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SHORT COMMUNICATION

Dose-dependent effect of dietary meat on endogenous colonic *N*-nitrosation

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Human male volunteers were studied in a metabolic facility whilst they were fed randomized controlled diets. In eight volunteers there was a significant increase in faecal apparent total *N*-nitroso compounds (ATNC) and nitrite excretion ($P < 0.0001$ and $P = 0.046$, respectively) when randomized doses of meat were increased from 0 to 60, 240 and 420 g/day over 10 day periods. Mean (± SE) faecal ATNC levels were 54 ± 7 μg/day when the diets contained no meat, 52 ± 11 μg/day when the diets contained 60 g meat/day, 159 ± 33 μg/day with 240 g meat and 199 ± 36 μg/day with 420 g meat. Higher concentrations of NOC were associated with longer times of transit in the gut ($r = 0.55$, $P = 0.001$) and low faecal weight ($r = –0.51$, $P = 0.004$). There was no significant decline in levels in individuals fed 420 g meat for 40 days. The exposures found on the higher meat diets were comparable with other sources of *N*-nitroso compounds (NOC), such as tobacco smoke. Many NOC are known large bowel initiators and promotors in colon cancer, inducing G→A transitions in codons 12 and 13 of K-*ras*. Endogenous NOC formation, combined with prolonged transit times in the gut, may explain the epidemiological associations between high meat/low fibre diets and colorectal cancer risk.

The large intestine is rich in nitrogenous residues and nitrosating agents from protein metabolism and dissimilatory nitrate metabolism, respectively (1). The amount of nitrogenous residues entering the colon are known to increase with increasing protein intake (2). These residues are made available for *N*-nitrosation by colonic bacteria via nitrite and nitrate reductases (3–5). Large intestinal *N*-nitrosation does not occur in germ-free rats given nitrate as a nitrosating agent, but it has been shown to occur in rats possessing a conventional flora, showing that bacteria are necessary for large intestinal *N*-nitrosation to occur (6). In humans large intestinal *N*-nitrosation has previously been shown to increase following dosage with nitrate (7). The potential for large intestinal *N*-nitrosation could be important for carcinogenesis since many classes of *N*-nitroso compounds (NOC) have been identified, including nitrosamines, nitrosamides and nitrosoguanidines, some of which are alkylating agents known to induce GC→AT transitions at the second base of codon 12 or 13 of the K-*ras* gene (8). This mutation is common in colorectal cancer cases expressing K-*ras* mutations (8).

Abbreviations: ATNC, apparent total *N*-nitroso compounds; MTT, mean transit time; NOC, *N*-nitroso compounds; PABA, *p*-aminobenzoic acid.

Large intestinal *N*-nitrosation has previously received little attention due to analytical difficulties. The development of a group-selective method for total NOC detection, however, has allowed NOC detection in several biological fluids, including faeces (9). NOC detected by this method are referred to as apparent total *N*-nitroso compounds (ATNC) because the method may be susceptible to false positives from *S*-nitrosothiols and nitrolic acids (10). Meat is a source of nitrogenous residues entering the large intestine which are available for large intestinal *N*-nitrosation. We have therefore investigated whether increasing doses of red meat result in increased large intestinal *N*-nitrosation in human volunteers. A demonstration of such an effect would support epidemiological associations between red meat intake and colon cancer (11,12). Red meat was chosen because an initial study indicated that red, rather than white, meat initiated large intestinal *N*-nitrosation (13). We also show that levels of large intestinal *N*-nitrosation remain high when red meat is given over a 40 day period of time and that longer residence times within the colon are associated with higher levels.

Permission for the studies was given by the Dunn Nutrition Unit Ethics Committee and each volunteer signed a consent form after receiving a detailed explanation of the study protocol and aims. The two studies were carried out in a metabolic suite at separate times where all food and drink was provided and all specimens collected. Only foods and drinks which were provided by the diet technicians, weighed to the nearest gram from a specifically designed diet, were consumed. Subjects remained within the suite for breakfast and dinner and body weights were monitored to ensure a constant weight throughout. Lunches were pre-packed. In order to keep nitrate intake constant, deionized water was given throughout for drinking and used in cooking and low nitrate vegetables were used. All food was bought from the same batch and stored for later use throughout the study in order to minimize day-to-day variation.

To determine the effect of different doses of meat, eight healthy male volunteers (aged 36–49 years) were studied over four 10 day dietary periods. Four doses of meat were studied, 0, 60, 240 and 420 g/day [given as 0–100 g roast beef at lunch and 0–320 g beef (lasagne or steak) or pork (sweet and sour pork) at dinner]. The protein contents of the diets were 42, 58, 100 and 167 g/day or 8, 11, 19 and 28% total energy, respectively (14). A glucose polymer drink and cream were substituted for meat in the low meat diets to equalize the energy content. All diets were constant in fat (28% total energy) and non-starch polysaccharide (13 g/day) and adjusted for the energy needs of each subject with extra bread, low fat margarine and marmalade (14).

For each study diets were randomized using a crossover design and each subject acted as their own control. Faecal samples were collected daily and were weighed, X-rayed and stored at –20°C. Recovery of radio-opaque faecal markers was noted and used to monitor compliance and to calculate mean

Table I. Effect of different doses of red meat on parameters of colonic metabolism

	Meat intake g/day				*P* value
	0	60	240	420	
n	8	8	8	8	
MTT (h)	55.8 ± 10.1	62.1 ± 12.1	64.5 ± 14.1	66.5 ± 17.1	0.82
Mean faecal weight (g/day)	136.4 ± 19.8	143.7 ± 19.0	122.8 ± 17.3	125.1 ± 15.7	0.48
Faecal ATNC (ng/g)	444.0 ± 59.4 416.0	374.0 ± 61.4 342.3	1516.1 ± 414.3 1195.4[a,c]	1980.8 ± 567.8 1566.8[b,c]	<0.0001
Faecal ATNC (μg/day)	53.7 ± 7.2 51.2	51.7 ± 10.6 46.5	159.2 ± 33.1 135.8[b,c]	198.8 ± 36.4 180.9[b,c]	<0.0001
Faecal nitrite (μg/g)	0.18 ± 0.04	0.11 ± 0.03	0.50 ± 0.20[d]	0.42 ±0.13[d]	0.023
Faecal nitrite (μg/day)	14.9 ± 4.9	8.7 ± 3.0	50.4 ± 24.5[f]	27.2 ± 9.8[g]	0.046
Urinary N (g/day)	8.03 ± 0.3	9.44 ± 0.4	13.33 ± 0.5[b,c]	19.92 ± 0.7[b,c,i]	<0.0001
Dietary N (g/day)	6.7	9.3	16.0	26.7	

Data are arithmetic means ± SEM. Geometric means are also presented for ATNC concentration and daily excretion results.
P value for dietary effects using two-way ANOVA.
[a]$P = 0.001$ when compared with the 0 g diet. [b]$P < 0.0001$ in comparison with the 0 g diet. [c]$P < 0.0001$ in comparison with the 60 g diet. [d]$P = 0.005$ and [e]$P = 0.025$ in comparison with the 60 g diet. [f]$P = 0.01$ and [g]$P = 0.04$ in comparison with the 60 g diet. [h]$P = 0.01$ in comparison with the 240 g diet. [i]$P < 0.0001$ in comparison with the 240 g diet.

transit time (MTT) (15). Of the total faecal markers administered 96% was recovered. Mean faecal weights were determined during the final 4 days of each diet and were corrected for faecal marker output by multiplication of mean daily weight by the ratio of marker output to marker input. Faecal samples collected on days 8–10 were processed within 20 min of excretion. Samples were diluted 4-fold with ultrapure deionized water, homogenized in a stomacher (Colworth 3500; Seward) and centrifuged at 4500 r.p.m. for 10 min. Each supernatant was filtered and stored at –20°C before being analysed for NOC and nitrite by the release of nitric oxide (NO) following chemical denitrosation of each compound via thermal energy analysis (16). The sample was then treated with sulphamic acid to remove nitrite and reinjected into the refluxing solvent to determine NO released from NOC only. Nitrite was calculated by the difference between the two results. During each analysis 160 ng *N*-nitrosodipropylamine was injected into the system as an internal standard to check recovery.

Each subject completed a 24 h urine collection on day 10 of each dietary period. Compliance with the 24 h collection was checked using the *p*-aminobenzoic acid (PABA) test (17). With the exception of one sample, all 24 h collections were complete, as PABA recoveries exceeded 85%. Urinary nitrogen was determined in complete collections, using a semi-automated Kjeldahl technique.

Results were log transformed (to the base 10) and analysed using Microsoft DATA DESK 4.0 for the Macintosh. Dietary effects were determined using two-way ANOVA with diet and subject as factors and probability results less than the 0.05 level were regarded as significant. Fischer's least significant difference tests were used to compare individual means when significant *F* values were found. Possible relationships between variables were calculated using Pearson product correlation coefficients. From repeat analyses of subjects on the high (420 g) meat diet, the within person standard deviation was 56 μg/day and setting $\alpha = 0.05$ and β as 0.2, the study had sufficient power to detect an 80 μg difference in ATNC between the 60 and 420 g diets with eight subjects.

Faecal ATNC and nitrite and urinary N excretion increased significantly with higher intakes of meat (Table I) and each of these correlated positively with dietary protein ($r = 0.75$, $P < 0.0001$, $r = 0.41$, $P = 0.02$ and $r = 0.95$, $P < 0.0001$). There were no significant differences in faecal ATNC and nitrite and urinary N excretion when meat intake increased from 0 to 60 g/day. ATNC excretion on the 240 and 420 g diets was significantly greater than on the low meat diets, i.e.

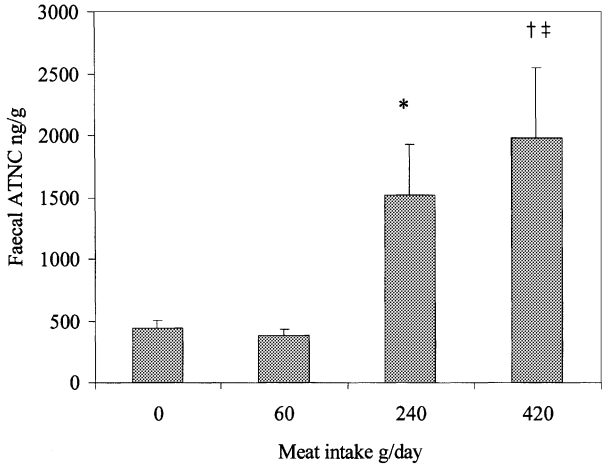

Fig. 1. Effect of increasing dose of meat on mean faecal ATNC concentration with SEM. *$P = 0.01$ in comparison with the 0 g diet; †$P < 0.0001$ in comparison with the 60 g diet; ‡$P < 0.0001$ in comparison with the 0 g diet.

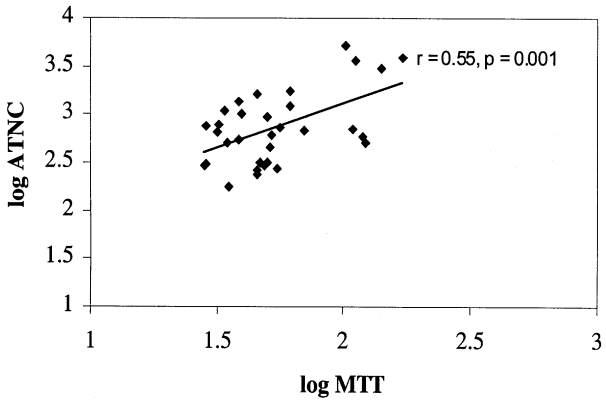

Fig. 2. Faecal ATNC concentration in relation to MTT.

0 and 60 g ($P < 0.0001$ for each comparison) (Figure 1). There was a consistent increase in ATNC with increased dose of meat for each subject. Inter-individual variation accounted for 15 ($P = 0.03$) and 35% ($P < 0.0001$) of the total variability calculated for daily ATNC excretion and ATNC concentration results with an individual range on the high meat diet of 93–427 μg/day (771–5103 ng/g). Mean faecal NO_2 excretion increased at meat intakes >60 g/day ($P = 0.023$ and $P = 0.046$ for NO_2 concentration and daily excretion, respectively). Daily NO_2 and ATNC excretion were positively associated ($r = 0.403$, $P = 0.022$). Diet accounted for 91% ($P < 0.0001$) of the total variability in daily urinary N excretion.

Overall, MTT for all eight subjects ranged from 28 to 171 h ($P < 0.0001$) between individuals and mean faecal weight from 62 to 238 g/day ($P < 0.0001$). Both parameters were negatively associated ($r = –0.73$, $P < 0.0001$). Faecal ATNC concentration was positively associated with MTT ($r = 0.55$, $P = 0.001$) (Figure 2) and inversely related to mean faecal weight ($r = –0.51$, $P = 0.004$). No significant associations between faecal nitrite excretion and MTT or mean faecal weight were evident ($P = 0.51$ and $P = 0.15$, respectively).

Previous studies have shown that increased intestinal nitrosation in response to meat occurs over a short period of time, 5 days (13). To check that 10 days was a sufficient time to investigate the effects of meat, four more volunteers were maintained in the metabolic suite for a 40 day period whilst consuming the 420 g meat diet using the same experimental conditions as above. Faecal samples were taken for ATNC determination as before on days 10, 20, 30 and 40. Mean (± SE) levels of ATNC were 149 ± 53, 148 ± 51, 223 ± 47 and 278 ± 107 μg/day, respectively. Mean levels did not decline with time. The difference between days 10 and 40 on paired *t* testing was not significant ($P = 0.460$).

Taking account of two previous studies from our laboratory, the influence of red meat on faecal ATNC excretion has now been shown in 24 healthy male volunteers, all of whom were studied in a metabolic suite where diet could be carefully controlled (13,18). The present study shows that this association is dose responsive. At the higher levels of meat consumption concentrations of ATNC were found to be of the same order of magnitude as the concentration of tobacco-specific NOC in cigarette smoke (19). Levels of ATNC in foods are low and a diet containing 600 g red meat/day contained only 13 μg ATNC/day (18). Faecal ATNC levels exceeded this value by as much as 30-fold for some subjects, showing that faecal ATNC excretion during the study was due to endogenous formation.

Endogenous formation appears to be the most potent source of human exposure to NOC following the reaction between nitrosating agents and nitrogenous substrates (19). *N*-nitrosation can occur under acid, neutral or inflammatory conditions and hence NOC are formed at a number of sites in the body (20). In the large intestine endogenous nitrosation is brought about by bacteria present in the large intestine, as has been shown by studies with germ-free animals (6). Even with the no meat diet endogenous nitrosation occurs, because even at low protein intakes there are significant dietary and non-dietary protein residues entering the large bowel from sloughed cells, mucin and enzymes which would be available for proteolysis and nitrosation (2). However, with only 60 g meat/day there was no significant elevation in ATNC or nitrite. Protein intakes were similar on the no and 60 g meat diets so that the amounts of protein residues available for nitrosation reaching the intestine would have been similar. The lack of effect of low doses of meat may be important in formulating public health recommendations for the consumption of meat in relation to cancer (11,12). At higher meat intakes arginine from meat may also influence *N*-nitrosation, as many mammalian cells produce NO by oxidation of the terminal guanido nitrogen of L-arginine to citrulline by NO synthase in the presence of oxygen and NADPH (21). 420 g of meat is calculated to provide ~7.3 g arginine, as compared with 1.04 g from 60 g meat (22).

Despite the highly controlled conditions of this study, there remained extensive individual variation in faecal ATNC excretion which might be attributable to individual variations in gut flora, with high responders harbouring high populations of nitrate- and nitrite-reducing bacteria. However, a major determinant of individual variation in levels was length of residence in the gut, as ATNC concentration correlated positively with MTT (Figure 2). A longer retention in the gut would favour increased ATNC formation due to more efficient bacterial protein degradation leading to increased nitrosatable substrates.

Ongoing work is investigating the genotoxic effects of faecal NOC levels. In preliminary work, seven other volunteers were maintained on the 420 g meat diet and faecal water isolated and genotoxicity assessed by Comet assay using the method of Venturi *et al*. (23). Samples from six of seven subjects were genotoxic, with mean tail moments of 16–24 units/sample (24). This is a comparatively high percentage genotoxicity, compared with the study of Venturi *et al.*, where only 43% of samples were genotoxic (23). A diet high in fat and meat but low in dietary fibre has also been shown to increase the genotoxic potential of faecal water collected from healthy human volunteers (25). Findings from the present study suggest that NOC produced in response to high meat diets may be one factor contributing to the genotoxic effects of these diets.

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