLOGY

Tissue samples from the caecum and rectum were fixed in buffered formalin. Paraffin embedded sections (5 µ) were stained by haematoxylin and eosin, Alcian blue (pH 2.5)-periodic acid Schiff (PAS), and the high iron diamine (HID)-alcian blue techniques.²³ The latter two methods allow identification of neutral mucus glycoproteins (PAS positive), non-sulphated sialomucins (Alcian blue positive), and sulphated sialomucins (HID positive). All samples were assessed histologically for morphological changes, inflammation, and alterations in mucus glycoprotein histochemistry, without knowledge of the treatment group.

STATISTICS

Each outcome measurement was compared between the four groups using the Kruskal-Wallis non-parametric one-way analysis of variance; when this was significant, differences from the control group were assessed by Mann-Whitney U tests. The change in serum nicotine concentrations from days 6 to 14 was assessed by the paired *t* test.

Results

The mean (SD) concentrations of serum nicotine (ng/ml) from two measurements on days 6 and 14 were 0.4 (0.2); 3.5 (1.1); 8.8 (2.3); and 16.2 (5.2) in the control and three nicotine groups respectively (Table I). Compared with day 6, values on day 14 had fallen by between 12 and 36%, and the difference reached significance in the medium dose nicotine group (*p* = 0.026, 95% confidence interval (CI) 0.5, 7.02). When animals were killed, an unexpected observation on opening the colon was that those given nicotine had softer stools than controls, and the change was most striking in the high dose group.

The thickness of the adherent mucus in the rectum differed highly significantly between the four groups (Kruskal-Wallis, *p* < 0.001): it was reduced (*p* = 0.0011) with low dose and increased (*p* = 0.035) with high dose nicotine (Table II).

TABLE I Plasma nicotine concentration (mean (SD), ng/ml) in control rabbits and three nicotine treatment groups given 0.5, 1.25, and 2 mg/kg/day for 14 days with measurements on days 6 and 14. Each group contained eight rabbits

Group	Day 6	Day 14	Mean (SD) of days 6+14
Control	0.3 (0.20)	0.4 (0.30)	0.4 (0.20)
Low dose	4.0 (1.2)	3.0 (1.4)	3.5 (1.1)
Medium dose	10.8 (3.2)	6.9 (2.7)	8.8 (2.3)
High dose	17.3 (5.3)	15.2 (6.1)	16.2 (5.2)

TABLE II Thickness of the visible adherent mucus on rectal mucosa (median (range)) with rates of papain resistant glycoconjugate (PRG) synthesis in control rabbits and three treatment groups given 0.5, 1.25, and 2 mg/kg/day of nicotine for 14 days. Mean synthesis rates are given as D[6-³H]-glucosamine dpm × 10³/g wet weight. Each group contained 8 rabbits. Kruskal-Wallis tests compare the four groups

Rectal mucosa	Control	Nicotine treatment			Kruskal-Wallis	
		Low dose	Medium dose	High dose	X ² ₃	p
Mucus thickness (µm)	36.0 (28.8–43.2)	21.6 (19.2–28.8)**	26.4 (19.2–40.8)	48.0 (31.2–52.8)*	19.59	<0.001
Synthesis of PRG (dpm × 10 ³ /g wet wt)	106.2 (94.2–136.6)	108.0 (65.9–131.7)	104.9 (43.6–139.9)	92.3 (46.2–141.9)	0.91	0.82

Significant differences from the control group are identified: *p<0.05, **p<0.01.

There was a steady progress of mucus thickness from low dose to high dose, with no overlap between the values recorded for these extreme doses (Fig 1; p<0.0002). There was a significant correlation between mean serum nicotine values in each animal given nicotine and the corresponding thickness of rectal mucus, r=0.71, p<0.001 (Fig 2). No significant change was found in the rate of synthesis of papain resistant glycoconjugates by rectal biopsy specimens in tissue culture (Table II).

Synthesis of several rectal eicosanoids showed inhibition which was statistically significant for 6-keto PGF_{1α}, PGF_{2α}, and 15HETE. There was again an inverse dose dependence with the greatest inhibition with the lowest dose of nico-

tine (Table III, Fig 3). Values for caecal synthesis of eicosanoids 6-keto PGF_{1α}, PGF_{2α}, PGE₂, PGD₂, TXB₂, HHT, 12HETE, and 15HETE showed no significant difference between controls and treated animals.

Faecal proteinase activity was very low, between 7 and 50 µmol N terminals/min/g dry weight for the different rectal and caecal samples; these values were at the limits of

sensitivity of the assay. Nicotine had no significant effect on the values.

No histological changes were detected in any of the treatment groups. In particular, nicotine administration was not accompanied by mucosal inflammation, changes in the morphology of the large intestinal epithelial cells, or changes in histochemical reactions of mucus glycoproteins in goblet cells.

Discussion

Subcutaneous nicotine changed the thickness of adherent rectal mucus with an inverse dose relationship – greatest inhibition was observed with the lowest dose. Rectal eicosanoids were reduced in each of the treatment groups and in some instances (6-keto PGF_{1α}, PGF_{2α}, 15HETE) also showed an inverse dose relationship. In contrast caecal eicosanoids showed no significant change and it was not technically possible to measure mucus thickness in the caecum. These changes in the rectum occurred in the absence of any morphological or histochemical change.

Although the groups were relatively small, several of the observed changes were significantly different from control values and all measurements were carried out without knowledge of the treatment group. The serum nicotine concentrations were in the lower end of the 5–60 ng/ml range typically found in smokers¹⁴; the average in the high dose group being about half the average concentration in smokers. The standard methods used to measure mucus thickness¹⁴ and tissue eicosanoids^{20–22} are well validated and known to give reproducible results.

Incorporation of radioactive glucosamine into papain resistant glycoconjugates was not changed in any of the nicotine groups. Previous studies have shown that a large proportion of this papain resistant glycoconjugate fraction is mucin^{15 16} and therefore it is reasonable to assume that no substantial changes in mucin biosynthesis occurred.

Changes in thickness of the adherent layer of mucus in the rectum after nicotine administration could theoretically be caused by differences

Figure 1: Thickness of adherent mucus on rectal mucosa in four groups of eight rabbits – control rabbits and three treatment groups given subcutaneous nicotine infusion in doses of 0.5, 1.25, and 2 mg/kg/day respectively for 14 days. Each animal is represented by a single mean value of readings in that animal.

Figure 2: Correlation between mean serum nicotine values (ng/ml) on days 6 and 14 with the rectal mucus thickness (µm) in 24 rabbits divided into three groups of eight and given doses of 0.5, 1.25, and 2 mg/kg/day of nicotine respectively for 14 days (r=0.71, p<0.001).

TABLE III Concentrations of rectal eicosanoids (median (range)) in controls and three nicotine treatment groups given 0.5, 1.25, and 2 mg/kg/day for 14 days. Prostaglandins (PG), thromboxane (Tx), hydroxy-5, 8, 10-heptadecatrienoic acid (HHT) and 15-hydroxyeicosatetraenoic acid (HETE) are given. Each group contained eight rabbits. Kruskal-Wallis tests compare the four groups

Eicosanoids (dpm/mg wet wt)	Control	Nicotine treatment			Kruskal-Wallis	
		Low dose	Medium dose	High dose	X ² ₃	p
6-keto PGF _{1α}	75.2 (15.4-120.7)	4.5 (0.8-38.4)**	16.9 (3.4-53.8)**	36.4 (5.3-93.6)	17.03	<0.001
PGF _{2α}	11.0 (1.9-39.0)	2.3 (1.1-5.0)*	3.6 (0.9-16.0)*	3.9 (0.5-23.2)	8.66	0.035
PGE ₂	3.9 (0.6-17.7)	1.8 (0-5.2)	2.0 (0-6.1)	2.4 (0-8.2)	2.05	0.56
PGD ₂	0.7 (0-2.1)	0.3 (0-1.2)	0.3 (0-3.1)	0.1 (0-3.7)	1.68	0.64
TxB ₂	0.2 (0-0.9)	0.2 (0-0.8)	0.0 (0-0.5)	0.1 (0-2.4)	0.81	0.85
HHT	26.1 (7.5-34.2)	8.2 (1.9-17.0)	15.9 (5.4-23.8)	10.4 (2.1-54.1)	7.75	0.052
15 HETE	19.9 (4.2-99.3)	0.7 (0-7.2)**	0.0 (0-9.2)**	5.1 (0-15.6)*	17.64	<0.001
12 HETE	1.1 (0-7.6)	0.0 (0-1.7)	0.4 (0-1.1)	0.0 (0-0.6)	5.18	0.16

Significant differences from the control group are identified: *p<0.05, **p<0.01.

in mucus synthesis or secretion rates, or faecal protease activity responsible for digestion of mucus.⁷⁻¹⁰ Because the results in relation to dose of nicotine we found were not initially hypothesised, it remains possible that they have arisen by chance. However, the consistency of a biphasic response for both eicosanoids and mucus makes this unlikely. The mechanisms by which low doses of nicotine are associated with reduced prostaglandin synthesis and nicotine with reversal at higher doses remain to be determined. While rates of glycoconjugate and therefore mucin biosynthesis seemed to remain unchanged, it does not follow that rates of mucus secretion were also constant. It was not possible to measure changes in the size of the intracellular preformed mucus pool which would indicate if rates of secretion were different from those of biosynthesis. Levels of faecal proteinase activity were uniformly low and would not explain the observed changes in thickness. Glycoconjugate synthesis was, of course, measured in vitro rather than in vivo using explants cultured for 24 hours: any nicotine present would probably dilute in the culture medium and one cannot entirely exclude a nicotine effect on synthesis rates. It is possible that the softer stools in those given nicotine, which were almost a slurry with high dose nicotine, could account for increased mucus thickness by reduced mechanical shear – but this would not explain the reduced thickness with low dose nicotine. Further measurements with higher doses of nicotine giving serum values similar to those observed in smokers of 20 cigarettes a day or so would be of particular

interest in clarifying this finding. Comparable measurements have not been made previously. Although Cope *et al*²⁵ showed that mucosal biopsy specimens taken at colonoscopy from patients with ulcerative colitis who do not smoke have reduced mucus production compared with tissue from non-smoking controls, but colitis patients who smoke have similar rates of production to controls. Recent measurements of faecal proteinase in man have identified values in normal control subjects and patients with ulcerative colitis, who have activities over three times those of control.^{26,27} We are not aware of previous proteinase values in herbivores, like the rabbit, but suspect that the almost total absence of proteinase may be related to the diet or anatomical features in rabbits, which have a large caecum in which carbohydrate is fermented and this may account for the observed low faecal proteolytic activity compared with man.

The uniform reduction in rectal eicosanoids, some of which were reduced with an inverse dose relationship, is difficult to explain. Similar changes were not observed in the caecum but data suggesting lower concentrations of eicosanoids have been obtained in man from rectal biopsy specimens in which healthy smokers were compared with non-smokers.²⁸ Parallel observations in the stomach have also shown reduced values in smokers.²⁹ Broncho-alveolar lavage fluids of female smokers and non-smokers have also shown a positive correlation between PGF_{2α} and TxB₂ levels and the number of ‘pack years’; in parallel with this the number of macrophages increased and gave a negative correlation to prostaglandin levels.³⁰

In patients with active ulcerative colitis, sulphated mucus glycoproteins are diminished³¹ and specific changes of mucus subfractions have been observed even in early phases of the disease.³² The bacterial enzymes which are relevant to mucus degradation include glycosidases, sulphatases, sialidases, acetyl esterases, and proteinases.^{11,33} Sulphatases have been shown in faeces from both normal subjects and patients with colitis.³⁴ Sialidases are also commonly present but probably have to act in synergy with the sialic acid 0-acetyl esterases, which have also been found in human faeces.²⁶ No convincing difference has been found between concentrations of the carbohydrate degrading enzymes in patients compared with controls, but increased concentrations of faecal proteinases have been reported in colitis.^{26,27}

The inter-relationship between mucosal eico-

Figure 3: Concentrations of 6-keto prostaglandin F_{1α} (6-keto PGF_{1α}) and PGF_{2α} in rectal tissue from four groups of eight rabbits – controls and those treated with subcutaneous nicotine infusion 0.5, 1.25, and 2 mg/kg/day for 14 days.

sanoid production and mucus synthesis has been explored in the stomach, where increases in eicosanoid values are associated with increased mucus thickness and synthesis.^{14,35} Such eicosanoid-induced increases in the thickness of the mucus barrier may play a role in protection of the gastric mucosa, particularly against pepsin damage.³⁶ Similar associations may apply in the rectum and may be relevant to the pathogenesis of ulcerative colitis. Increased endogenous secretion of mucosal prostaglandins may stimulate both synthesis and secretion of mucin, which may in turn enhance the surface barrier on the colonic mucosa. This is supported by the reduction in mucus thickness and prostenoid levels with the low dose of nicotine and the inverse dose relationship at higher nicotine levels. Observations on eicosanoids in colitis are somewhat disappointing and simply show high levels during phases of activity that return to normal with clinical remission.³⁷ It is difficult to attribute a role to agents which appear as indicators of acute inflammation but also have the potential of protecting mucosa from damage. Intraluminal PGE₂ has been shown to protect against colitis³⁸ in a rat model where colitis was induced by alcohol.

In recent years, the most striking epidemiological finding in relation to ulcerative colitis is the recognition that it is predominantly a disease of non-smokers¹ and risk of its development in ex-smokers is between four and five times the expected risk in the general population.² Elucidation of the mechanisms responsible for these observations may not establish nicotine as a 'treatment' but should open the way to new treatments that operate through similar mechanisms.

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