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Analytical Methods

Increased oxidative and nitrosative reactions during digestion could contribute to the association between well-done red meat consumption and colorectal cancer

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ABSTRACT

Uncured and nitrite-cured pork were subjected, raw, cooked (65 °C, 15 min) or overcooked (90 °C, 30 min), to an *in vitro* digestion model, which includes mouth, stomach, duodenum, and colon phases. Heating of uncured meat resulted in a pronounced increase in lipid and protein oxidation products throughout digestion. Nitrite-curing had an antioxidant effect during digestion, but this effect disappeared when the meat was overcooked, resulting in up to ninefold higher 4-hydroxy-2-nonenal concentrations compared with digested nitrite-cured raw and cooked pork. Colonic digesta contained significantly higher concentrations of the NOC-specific DNA adduct O^6-carboxy-methylguanine when pork underwent a more intense heating procedure, independent of nitrite-curing, depending strongly on the fecal inoculum used. Since processed meats are usually nitrite-cured, the present study suggests that overcooking processed meat is likely to result in the formation of genotoxic compounds during digestion and should, therefore, be avoided.

1. Introduction

Different independent meta-analyses have demonstrated a significant epidemiological association between colorectal cancer (CRC) risk and high consumption of red and processed meat (Chan et al., 2011). Moreover, evidence for increased CRC risk has been reported in several epidemiologic studies when the meats consumed have undergone a more intense heating process, such as well-done, fried, barbequed or grilled (Martínez et al., 2007; Rohrmann, Hermann, & Linseisen, 2009; Sinha et al., 1999). However, this association was not found in other studies (Ollberding, Wilkens, Henderson, Kolonel, & Le Marchand, 2012; Tabatabaei et al., 2011). The association between consumption of well-done red meat and CRC is usually explained by the formation of carcinogenic heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs). Santarelli, Pierre, and Corpet (2008) questioned the importance of these compounds as major determinants of CRC risk because chicken is a major contributor to HCA (Skog, Augustsson, Steineck, Stenberg, & Jägerstad, 1997) and PAH intake (Kazerouni, Sinha, Hsu, Greenberg, & Rothman, 2001), but chicken consumption is not associated with an increased CRC risk in epidemiological studies. Furthermore, Santarelli et al. (2008) argued the dose to induce carcinogenic effects in rodents and monkeys is 1000- to 100,000-times higher than the dose to which humans are normally exposed by consuming cooked meats. Finally, bread, cereals and grains contribute the most to daily intakes of PAHs through the diet (Kazerouni et al., 2001), while their consumption is not associated with an increased CRC risk. These inconsistencies indicate that other pathways might contribute to the epidemiological association between consumption of well-done red meat and CRC risk.

To date, the formation of cyto- and genotoxic lipid oxidation products, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), and *N*-nitroso-compounds (NOCs) catalyzed by

Abbreviations: ALA, α-linolenic acid; CFU, colony forming units; CRC, colorectal cancer; FA, fatty acids; FI, fecal inoculum; HCA, heterocyclic amines; 4-HNE, 4-hydroxy-2-nonenal; LA, linoleic acid; MDA, malondialdehyde; MUFA, monounsaturated fatty acids; LC *n*−3 PUFA, long chain *n*−3 polyunsaturated fatty acids; LC *n*−6 PUFA, long chain *n*−6 polyunsaturated fatty acids; ·NO, nitric oxide; NOCs, *N*-nitroso-compounds; O^6-C-MeG, O^6-carboxy-methylguanine; PAHs, polycyclic aromatic hydrocarbons; PCC, protein carbonyl compounds; ROS, reactive oxygen species; SFA, saturated fatty acids.

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heme-Fe during digestion, are considered to be the most plausible factors contributing to increased risk of developing CRC (Corpet, 2011). A high consumption of red meat by humans has been shown to enhance NOC formation, which is associated with increased colonic formation of the NOC-specific DNA adduct O^6-carboxymethylguanine (O^6-C-MeG) (Lewin et al., 2006).

Heating of meat can induce a series of reactions that are able to enhance lipid and protein oxidation, including inactivation of antioxidant enzymes (e.g. glutathione peroxidase), an increase in free Fe^{2+} through destruction of the heme-porphyrin moiety, and the release of O_2 from oxymyoglobin with production of H_2O_2 (Kanner, 1994). Nitrite-curing of meat is a common meat processing procedure that induces color formation, inhibits growth of *Clostridium botulinum* and delays oxidative rancidity. Among other antioxidant mechanisms, nitrite may act as a precursor of the heat-stable NO-heme, through which the release of Fe^{2+} during heating is inhibited. Because of this, nitrite-curing interacts with the destruction of heme-Fe during heating of meat and, hence, an effect on the oxidation and nitrosation pathway during digestion is suspected. Thus, we aimed to investigate the modulating effects of heating and nitrite-curing of meat on markers of lipid oxidation (MDA, 4-HNE, simple aldehydes) and protein oxidation (protein carbonyl compounds) during *in vitro* digestion. Many NOCs are alkylating agents that can induce carboxymethylation of DNA. Therefore, we also measured formation of the NOC-specific DNA adduct O^6-C-MeG during *in vitro* digestion. Previously, using the same *in vitro* approach, we demonstrated the role of heme-Fe and fat content on oxidation processes and O^6-C-MeG formation during the digestion of meat as well as the inhibitory effect of nitrite-curing on oxidation (with no effect on O^6-C-MeG formation) (Vanden Bussche et al., 2014; Van Hecke, Vanden Bussche et al., 2014; Van Hecke, Vossen et al., 2014).

2. Materials and methods

2.1. Experimental setup

Uncured and nitrite-cured pork products, containing 5% total fat, were subjected, raw, cooked (65 °C, 15 min) or overcooked (90 °C, 30 min), to an *in vitro* digestion model. Each incubation run included all treatments in quadruplicate from which two replicates underwent digestion as far as the duodenum and two until the colon. Each incubation was performed three times with fecal inoculum (FI) originating from three different individuals.

2.2. Preparation of the meat samples

Commercially available lean meat samples from porcine m. *Longissimus dorsi* were purchased at the local supermarket. The loin was chopped manually into cubes of approximately 1–2 cm^3. Subcutaneous pork fat from one batch was added to the chopped meat to obtain a targeted total fat content of 5%. Meat samples with added fat were first ground using a grinder (Omega T-12) equipped with a 10 mm plate and, subsequently, a 3.5 mm plate. After grinding, the meat was cured by adding 20 g of 0.6% nitrite salt/kg meat, corresponding to an added concentration of 120 mg of nitrite/kg meat. Next, the samples were heated in a warm water bath for 15 min after the core temperature had reached 65 °C (cooked) or for 30 min after the core temperature had reached 90 °C (overcooked). Raw and heated meat samples were homogenized in three 5 s bursts using a food processor (Moulinex DP700), vacuum packed and stored at −20 °C until the start of the incubation experiments.

2.3. In vitro digestion model

The *in vitro* digestions consisted of an enzymatic digestion simulating the mouth, stomach, and duodenum gastrointestinal tract compartments, followed by simulation of colonic fermentation, as previously described by Van Hecke, Vanden Bussche et al. (2014). In brief, meat samples (4.5 g) were incubated sequentially for 5 min with 6 mL of mimicked saliva, 2 h with 12 mL of gastric juice, and 2 h with 2 mL of bicarbonate buffer (1 mol/L, pH 8.0), 12 mL of duodenal juice and 6 mL of bile juice. These enzymatic incubations were performed in quadruplicate. After completion, two replicates were diluted with 44 mL H_2O to obtain the same solid/liquid ratio as in the colonic digestion (see further) and homogenized with an Ultraturrax (9500 rpm). The remaining two replicates underwent an additional colonic fermentation stage by adding 22 mL simulation of human intestinal microbial ecosystem (SHIME) medium and 22 mL of human FI (for preparation see Section 2.4) to the digesta. An anaerobic environment was obtained by flushing with N_2 for 30 min and controlled using resazurin saturated test strips. Subsequently, the vessels were incubated for 72 h while stirring at 37 °C. To evaluate the rate of bacterial fermentation, total anaerobic bacteria were counted after 72 h of fermentation. One mL digesta was serially diluted (10-fold) using a sterile peptone solution (1 g/L peptone, 0.4 g/L agar, 8.5 g/L NaCl and 0.5 g/L cysteine), after which 0.1 mL was added to an RCM-plate (reinforced clostridial medium). After 48 h of incubation at 37 °C, CFU were counted and expressed as $\log_{10}$ CFU/mL digesta. Duodenal and colonic digestion samples were homogenized by Ultraturrax at 9500 rpm and stored at −20 °C and −80 °C pending analysis. Undigested control samples were obtained in duplicate by homogenizing 4.5 g meat in 82 mL H_2O, mimicking the liquid/solid ratio in the digested samples.

2.4. Preparation of human fecal inoculum (FI)

Fresh fecal material was collected from three volunteers and treated as previously described (Van Hecke, Vanden Bussche et al., 2014). Fecal donors were without known gastrointestinal diseases and had not taken antibiotics for at least six months prior to sampling. All volunteers were male, non-vegetarians on a Western-type diet, and aged 49, 26 and 38 years, respectively. Before use in the colonic fermentation phase, the bacterial inoculum was cultured for 24 h at 37 °C to obtain an active microbial culture. To this end, FI was diluted with BHI broth (37 g/L Brain Heart Infusion and 0.5 g/L cysteine) in a 1/9 ratio. Subsequently, anaerobic conditions in the flask were reached by flushing the headspace with N_2 for 1 h.

2.5. Chemical composition of the meat samples

Meat samples were analyzed for dry matter, crude protein and crude fat contents according to the relevant ISO 1442-1973, ISO 937-1978 and ISO 1444-1973 methods. Lipids were extracted using chloroform/methanol (2/1; v/v) (Folch, Lees, & Sloane-Stanley, 1957) and, subsequently, fatty acids (FA) were analyzed as previously described (Raes, De Smet, & Demeyer, 2001). Residual nitrite concentrations were measured colorimetrically (Thermo Fisher Scientific model G10S UV–Vis) at 538 nm after diazotization with sulfanilamide and *N*-(1-naphthyl)-ethylenediamine dihydrochloride (ISO 2918-1975). Nitrite concentrations were calculated based on a standard curve obtained with sodium nitrite and expressed as mg nitrite/kg meat. Hematin was determined colorimetrically using the method of Hornsey (1956) and converted to heme-Fe using the formula heme-Fe = hematin × atomic weight