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

Amanda Jane Cross, Jim R. A. Pollock, and Sheila Anne Bingham

Medical Research Council, Dunn Human Nutrition Unit, Wellcome Trust/MRC Building, Cambridge CB2 2XY [A. J. C., S. A. B.], and Pollock and Pool Ltd., Reading RG5 4DX [J. R. A. P.]. United Kingdom

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 NOCs2 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 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 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


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 $\sim 20^\circ$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 diet 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 $\sim 20^\circ$C before being analyzed for NOC and nitrate 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 $\mu$g/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 $\sim 20^\circ$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 $\mu$g/day, and setting $\alpha = 0.05$ and $\beta$ as 0.2, the study had sufficient power to detect 65- and 75- $\mu$g 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-
HAEM IS RESPONSIBLE FOR ENDOGENOUS N-nITROSATION

The direction of an increase with increasing red meat is consistent in
metabolic suite where diet could be carefully controlled (2–4, 7, 8). The
direction of an increase with increasing red meat is consistent in
metabolism could be carefully controlled (2–4, 7, 8).

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

Table 1 Mean (±SE) faecal ATNC (µg/kg or µg/day) as NNO and nitrite for the two
dietary protocols

A. Protocol 1

<table>
<thead>
<tr>
<th></th>
<th>Low red meat</th>
<th>High red meat</th>
<th>Vegetarian</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATNC (µg/kg)</td>
<td>n = 12</td>
<td>n = 12</td>
<td>n = 12</td>
</tr>
<tr>
<td></td>
<td>301.6 ± 48.3</td>
<td>1279.5 ± 238.9</td>
<td></td>
</tr>
<tr>
<td>ATNC (µg/day)</td>
<td>n = 12</td>
<td>n = 12</td>
<td>n = 12</td>
</tr>
<tr>
<td></td>
<td>42.1 ± 5.3</td>
<td>190.1 ± 21.6</td>
<td></td>
</tr>
<tr>
<td>Nitrite (µg/kg)</td>
<td>n = 12</td>
<td>n = 12</td>
<td>n = 12</td>
</tr>
<tr>
<td></td>
<td>221.3 ± 37.0</td>
<td>578.0 ± 104.3</td>
<td></td>
</tr>
<tr>
<td>Nitrite (µg/day)</td>
<td>n = 12</td>
<td>n = 12</td>
<td>n = 12</td>
</tr>
<tr>
<td></td>
<td>39.7 ± 8.5</td>
<td>90.1 ± 15.0</td>
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B. Protocol 2

<table>
<thead>
<tr>
<th></th>
<th>Low red meat</th>
<th>Haem supplement</th>
<th>Inorganic iron supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATNC (µg/kg)</td>
<td>n = 9</td>
<td>n = 9</td>
<td>n = 6</td>
</tr>
<tr>
<td></td>
<td>766.4 ± 232.6</td>
<td>1438.0 ± 344.8</td>
<td>852.3 ± 392.93</td>
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<tr>
<td>ATNC (µg/day)</td>
<td>n = 9</td>
<td>n = 9</td>
<td>n = 6</td>
</tr>
<tr>
<td></td>
<td>77.5 ± 9.0</td>
<td>156.8 ± 22.7</td>
<td>60.7 ± 9.5</td>
</tr>
<tr>
<td>Nitrite (µg/kg)</td>
<td>n = 9</td>
<td>n = 9</td>
<td>n = 6</td>
</tr>
<tr>
<td></td>
<td>267.6 ± 31.3</td>
<td>712.4 ± 161.9</td>
<td>388.8 ± 116.2</td>
</tr>
<tr>
<td>Nitrite (µg/day)</td>
<td>n = 6</td>
<td>n = 6</td>
<td>n = 6</td>
</tr>
<tr>
<td></td>
<td>33.5 ± 4.2</td>
<td>94.9 ± 29.7</td>
<td>43.4 ± 19.5</td>
</tr>
</tbody>
</table>

* P = 0.001 low versus high red meat diet.
* P < 0.001 vegetarian versus high meat diet.
* P = 0.006 low meat versus haem diet.
* P = 0.004 inorganic iron versus haem diet.
* P < 0.0001 low versus high red meat diet.
* P = 0.004 low meat versus haem diet.
* P = 0.001 inorganic iron versus haem diet.
* P = 0.007 low versus high red meat diet.
facultative and anaerobic bacteria from healthy humans, including those from feaces, 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-nitrosohaemoglobin 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 Nω-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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References

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