N-Nitroso compounds in the gastrointestinal tract of rats and in the feces of mice with induced colitis or fed hot dogs or beef

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Because colonic N-nitroso compounds (NOC) may be a cause of colon cancer, we determined total NOC levels by Walters’ method in the gastrointestinal tract and feces of rodents: (i) feces of C57BL mice fed chow and semi-purified diets contained 3.2 ± 0.4 and 0.46 ± 0.06 NOC/g, respectively (P < 0.01, mean ± SD). (ii) NOC levels for gastrointestinal contents of three groups of Sprague-Dawley rats fed chow diet were 0.9 ± 0.05 (diet), 0.2 ± 0.0 (stomach), 0.3–0.4 (small intestine), 0.7–1.6 (cecum and colon) and 2.6 ± 0.6 (feces) nmol/g. NOC precursor (NOCP) levels (measured as NOC after mild nitrosation) for two rat groups fed chow diet showed a 16-fold increase from stomach to proximal small intestine (mean, 6.2 µmol/g), and a 1.7-fold increase from distal colon to feces (mean, 11.6 µmol/g). (iii) Eight Min and five C57BL/6J mice received 4% dextran sulfate sodium in drinking water on days 1–4 to induce acute colitis. This increased fecal NOC levels 1.9-fold on day 5 in both strains (P < 0.04), probably due to NO synthase-derived nitrosating agents in the colon. (iv) Following studies on humans fed beef [Hughes et al. (2001) Carcinogenesis, 22, 199], Swiss mice received semi-purified diets mixed with 18% of beef plus pork hot dogs or sautéed beef for 7 days. On day 7, individual 24-h fecal NOC outputs were determined. In three hot dog and two beef groups with 5 mice/group, mean fecal NOC output/day was 3.7–5.0 (hot dog) and 2.0–2.9 (beef) times that for control groups fed semi-purified diet alone (P < 0.002 for each of combined groups). These groups showed little change in fecal NOCP output. (v) Initial purification of rat fecal NOCP by adsorption–desorption and HPLC is described. Results should help evaluate the view that colonic NOC causes colon cancer associated with colitis and ingestion of red and nitrite-preserved meat.

Introduction

N-Nitroso compounds (NOC) include nitrosamines and nitrosoamides. They are produced by the reaction of nitrite and nitrogen oxides with secondary amines and N-alkylamides, i.e. with ‘NOC precursors’ (NOCP). We report here studies on NOC and NOCP in the gastrointestinal tract (GIT) and feces of rats and mice. A basic question was to determine whether NOC in the GIT and feces arise mainly from unabsorbed dietary NOC or by in vivo nitrosation of NOCP in the GIT. Carcinogenic NOC may be involved in the etiology of several types of cancer, including those of the stomach and esophagus (1). In 1996 Bingham et al. (2,3) proposed that NOC in colonic contents are a cause of colon cancer. In earlier studies involving the determination of total NOC in human feces by Walters’ method (4), mean total NOC levels were 8.4 nmol/g of human feces compared with 1.0–1.5 nmol/g of fasting gastric juice (5), and feeding nitrate raised mean fecal NOC levels from 109 (with a low nitrate diet) to 7.0 nmol NOC/g (6). In the only related animal study, the colonic contents of conventional and germ-free rats fed a semi-purified (SP) diet showed mean values of 0 and 0.9 µmol NOC/kg, respectively, and colonic NOC levels in conventional but not germ-free rats rose (to a mean of 6.6 µmol/kg) when 500 mg nitrate/liter drinking water was fed (7).

In 2001 we described improvements and simplifications of the Walters’ method for determining total NOC and described a method for measuring NOCP by nitrosation of aqueous food extracts under mild conditions (110 mM nitrite, pH 1.5–2.0, incubation for 1 h at 37°C), addition of sulfamic acid to destroy excess nitrite and analysis for NOC (8). Nitrosation under these conditions gave poor NOC yields when the NOCP were simple secondary amines and N-alkylureas, but high (≥45%) NOC yields when the NOCP was a rapidly nitrosated amine such as morpholine, or was N-1-fructosyl valine. We examined hot dogs (franks, frankfurters, wiener and sausages) because they are a widely consumed nitrate-preserved meat product, with US sales of >800 million pounds in 2001 (9). We purified the NOCP in hot dogs by adsorption–desorption and HPLC (10). After the purified fractions were nitrosated and treated with sulfamic acid, they were directly mutagenic for Salmonella typhimurium TA-100, with up to a 4-fold increase in mutagenicity ([10] and unpublished studies). In part, because some N-nitrosoglycosyl amine are direct mutagenic (11,12), we suggested that the NOCP in hot dogs are N-glycosyl amino acids (Figure 1) and N-glycosyl peptides (10).

Red meat (beef, pork and mutton) and, especially, processed red meat (most of which is probably preserved with nitrite) are probable risk factors for colon cancer (14). A recent meta-analysis of 13 studies concluded that the risk of colorectal cancer was increased 12–17% by daily ingestion of 100 g red meat and was increased by a mean of 49% on daily ingestion of 25 g processed meat (15). Another recent review reached a similar conclusion (16), but a third review disputed this conclusion and emphasized that meat is a nutritionally important component of most Western diets (17). Fecal NOC excretion in humans increased 3.7-fold when 420 g/day of beef was consumed, did not decline even after this diet was consumed for 40 days, showed a dose–response relationship for the amount of beef consumed and was not affected when chicken

Abbreviations: DSS, dextran sulfate sodium; Exp., Experiment; GIT, gastrointestinal tract; INOS, inducible nitric oxide synthase; NO, nitric oxide; NOC, N-nitroso compounds; NOCP, NOC precursors; SP, semi-purified.
derivatives can rearrange to fructosyl derivatives by the Amadori reaction (28).

Some of our results were presented at national meetings.

Inflammatory bowel disease, colitis have been developed in mice and the small bulk of fecal NOC and NOCP because the most useful models of inflammatory bowel disease, colonic iNOS may (24). During inflammation, nitric oxide synthase (iNOS) may produce excess NO, which is oxidized to nitrogen oxides and nitrite, which in turn could react with NOCP in colonic contents to produce NOC. These NOC could initiate colon cancer. Acute and chronic colitis were induced in mice by treating mice for 4 days with 4% DSS (26). We applied the latter treatment to Min (multiple intestinal neoplasia) and their wild-type C57BL/6J mice, because Min mice spontaneously develop adenomas of the small intestine and, to some extent, the colon (27), and these tumors could be induced by NOC formed in the intestines.

Rats were used here to study NOC and NOCP levels in the GIT because it was easier to obtain useful amounts of GIT contents from these animals than from mice. Mice were used to study the effects of colitis and of feeding hot dogs and beef on fecal NOC and NOCP because the most useful models of colitis have been developed in mice and the small bulk of their feces made it easy to dry and analyze 24-h samples. Some of our results were presented at national meetings (28-30).

Materials and methods

Animals, diets and general procedures

We used male Sprague–Dawley rats and male mice of various strains, with both species aged 6–9 weeks, except in Experiment (Exp.) 4. The animals were obtained from the National Institutes of Health unless mentioned otherwise. To prevent coprophagy (eating the feces), in most tests the animals were kept in cages fitted with stainless steel wire grids mounted 2.5 cm from the bottom of the cages, with absorbent paper under the grids to soak up split water and urine. Feces excreted over 24 h (except in Exp. 1) were collected with tweezers after removing most of the split diet with a kitchen strainer.

The diets were supplied by Harlan Teklad (Madison, WI) except in Exp. 4. The chow diet was pelleted Sterilizable Rodent Diet W-8656, which contained 24.5% (by weight) of protein, 4.4% fat, 3.8% fiber, 7.4% ash, 47% nitrogen-free extract and added vitamins and minerals. Pelleted TD-98061 semi-purified (SP) diet was based on the AIN-76A diet (31), with replacement of sucrose by maltodextrin to mimic human diets more closely and still be able to pellet the mixture. TD-01407 SP diet was powdered and was a modification of the AIN-93G diet (32). The TD-98061 diet was pelleted, respectively, in 2-kg, 200 and 214 casein, 378 and 417 cellulose, 150 and 161 maltodextrin, 120 and 0 sucrose, 0 and 61 dextrose, H2O, 50 and 0 corn oil, 0 and 24 soybean oil, 50 and 61 cellulose, 35 and 0 AIN-76 mineral mixture, 0 and 43 AIN-93G-MX mineral mixture, 10 and 0 AIN-76A vitamin mixture, 0 and 12 AIN-93-VX vitamin mixture, 3 and 0 m-methionine, 2 and 0 CaCO3, 2 and 3 choline bitartrate, and 0 and 0.017 2-butyldihydroxyquinone. Energy in Exp. 4, the animals received tap water for drinking; this contained ~3 mg nitrate (as NO3−)/l according to our local water company. In chemical experiments, suspensions were mixed with a Vortex-Genie mixer (Fisher Scientific, Pittsburgh, PA).

Analysis for NOC and NOCP

Samples were stored at ~15°C for up to 3 weeks before analysis. For all analyses, up to 1 g of diet, GIT contents or feces were weighed, dried overnight at room temperature and <1 Torr (this brought the samples to constant weight) and weighed again. Mouse fecal pellets were then freed of adhering diet by gentle rubbing with tissue paper. We analyzed several pellets of rats and mice or individual rat fecal pellets in Exp. 1: 24-h fecal collections from mice in Exps 2, 4 and 5; and 1 g of diet, homogenized sections of the GIT contents or several fecal pellets totaling ~1 g from rats in Exp. 3. The samples were soaked for 30 min in a measured volume (6–10 ml) of distilled water, vortexed, blended for 2 min in a motor-driven Polytron, centrifuged at 2000 g for 15 min, and acidified with excess sulfamic acid, diluted 100 times with distilled water and analyzed with excess sulfamic acid in water to destroy any nitrite present. After the mixture was kept for 15 min at room temperature and up to 4 h on ice, 100–200 µl samples were injected into a modified Waters’ system for determining total NOC, in which NOC are decomposed to NO by an HBr/HCl/HOAc/EtOAc mixture refluxing under reduced pressure at 28°C (8,33,34). The NO is swept in a stream of argon through seven wash bottles to dry and remove acid from the gas stream, and is then passed into a Thermal Energy Analyzer to determine the NO. N-Nitrosoproline (0.1 nmol) was injected as a standard once per hour. To determine NOCP (8), samples of supernatant A were nitrosated by treatment with excess sulfamic acid, diluted 100 times with distilled water and analyzed for NOC as just described. Analyses for NOC and NOCP are based on duplicate and single analyses, respectively, of each extract. Duplicate analyses generally agreed within 10%.

Exp. 1: initial analyses of rat and mouse feces

Rats and Swiss mice were fed chow or TD-98061 SP diets for 1 week. Samples composed of several fecal pellets were then collected and the undried samples were analyzed for NOC. In other tests, dried individual fecal pellets were taken from 24-h fecal collections from individual rats and were analyzed for NOC.

Exp. 2: fecal NOC in mice transferred from chow to SP diet

Four cages of five mice, two with IL10−/− and two with their wild-type C57BL/6J mice (both from Jackson Laboratories, Bar Harbor, Maine) were maintained on chow diet for 7 days. Then, starting on day 1, 24-h fecal samples were collected daily for 6 days and analyzed for NOC. The chow diet was continued until 10 a.m. on day 2, when the diet was switched to the TD-98061 SP diet.

Exp. 3: NOC and NOCP in GIT contents and feces of rats

Rats were fed tap water and pelleted chow diet or powdered TD-98061 SP diet for at least 1 week and were then killed with CO2. The contents of different GIT sections were collected, combined for all rats on the same diet, weighed and well mixed. The total collection (if <1 g) or 1 g samples thereof were analyzed for NOC or NOCP. The lengths of the GIT sections designated as proximal, middle and distal small intestine and proximal and distal colon were ~70, 70, 15, 8 and 8 cm, respectively. Each test involved two to three rats fed chow diet and the same number fed SP diet. In two of these tests, daily dietary intake and fecal output were determined for 2–4 days before death.

Exp. 4: fecal NOC in mice with acute colitis

Female Min and wild-type C57BL/6J mice from breeding colonies at Fox Chase Cancer Center were treated there when they were 60–70 days old. They were fed Purina Rodent chow 5013 (PMI-Nutrition, Brentwood, MO). A 4% solution in distilled water of DSS (molecular weight 36 000–44 000 Da, ICN, Costa Mesa, CA) was administered as drinking water on days 1–4. Body weight, stool consistency and results of a fecal occult blood test were recorded daily. During days 5 and 7, feces were collected for 24 h while the...
mice were kept in metabolic cages and fasted. The feces were frozen, mailed on dry ice to Omaha and analyzed for NOC.

**Exp. 5: fecal NOC and NOCP in mice fed hot dogs and beef**

The TD-01407 SP diet was designed so that addition of hot dog or beef at 18% by weight of the final diet brought the fat and protein contents to 5.6–7.9 and 17.5–21.3% by weight, respectively (Table I). According to their labels, the hot dogs were manufactured from beef and pork, and contained 26 g fat, 1.2 g protein and 1.7 g sugar/100 g (based on the reported composition of a 57 g hot dog). The hot dogs, which are cooked during manufacture, were mixed in the diet without further cooking. The beef was labeled as containing 26 g fat, 12 g protein and 1.7 g sugar/100 g (based on the reported composition of a 26 g beef sample).

In each test, two cages of five Swiss mice were weighed. One cage received the hot dog or beef diet and the other cage received the corresponding control diet. The diets were supplied for 6 days in glazed clay pots (4.4 cm high, diameter 6 cm at bottom and 3.5 cm at top). On day 7 the mice were placed singly in cages each supplied with one pot of the same diet as before. The SD-01407 SP diet contained 3.2 g fat/100 g, normally contains 29 g protein/100 g (35) and was sautéed in the diet without further cooking. The beef was labeled as containing 18 g fat/100 g, normally contains 25 g protein/100 g (35) and was sautéed (browned in a pan without added fat). The hot dog or beef (180 g) was blended for several minutes in a Black and Decker Quick ’N Easy Plus food processor (Hampstead, MD) and 820 g of the SP diet was gradually blended in. For the control diets (Table I), soy oil and casein were blended with the SP diet. In each test, two cages of five mice were weighed. Each cage received the hot dog or beef diet and the other cage received the corresponding control diet. The diets were supplied for 6 days in glazed clay pots (4.4 cm high, diameter 6 cm at bottom and 3.5 cm at top). On day 7 the mice were placed singly in cages each supplied with one pot of the same diet as before. The feces were collected for 24 h, the mice were reweighed (there was little change in weight over 7 days) and the feces analyzed. In each test, one sample of each diet was analyzed for NOC and NOCP. Food consumption was measured in one of the hot-dog tests.

### Table I. Composition of meat and control diets fed to mice in Exp. 5

<table>
<thead>
<tr>
<th>Components of diet</th>
<th>Hot dog diet</th>
<th>Control for hot dog diet</th>
<th>Beef diet</th>
<th>Control for beef diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP diet TD-10407</td>
<td>820</td>
<td>917</td>
<td>820</td>
<td>909</td>
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<tr>
<td>Casein</td>
<td>0</td>
<td>26</td>
<td>0</td>
<td>51</td>
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<td>Soy oil</td>
<td>0</td>
<td>57</td>
<td>0</td>
<td>40</td>
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<td>Hot dog or beef</td>
<td>180</td>
<td>180</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Protein and fat in diets</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein in SP diet</td>
<td>153</td>
<td>171</td>
<td>153</td>
<td>169</td>
</tr>
<tr>
<td>Protein in added casein</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>Protein in hot dog or beef</td>
<td>22</td>
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<td>52</td>
<td>0</td>
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<tr>
<td>Total protein</td>
<td>175</td>
<td>191</td>
<td>205</td>
<td>213</td>
</tr>
<tr>
<td>Fat in SP diet</td>
<td>21</td>
<td>22</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>Fat in added soy oil</td>
<td>0</td>
<td>57</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>Fat in hot dog or beef</td>
<td>47</td>
<td>0</td>
<td>36</td>
<td>0</td>
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<tr>
<td>Total fat</td>
<td>68</td>
<td>79</td>
<td>56</td>
<td>62</td>
</tr>
</tbody>
</table>

*Based on the wet (undried) weights of each component.*

### Results

#### Stability of NOC and NOCP in mouse feces

Feces were routinely collected for 24 h and dried under vacuum overnight, both at room temperature. Some samples were stored for up to 3 weeks at −15°C before analysis. To examine the stability of fecal NOC and NOCP under these conditions, two cages of mice were fed the hot dog diet for 6 days. Feces were collected from each cage (samples 1 and 2) and dried by our standard method, and ground to a powder with a mortar and pestle. Samples (200–300 mg) of each of the two batches were stored for 3 weeks at −70°C (control samples 1 and 2) or at −15°C (samples 3 and 4), or were left for 24 h at room temperature and then stored at −70°C (samples 5–8). We prepared samples 1, 3, 5 and 6 from batch 1 and the remaining samples from batch 2. Samples 1 and 2 showed 8.0 and 12.7 nmol NOC, and 4600 and 4700 nmol NOCP/g feces, respectively. Sample 3 showed 98% for NOC, and samples 3 and 4 showed 86 and 120% for NOCP of the mean values for the corresponding control samples. Samples 5–8 showed 76 ± 7% for NOC and 118 ± 18% for NOCP of the mean results for samples 1 and 2 (mean ± SD). Some of this variability may have been due to non-homogeneity of the ground feces, a problem that did not arise in the main experiments where entire 24-h fecal collections were analyzed without grinding. These results indicate that NOC and NOCP were stable for 3 weeks at −15°C and that NOC, but not NOCP, decomposed by ~25% when mouse feces were kept for 24 h and evaporated, both at room temperature. This is far less than the several-fold changes observed in most of the reported experiments.

It remained possible that a fraction of the fecal NOC or NOCP decomposed extensively during the initial 24-h collection, even though storage for a second day at room temperature caused only limited decomposition of NOC. Such a finding would be analogous to the report of an unstable NOC fraction in human gastric juice (36). Four batches of feces were collected at room temperature during only 6–8 h, stored overnight at −15°C, vacuum dried for 36 h at 0°C to constant weight and ground as before. From each batch, one 90–150 mg sample (control samples 9–12) was stored for 3 weeks at −70°C and another similar sample (samples 13–16) was stored at room temperature for 24 h and then for 20 days at −70°C. Samples 9–12 showed 12 ± 7 nmol NOC and 3600 ± 1000 nmol NOCP/g. The results for samples 13–16 were 118 ± 12% for NOC and 82 ± 29% for NOCP of the mean levels for samples 9–12, and hence do not indicate extensive instability of NOC or NOCP in freshly collected mouse feces stored for 24 h at room temperature.

### Exp. 6: partial purification of NOCP in rat feces

Solutions were concentrated in a rotary evaporator at <25 Torr and <40°C (solutions in organic solvents) or <50°C (aqueous solutions). Feces were collected from four rats maintained on chow diet. Dried feces (45 g) were ground with 300 ml water in a Waring blender for 15 min. The mixture was kept for 4 h at room temperature with occasional blending, centrifuged for 30 min at 6000 g and 4°C, filtered through Whatman no. 1 paper and evaporated to 150 ml. The concentrate was mixed with 200 ml silica gel (Merck, grade 60, 70–230 mesh, Aldrich, Milwaukee, WI). A mixture of the resulting wet solid and 300 ml acetonitrile (MeCN) was stirred occasionally for 30 min. The supernatant (425 ml) was decanted. The sediment was extracted similarly with 300 ml methanol (MeOH). The MeCN and MeOH extracts from the silica gel were each evaporated to dryness and dissolved in 5 ml water. The aqeous solutions were stirred for 5 min with 25 ml of the acidic form of a cation exchange resin (50W–X8, Bio-Rad, Hercules, CA) at pH 2. The resin was stirred (i) for 5 min with 300 ml water, which remained at pH 2 and was decanted and discarded, (ii) with 2 N NH₂OH until the pH reached 9.0 and (iii) with 300 ml water adjusted to pH 9 with NH₄OH. The pH 9 solution derived from the MeOH extract was evaporated and dissolved in 5 ml water. Of this solution, 50 µl was diluted with water to 2 ml. Of the diluted solution, 250 µl was injected onto a Rexchrom 25×1 cm amino HPLC column (Regis, Morton Grove, IL), which was eluted with MeCN–water 7:3 at 3 ml/min. The eluate was monitored at 260 nm. Ten 2-ml fractions were collected and analyzed.

### Statistics

The Wilcoxon rank order test was used to determine the significance of differences between groups.
Exp. 2: fecal NOC levels in mice transferred from chow to SP diet

IL10(−/−) and their wild-type C57BL/6J mice were examined here because IL10(−/−) mice can spontaneously develop colitis (37). We determined how rapidly the fecal NOC level would change after these mice were transferred from chow to SP diet. Similar to our findings in rats, SP diet gave feces with only ~25% of the NOC level obtained with chow diet (Table II). It took 3 days after the diets were switched (from days 2 to 5) for fecal levels to drop to the value for SP diet. No differences were seen between the two mouse strains, perhaps because colitis does not always occur in IL10(−/−) mice on a C57BL/6J background (37). [IL10(−/−) mice on a C5H background have just been reported to develop colitis more consistently (38).] Hence we did not use IL10(−/−) mice for studying the effect of colitis on fecal NOC excretion (see Exp. 4).

Exp. 3: NOC and NOCP in GIT contents and feces of rats

NOC and NOCP were determined in the diet, feces and contents of individual GIT sections of rats fed chow and SP diets (Figure 2). NOC and NOCP concentrations are presented per gram wet weight (weight before drying) rather than per gram dry weight because the GIT mucosa is exposed to the undried contents. In two tests, mean dietary intake per rat per day was 25 and 28 g for chow diet and 23 and 26 g for SP diet, and mean fecal output per rat per day was 13 and 21 g for chow diet and 4.7 and 5.2 g for SP diet. The wet weights of all GIT sections were higher for chow than for SP diet (Figure 2A). The dry/wet weight ratio (Figure 2B) remained ~0.23 in the upper GIT, but increased sharply in the colon and feces. In three tests each on rats fed chow and SP diets, NOC concentration was somewhat lower in the stomach than in the diet and then increased steadily towards the distal GIT, with especially large increases for chow diet just beyond the distal small intestine, and for both diets in the colon and feces (Figure 2C). NOC levels were higher with chow than with SP diet, with chow/SP ratios for nmol NOC per gram wet weight of 6.3 for the diet, 1.5–3.3 for the GIT contents and 3.8 for feces.

In two tests each on rats fed chow and SP diets, NOCP levels per gram wet weight were similar in the stomach and diet, rose by a surprising mean of 16-fold for chow diet and 2.6-fold for SP diet in the proximal small intestine, fell in the cecum (especially for SP diet) and (for chow diet) rose in the distal colon and feces, with a mean 1.7-fold increase from distal colon to feces (Figure 2D). The two tests gave similar results. The difference between the NOCP levels in the stomach and proximal small intestine was significant (P = 0.03) when the results for the chow and SP diets were combined. Mean NOCP concentrations for rats on chow diet were four (in the distal colon) and 23 times (in the feces) higher than those for rats on SP diet. The differences between the results for the two diets were significant for the combined distal colon and feces, with P = 0.005 for NOC and 0.03 for NOCP.

Exp. 4: fecal NOC in mice with acute colitis

Min and their wild-type C57BL/6J mice were treated on days 1–4 with DSS to induce acute colitis (26). Chow diet was fed here because previous studies by one of us (M.L.C.) showed that DSS was highly toxic in mice fed an AIN-76A SP diet. Thus, only half of a group of Swiss-Webster mice that were given this diet survived DSS treatment for 7 days, whereas all mice of this strain that were fed chow diet survived the DSS treatment. The samples of the chow diet used here contained 0.43 ± 0.06 nmol NOC/g and 4900 ± 700 nmol NOCP/g. In the present test, the DSS-treated mice showed gross signs of acute colitis, i.e. loss of body weight, blood in the stools and diarrhea. Two of the eight DSS-treated Min mice died on day 8. Mean body weights on days 1, 5 and 7 were, respectively, 16, 16 and 13 g for the DSS-treated Min mice, and 18, 18 and 16 g for the DSS-treated C57BL/6J mice. Fecal blood was detected in all DSS-treated mice on both days 5 and 7. Stool consistency on days 5–7 was soft in almost all the DSS-treated mice. Feces from days 5 and 7 were analyzed for NOC. The results (Table III) are presented as fecal NOC concentration rather than output per day because the mice were fasted overnight (to facilitate urine collection for another purpose), which reduced fecal output, and the diarrhea may have prevented collection of all the feces. On day 5, the DSS-treated mice of both strains showed significant (P = 0.04; mean, 1.9-fold) increases in fecal NOC concentration compared with untreated mice of the same strains. On day 7, the DSS-treated mice of both strains showed a consistent but smaller mean increase of 1.6-fold in fecal NOC level, with P = 0.07.

Exp. 5: fecal NOC and NOCP in mice fed hot dogs and beef

The beef and hot dog diets (Table I) were eaten freely from day 1 of each test, but it took 1–2 days for the mice to eat freely of the control diets. NOC concentrations in the hot dog and sautéed beef used to prepare these diets were 0.4–0.8 nmol/g, consistent with the NOC content of the complete diets. Mean body weights in each test were 26–30 g. The results (Table IV) are quite striking. Fecal NOC excretion (in nmol/day) showed mean values that were 3.7, 5.0 and 3.8 times higher for the three tests of hot dog diet, and 2.0 and 2.9 times higher for the two tests of beef diet, as compared with control groups in the same test. The differences in fecal NOC excretion (nmol/mouse/day) between meat and control groups of the same test were significant, with P < 0.002 for each of the three hot dog tests, 0.01 and 0.06 for each of the two beef tests, <0.0001 for the combined hot dog tests, and 0.0013 for the combined beef tests (the results for the combined tests were obtained after expressing the result for each treated and control mouse as a percentage of the mean control value in the individual test). Because the amount of feces excreted in 24 h did not differ much between the groups (Table IV), fecal NOC concentration paralleled fecal NOC output/day. The differences between fecal NOC concentration in the treated and control groups showed P values of 0.012 for each of the three hot dog groups, 0.06 and 0.02 for the two beef groups, and <0.002 for the combined hot dog and the combined beef groups.

Mean fecal NOC output was 33–41% (for the groups fed the hot dog and beef diets) and 15–69% (for the control groups) of mean dietary NOC intake (both intake and output were expressed as nmol/mouse/day) (Table IV). The estimation of dietary NOC intake assumes that the mice ate 5 g diet/day, which is standard for this species (39) and is similar to consumptions of 4.5–5.5 g/day observed in one of our tests. Whereas the entire 24-h fecal collection was homogenized and analyzed, only single food samples were analyzed in most of these tests. This and the low NOC levels in the control diets probably explain the wide variations in fecal NOC output expressed as percentages of dietary NOC. In one test each of the hot dog and beef diets, fecal NOCP was determined in addition to NOC (Table IV). Mean NOCP outputs in the treated and control groups were 1100–2000 nmol/day, ~1000...
Fig. 2. Analysis of diet, GIT sections and feces for NOC and NOCP in rats fed chow and SP diets. (A) Wet weights; (B) dry weights as percentages of the wet weights; (C) NOC concentration as nmol NOC/g wet weight; (D) NOCP concentration as µmol NOCP/g wet weight. Results are given for the different GIT sections and (except in A) for the diet and feces. Note that NOC are expressed in nmol/g and NOCP in µmol/g. These figures show the mean and SD for three tests each on rats fed chow and SP diet. The ‘GIT sections’ are: 1, diet; 2, stomach; 3–5, proximal, middle and distal small intestine, respectively; 6, cecum; 7, appendix; 8, proximal colon; 9, distal colon; and 10, feces. T-bars show standard of deviations.
Table II. Fecal NOC in male mice transferred from chow to SP diet

<table>
<thead>
<tr>
<th>Day</th>
<th>NOC (nmol/g feces)</th>
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<td></td>
<td>IL-10(−/−) mice</td>
</tr>
<tr>
<td>1</td>
<td>2.3, 2.5</td>
</tr>
<tr>
<td>2</td>
<td>2.2, 2.3</td>
</tr>
<tr>
<td>3</td>
<td>1.3, 1.3</td>
</tr>
<tr>
<td>4</td>
<td>0.6, 0.8</td>
</tr>
<tr>
<td>5</td>
<td>0.5, 0.5</td>
</tr>
<tr>
<td>6</td>
<td>0.5, 0.5</td>
</tr>
</tbody>
</table>

*aMice were switched to SP diet on 10 am of day 2. Results are shown for two cages of each mouse strain.

Table III. NOC in feces of mice treated with 4% DSS in drinking water on days 1–4

<table>
<thead>
<tr>
<th>Mice</th>
<th>No. of mice</th>
<th>DSS</th>
<th>NOC (nmol/g feces, mean ± SD)</th>
<th>Significance (P &lt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 5</td>
<td>Day 7</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>7</td>
<td>−</td>
<td>20 ± 17</td>
<td>18 ± 9</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>5</td>
<td>+</td>
<td>38 ± 18</td>
<td>29 ± 12</td>
</tr>
<tr>
<td>Min</td>
<td>8</td>
<td>−</td>
<td>22 ± 9</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>Min</td>
<td>8</td>
<td>+</td>
<td>41 ± 16</td>
<td>42 ± 14</td>
</tr>
</tbody>
</table>

Exp. 6: partial purification of NOCP in rat feces

NOCP were studied here rather than NOC because they are about 1000 times more abundant in rat feces than the NOC (see Exp. 1) and identification of the NOC would probably be easy once the NOCP were identified. NOCP from the feces of rats fed chow diet were purified as summarized in Figure 3, which also gives the NOCP yield at each stage of the purification. The method involved batchwise adsorption on silica gel, desorption with MeCN and then MeOH (these contained 44 and 56%, respectively, of the total eluted NOCP), followed by batchwise adsorption onto cation exchange resin in its H⁺ form and elution from the resin at pH 9. Cation exchange resin was used because, if the NOCP are secondary amines, which is probable, they should be adsorbed on the resin in their protonated form (RNH₂R⁺) and eluted from the resin in their basic form (RNHR⁺). In the final step, HPLC on an amino column of the pH-9 eluate from the MeOH fraction (Figure 4) showed a principal NOCP peak in fractions 2–3 and three minor NOCP peaks, and UV absorption at 260 nm that coincided with two of the NOCP peaks.
Table IV. Dietary intake and fecal output of NOC and NOCP in mice fed hot dog and beef diets 

<table>
<thead>
<tr>
<th>Test no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test diet</td>
<td>Hot dog</td>
<td>Hot dog</td>
<td>Hot dog</td>
<td>Beef</td>
<td>NOC</td>
<td>Hot dog</td>
<td>NOC</td>
</tr>
<tr>
<td>NOC (nmol/g)</td>
<td>0.35</td>
<td>0.32</td>
<td>0.23</td>
<td>0.18</td>
<td>0.39</td>
<td>1400</td>
<td>113</td>
</tr>
<tr>
<td>NOCP (nmol/g)</td>
<td>1.77</td>
<td>1.62</td>
<td>1.15</td>
<td>0.92</td>
<td>1.94</td>
<td>7000</td>
<td>565</td>
</tr>
</tbody>
</table>

Discussion

Our studies used both chow and SP diets because chow diets are better surrogates for human diets and are more suitable for inducing colitis with DSS (Exp. 4), whereas SP diets are more defined and showed lower NOC levels (Figure 2C) and smaller variations in NOC content of the diet and feces than did chow diet. Our results were mostly consistent within each experiment but varied extensively between experiments, especially when chow diet was fed. Thus, with chow diet the mean fecal NOC levels in nmol NOC/g were 0.44 for rats in Exp. 1, 2.4 for mice in Exp. 2 (Table II), 3.1 for rats in Exp. 3 (Figure 2C) and 18–20 for mice in Exp. 4 (Table III), where the source of the diet differed from that for the other experiments. For animals fed one of the SP diets, mean fecal results in nmol NOC/g were 0.46 for mice in Exp. 1, 0.5–0.7 for mice in Exp. 2 (Table II), 0.69 for rats in Exp. 3 (Figure 2C) and 0.8–2.0 for mice in Exp. 5 (Table IV). The mean fecal NOC levels for rats and mice fed chow diet are in the same range as the 8.4–9.5 nmol NOC/g reported for the feces of humans fed control diets (6,18). The low fecal NOC level with SP diet may explain why Massey et al. (7) did not detect NOC in the colons of rats fed SP diet. NOC levels also varied, with mean values for nmol NOC/g feces of 430 for mice fed chow diet in Exp. 1, 11 600 and 510 for rats fed chow and SP diet, respectively, in Exp. 3 (Figure 2D), and 11 600 and 510 for rats fed chow diet in Exp. 1, 2.4 for mice in Exp. 2 (Table II), 3.1 for rats in Exp. 3 (Figure 2C) and 0.8–2.0 for mice in Exp. 5 (Table IV).

The tap water containing ~3 mg nitrate (as NO$_3^-$) per liter was fed in all tests except Exp. 4, where distilled water was given. This nitrate was probably insufficient to produce significant amounts of NOC in vivo, e.g. lung adenomas were induced in strain A mice by feeding piperazine (a readily nitrosated amine) together with as little as 250 mg NaNO$_2$ per liter, but no lung tumors were induced by feeding piperazine with 12.3 g NaNO$_3$/liter drinking water (40), suggesting that nitrate is not reduced extensively to nitrite in mice and rats, unlike the situation in humans (41).

The results in Exp. 3 (Figure 2) extend the sections of the GIT known to contain NOC from the previously studied stomach (33) and colon (7) to the entire GIT, and suggest that at least some fecal NOC arise from dietary NOC that pass unabsorbed through the GIT. Most likely, NOC concentration increases on descending the GIT because the GIT mucosa absorbs water and digested food components, but not some of the NOC. In support of this view, percent dry weight increased 2.7-fold and NOC level increased by a similar 3.3-fold on proceeding from the proximal colon to the feces in rats fed chow diet (Figure 2B and C). For SP diet, the corresponding increases were 1.37- and 1.92-fold. Mean NOC level/g wet weight in the distal colon was 82 and 92% of that in the feces for rats fed chow and SP diet, respectively (Figure 2C). Hence, at least in rats, fecal NOC level is a good indicator of NOC level in the distal colon. If NOC are a cause of colon cancer, the low NOC level in the small intestine relative to that in the colon helps explain why the incidence of small intestine cancer in the USA is only 5% of that for colorectal cancer (42).

The large NOCP peak in the proximal small intestine (duodenum) with both chow and SP diets (Figure 2D) suggests that NOCP is secreted into or produced in this section of the GIT known to contain NOCP. In support of this view, percent dry weight increased 2.7-fold and NOC level increased by a similar 3.3-fold on proceeding from the proximal colon to the feces in rats fed chow diet (Figure 2B and C). For SP diet, the corresponding increases were 1.37- and 1.92-fold. Mean NOC level/g wet weight in the distal colon was 82 and 92% of that in the feces for rats fed chow and SP diet, respectively (Figure 2C). Hence, at least in rats, fecal NOC level is a good indicator of NOC level in the distal colon. If NOC are a cause of colon cancer, the low NOC level in the small intestine relative to that in the colon helps explain why the incidence of small intestine cancer in the USA is only 5% of that for colorectal cancer (42).

The large NOCP peak in the proximal small intestine (duodenum) with both chow and SP diets (Figure 2D) suggests that NOCP is secreted into or produced in this section of the GIT, and is absorbed or degraded in the distal small intestine. The source of the duodenal NOCP could be bile salts secreted in the bile or N-glycosyl amino acids (Figure 1) produced by reactions between monosaccharides and amino acids under the neutral or slightly basic conditions of the duodenum (43). NOCP concentrations from the cecum to the feces were far higher for chow than for SP diet, e.g. 23-fold higher for feces (Figure 2D). From the proximal colon to the feces, percent dry matter increased 2.72-fold with chow diet and 1.37-fold with SP diet (Figure 2B), but NOCP level increased only 2.03-fold with chow diet and fell 62% with SP diet (Figure 2D), indicating some loss of NOCP in the colon and/or feces.

In Exp. 2 the 3 days required for mouse fecal NOC to drop after the switch from chow to SP diet (Table II) probably reflects transit time through the GIT. The finding that IL10(−/−) mice on a C57BL/6J background did not show raised NOC
levels, despite their tendency to develop colitis (37), may be due to variability in the extent of colitis (38). In Exp. 4 we used DSS to induce acute colitis in Min and their wild-type C57Bl/6J mice, and found that fecal NOC levels almost doubled on the day after DSS treatment was stopped (Table III). This effect may be due to increased levels of nitrosating agents formed by colitis-associated iNOS (see Introduction). In support of this view, ulcerative colitis patients showed increased levels of iNOS in the colonic mucosa (24) and of nitrite and NO in the colonic lumen (44,45). Our results support the view that colonic NOC are a cause of colon cancer associated with colitis or indicate the presence of other agents, e.g. N$_2$O$_3$ formed by the oxidation of NO, that induce colon cancer.

In Exp. 5 the most striking result was the differences in daily fecal outputs of NOC between the hot dog and beef groups and the control groups (Table IV). Beef increased fecal NOC excretion in mice by a mean of 2.5-fold (Table IV), compared with 3.7-fold in the study on beef fed to humans (18). Hot dogs increased NOC output by a mean of 4.5-fold. The simplest explanation of these findings is that the raised fecal NOC output in the beef and, especially, the hot dog groups was due to the higher NOC content of the beef and hot dog diets, and fecal NOC represented the fraction (15–69% according to Table IV) of ingested NOC that was not absorbed from or degraded in the GIT. The concentration of NOC in the feces was far higher than that in the diet, e.g. in test 1 of the hot dog diet, fecal NOC concentrations were 5.58 and 0.98 nmol/g in the hot dog and control groups, respectively, compared with dietary NOC levels of 0.80 and 0.38 nmol/g, respectively, in the same groups (Table IV). This difference was more than counterbalanced by the small amount of feces, e.g. in test 1, fecal output was 210 mg/day whereas diet consumption was assumed to be 5 g/day. The mean of 2.4–7.3 nmol NOC/g feces for the mice fed beef and hot dogs mixed with SP diet (Table IV) were somewhat higher than those for mice fed chow diet, which showed 2.2–2.3 nmol NOC/g feces (Table II).

Huges et al. (18) did not measure NOC intake in their beef diets. However, we reported (8) that fresh meat, including beef, contains ~0.5 μmol NOC/kg and that heating hot dog extracts at 100°C slightly lowered their NOC content (8). On this basis, the diet with 420 g beef/day fed by Huges et al. contained 0.21 μmol NOC/day, far less than the average fecal excretion of 4.52 μmol NOC/day [(18) and S.Bingham, personal communication]. Hence, in the test on humans, >90% of the excreted NOC may have been produced in vivo.

In Exp. 6, the behavior of rat fecal NOCP during their partial purification (Figures 3 and 4) suggests that these NOCP are similar to those in hot dogs, as they were purified by similar methods. The purified fractions were not weighed, so that activity (μmol NOCP/g) could not be determined. However, purification of the hot dog NOCP showed a 19-fold increase in NOCP concentration (paper in preparation). Most of the fecal NOCP may be N-glycosyl amino acids and peptides for reasons presented elsewhere for the hot dog NOCP (10,30). The only observed difference between fecal and hot dog NOCP was that the MeCN and MeOH fractions from the silica gel adsorption step contained, respectively, 45 and 55% in the fecal study and 32 and 68% in the hot dog study of the eluted NOCP [(10,30) and paper in preparation]. This difference could have arisen because most of the hot dog NOCP was highly hydrophilic glycosyl peptides, whereas a larger proportion of the fecal NOCP were less hydrophilic glycosyl amino acids. UV absorption at 260 nm of the HPLC eluate fractions was followed because it could be used to identify fractions from different HPLC runs when they are combined. In Figure 4, the coincidence of the major NOCP peak (fraction 2) with a major peak of UV absorption probably does not mean that this NOCP was UV-absorbing as the NOCP was probably a minor component of this fraction.

In conclusion, our results support the view that colonic NOC are a cause of colitis-associated colon cancer and, as proposed by Bingham et al. (2), of sporadic colon cancer linked with consumption of red and processed meat. It remains to establish in rodent models and in humans whether fecal NOC represent unabsorbed dietary NOC, as suggested by Exp. 5 on the meat diets and by the finding in Exp. 3 that NOC occur throughout the GIT; or whether some fecal NOC arise by in vivo nitrosation, as suggested by Exp. 4 on colitis and by the Hughes experiment (18). To help establish whether NOC are a significant factor for the etiology of colon cancer, it is urgent to identify the NOC in feces and to establish whether they are absorbed from the GIT and are genotoxic and carcinogenic in the colon.

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References


