*Advances in Brief*

# Haem, not Protein or Inorganic Iron, Is Responsible for Endogenous Intestinal *N*-Nitrosation Arising from Red Meat

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## Abstract

Many *N*-nitroso compounds (NOC) are carcinogens. In this controlled study of 21 healthy male volunteers, levels of NOC on a high (420 grams) red meat diet were significantly greater ($P = 0.001$) than on a low (60 grams) meat diet but not significantly greater when an equivalent amount of vegetable protein was fed. An 8-mg supplement of haem iron also increased fecal NOC ($P = 0.006$) compared with the low meat diet, but 35-mg ferrous iron had no effect. Endogenous *N*-nitrosation, arising from ingestion of haem but not inorganic iron or protein, may account for the increased risk associated with red meat consumption in colorectal cancer.

## Introduction

Red and processed meat intake is associated with increased risk of colorectal cancer (1). Our previous studies in humans have established that red but not white meat stimulates endogenous intestinal *N*-nitrosation and that there is a dose response (2–4). This could be important for carcinogenesis in the large bowel because many classes of NOCs[2] have been identified, including nitrosamines, nitrosamides, and nitrosoguanidines, most of which are known carcinogens. After eating meat, the large intestine is rich in nitrogenous residues and nitrosating agents from protein metabolism and bacterial dissimilatory nitrate metabolism. In the present study, we show that it is haem iron, not protein residues or inorganic iron, that stimulates endogenous NOC production.

## Materials and Methods

Two studies were carried out with volunteers living in a metabolic suite where all food and drink was provided and all specimens collected. The Cambridge Local Research Ethics Committee gave permission for the studies, and each volunteer signed a consent form after receiving a detailed explanation of the study protocol and aims. All volunteers were given a medical and completed a health and lifestyle questionnaire; subjects with a history of gastrointestinal disease or recent antibiotic use were excluded. Only foods and drinks provided by diet technicians, and weighed to the nearest gram from a specifically designed diet, were consumed. Body weights were monitored to ensure a constant weight throughout. 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 to minimize day-to-day variation. All studies were conducted as randomized, cross-over designs, and each subject acted as their own control. All diets were adjusted for energy needs of each subject with extra bread, low fat margarine, and marmalade.

To determine the effects of protein in Protocol 1, 12 healthy male volunteers (age range of 25–74 years) were studied over three 15-day periods. A 60-gram red meat, 420-gram red meat, and vegetarian diet containing the same amount of protein as the 420-gram red meat diet were studied. The vegetarian diet had the meat substituted with egg, peanuts, low fat cheese, kidney beans, and green lentils. The rest of the diet was balanced to match the energy, fat, and fiber content of the other two diets; in particular, white bread was used instead of wholemeal bread. Each diet was constant in fat (30% total energy) and fiber (as nonstarch polysaccharides 23–26 grams). The 60-gram meat diet contained 65-gram protein, and the high meat and vegetarian diets contained 143–150-gram protein. A glucose polymer drink and cream were substitutes for meat during the low meat diets to equalize the energy content. To determine the effects of haem and inorganic iron in Protocol 2, 9 healthy male volunteers (age range of 24–74 years) were studied, also over three 15-day periods. A 60-gram red meat diet (containing 9.9 mg/day iron) was used throughout. A supplement of 7.8-mg haem iron, as 50-gram liver pate and 70-gram blood sausage, to match the iron content of the 420-gram red meat diet (17.7 mg/day) was given in a second dietary period. A daily 300-mg ferrous gluconate tablet (35 mg of ferrous iron) supplement was given in a third. Protein contents of the three diets were 66, 76, and 66 grams, respectively, per day.

Fecal samples were collected daily, weighed, X-rayed, and stored at $-20°C$. Recovery of radio-opaque fecal markers was noted and used to monitor compliance and calculate Mean Transit Time (5). Mean fecal weights were determined during the final 4 days of each diet and corrected for fecal marker output by multiplication of mean daily weight by the ratio of marker output to marker input. Previous studies have shown that increases in fecal NOC occur within 5 days of dietary change (4), and to allow for adaptation after dietary cross-overs, samples from the first 10 days (equivalent to three to five transits through the gut) of each dietary arm were not analyzed for NOC. Fecal samples collected on days 10, 13, and 15 were immediately frozen on dry ice and processed within 48 h. Samples were diluted 4-fold with ultra-pure deionized water, homogenized in a stomacher (Colworth 3500, Seward), and centrifuged at 4500 rpm for 10 min. Each supernatant was filtered and stored at $-20°C$ before being analyzed for NOC and nitrite by the release of NO after chemical denitrosation of each compound via Thermal Energy Analysis (6). Results for NOC are presented as ATNC expressed as the concentration of the common unit of structure, NNO, as µg/kg. The sample was then treated with sulfamic 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 of *N*-nitroso dipropylamine was injected into the system as an internal standard to check recovery. Acidified supernatants were stored at $-20°C$ and analyzed for ammonia (Ammonia diagnostic kit 171; Sigma, Poole, United Kingdom).

Statistical analysis was carried out using Excel for Microsoft Office 2001 and SPSS version 10.0. Two-way ANOVA was used to determine the effects of diet and differences between individual responses. When an effect of diet was apparent by two-way ANOVA, paired Student's *t* tests were carried out. Pearson's product moment correlation coefficient was used to detect relationships between variables. We also analyzed the data treating "volunteer" as a random effect. There were no differences in the effect of diet between these two statistical models. Two-tailed probability results $< 0.05$ significance level were regarded as significant. From repeat analyses on subjects on high (420 grams) meat diets, the within-person SD was 56 µg/day, and setting $\alpha = 0.05$ and $\beta$ as 0.2, the study had sufficient power to detect 65- and 75-µg differences in ATNC between study periods with 12 and 9 subjects, respectively.

The recoveries of fecal markers (97.9 and 97.7% for Protocols 1 and 2, respectively) and correlation between dietary nitrogen and 24-h urinary nitro-

gen ($r = 0.986$ and 0.709 for Protocols 1 and 2, respectively), from samples collected on days 10, 13, and 15, suggest a high degree of dietary compliance.

Three volunteers did not wish to take part in the full protocol, so these individuals were randomized to the low meat and low meat supplemented with haem dietary periods. Therefore, results for inorganic iron are only available for 6 subjects.

## Results and Discussion

Table 1 shows that in Protocol 1, fecal ATNC concentration was significantly greater on the high meat diet compared with the low meat diet ($P = 0.001$) but that the difference in ATNC concentration between the low meat and vegetarian diet was not significant ($P = 0.2$). Fig. 1 shows that there was individual variation in the extent of response to red meat but that all individuals had increased fecal ATNC levels on the high red meat diet. The low individual ATNC levels on the vegetarian diet were similar to those found in response to the low meat diet. The table also shows that, in Protocol 2, haem had a highly significant effect on increasing fecal ATNC output when compared with the low meat diet ($P = 0.006$). However, inorganic iron had no effect. Fig. 2 shows that all individuals had increased fecal ATNC levels on the high haem diet, but all individuals had low levels of fecal ATNC on the inorganic iron diet. The individual variation in extent of response to the high haem diet is also apparent in Fig. 2.

The table shows that differences in fecal nitrite between the low and high red meat diet in Protocol 1 were significant, but there were no other differences between diets. There were no significant differences in fecal weight or Mean Transit Time between study periods, apart from an increase in fecal weight from $165.4 \pm 22.8$ to $211.4 \pm 27.2$ grams/day ($P = 0.018$) between the low meat and vegetarian diets in Protocol 1. Fecal ammonia was higher on the high protein red meat and vegetarian diets than on the low protein low red meat diet; $32.4 \pm 4.2$ and $26.3 \pm 4.4$ compared with $20.3 \pm 3$ mmol/liter ($P = 0.007, 0.062$), and $5.3 \pm 0.7$ and $5 \pm 1$ compared with $2.9 \pm 0.4$ mmol/day ($P = 0.011, 0.015$), respectively. However, there were no differences in fecal ammonia between the high protein diets of Protocol 1 or the low iron and iron-supplemented diets of Protocol 2.

Taking account of five previous studies from our laboratory, the influence of red meat on fecal ATNC excretion has now been shown in >60 healthy male volunteers, all of whom were studied in a
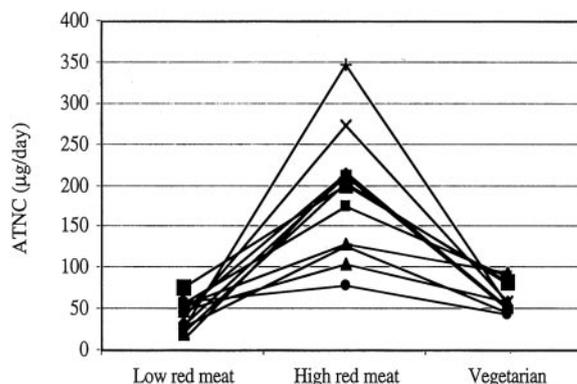


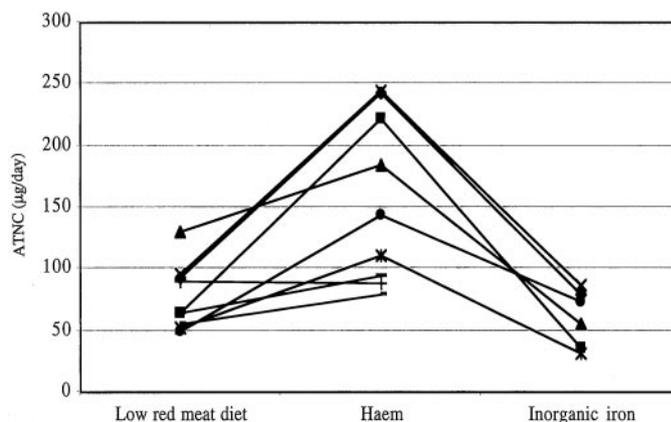Fig. 1. Protocol 1, mean faecal ATNC excretion for each dietary period (*lines* represent individuals).



Fig. 2. Protocol 2, mean faecal ATNC excretion for each dietary period (*lines* represent individuals).

metabolic suite where diet could be carefully controlled (2–4, 7, 8). The direction of an increase with increasing red meat is consistent in nearly all individuals. Furthermore, there is a dose response, which occurs at normal levels of 120-, 240-, and 420-gram red meat/day (2, 3). At the higher levels of red meat consumption, concentrations of ATNC are as the same order as the concentration of tobacco-specific NOC in cigarette smoke (4). We have shown previously that fermentable carbohydrate does not change fecal ATNC output (4, 7, 8) and that it is red, not white, meat which is responsible for the effect (2). It has been established that ATNC levels arise endogenously, because high red meat diets containing 600-gram meat per day provide only 13 $\mu$g of preformed ATNC per day (7).

We postulated that an increase in meat consumption would increase the amount of nitrogen residue reaching the large intestine, so that the substrates for nitrosating agents from protein metabolism and bacterial dissimilatory nitrate metabolism, and hence NOC levels, would increase (9). Fecal ammonia, also implicated in carcinogenesis, increased in this study in response to increased meat, as expected (9). To determine whether the reason for the increase in endogenous *N*-nitrosation arising from increased meat consumption was attributable to protein, we fed 143–150 grams of protein from mainly red meat or vegetarian sources in Protocol 1. However, endogenous NOC production increased when subjects were on the red meat diet, but the protein from vegetarian sources had no effect.

Our previous work was based on the supposition that *N*-nitrosation was brought about by bacteria colonizing the large intestine, because a study with germ-free rats showed that a normal microbial flora was required for endogenous *N*-nitrosation to occur (10). A number of

Table 1 *Mean ($\pm SE$) faecal ATNC ($\mu$g/kg or $\mu$g/day) as NNO and nitrite for the two dietary protocols*

### A. Protocol 1

|  | Low red meat diet $n = 12$ | High red meat diet $n = 12$ | Vegetarian diet $n = 12$ |
|---|---|---|---|
| ATNC ($\mu$g/kg) | $301.6 \pm 48.3$ | $1279.5 \pm 238.9^a$ | $349.6 \pm 46.8^b$ |
| ATNC ($\mu$g/day) | $42.1 \pm 5.3$ | $190.1 \pm 21.6^e$ | $63.3 \pm 5.3^b$ |
| Nitrite ($\mu$g/kg) | $221.3 \pm 37.0$ | $578.0 \pm 104.1^h$ | $300.7 \pm 69.6$ |
| Nitrite ($\mu$g/day) | $39.7 \pm 8.5$ | $90.1 \pm 15.0$ | $70.0 \pm 24.6$ |

### B. Protocol 2

|  | Low red meat diet $n = 9$ | Haem supplement $n = 9$ | Inorganic iron supplement $n = 6$ |
|---|---|---|---|
| ATNC ($\mu$g/kg) | $766.4 \pm 232.6$ | $1438.0 \pm 344.8^c$ | $852.3 \pm 392.9^d$ |
| ATNC ($\mu$g/day) | $77.5 \pm 9.0$ | $156.8 \pm 22.7^f$ | $60.7 \pm 9.5^g$ |
| Nitrite ($\mu$g/kg) | $267.6 \pm 31.3$ | $712.4 \pm 161.9$ | $388.8 \pm 116.2$ |
| Nitrite ($\mu$g/day) | $33.5 \pm 4.2$ | $94.9 \pm 29.7$ | $43.4 \pm 19.5$ |

[a] $P = 0.001$ low *versus* high red meat diet.
[b] $P < 0.001$ vegetarian *versus* high meat diet.
[c] $P = 0.006$ low meat *versus* haem diet.
[d] $P = 0.004$ inorganic iron *versus* haem diet.
[e] $P < 0.0001$ low *versus* high red meat diet.
[f] $P = 0.004$ low meat *versus* haem diet.
[g] $P = 0.001$ inorganic iron *versus* haem diet.
[h] $P = 0.007$ low *versus* high red meat diet.

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facultative and anaerobic bacteria from healthy humans, including those from feces, are able to catalyze the formation of NOCs at neutral pH via nitrate reductase (11, 12). The activity of this enzyme has been positively correlated with nitrosating ability (13), shown to vary ≤8-fold among individuals (14), and could thus explain individual variability in fecal ATNC levels. In this study, ATNC levels in some people were increased by as much as seven times over baseline values, whereas other levels only increased by ~1.5 times (Figs. 1 and 2).

Red meat also contains iron, which is an integral part of bacterial nitrate reductase and could also explain the effect of red meat. Rats harboring a human fecal flora in the intestine and fed human diets showed a 3-fold increase in fecal nitrate reductase activity with a 3-fold increase in meat consumption (15). However, in Protocol 2, supplements of either haem iron or inorganic ferrous iron showed that only haem iron increased endogenous *N*-nitrosation. *N*-nitrosohaemo-globin and *N*-nitrosomyoglobin can be formed from the reaction of nitrite with hemoglobin and myoglobin (16). NO has also been shown to react directly with hemoglobin and myoglobin to produce NOCs (17). More specifically, the reaction of a haem containing mutant cytochrome-*c*-peroxidase with peroxide gave a product capable of oxidizing *N*-hydroxyguanidine or *N*ω-hydroxyarginine, resulting in the NOC *N*-nitrosoarginine (18). The finding that haem has an independent effect suggests that chemical catalysis, in addition to bacterial *N*-nitrosation, is responsible for the dose-dependent effect of red meat on increasing endogenous intestinal *N*-nitrosation. Should the NOCs formed endogenously in the intestine as a result of haem consumption be shown to be mutagenic or carcinogenic, this might explain the association between red meat consumption and large bowel cancer risk.

## Acknowledgments

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