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DNA adductomics to study the genotoxic effects of red meat consumption with and without added animal fat in rats

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ABSTRACT

Digestion of red and processed meat has been linked to the formation of genotoxic *N*-nitroso compounds (NOCs) and lipid peroxidation products (LPOs) in the gut. In this study, rats were fed a meat based diet to compare the possible genotoxic effects of red vs. white meat, and the interfering role of dietary fat. To this purpose, liver, duodenum and colon DNA adductomes were analyzed with UHPLC-HRMS. The results demonstrate that the consumed meat type alters the DNA adductome; the levels of 22 different DNA adduct types significantly increased upon the consumption of beef (compared to chicken) and/or lard supplemented beef or chicken. Furthermore, the chemical constitution of the retrieved DNA adducts hint at a direct link with an increase in NOCs and LPOs upon red (and processed) meat digestion, supporting the current hypotheses on the causal link between red and processed meat consumption and the development of colorectal cancer.

1. Introduction

DNA adduct formation is the result of the attack of nucleophilic sites in DNA by endo- or exogenous electrophilic molecules. As such, the DNA building blocks, i.e. the guanine (G), cytosine (C), adenine (A) and thymine (T) nucleobases can be altered both structurally and functionally. In the absence of a timely detoxification of the initiating genotoxin and/or repair of the resulting DNA adduct, DNA adduct formation can lead to mutations and chemically induced carcinogenesis (Poirier, 2004). Hence, investigation of DNA adduct formation can provide valuable information on exposure to both environmental and endogenous chemicals with genotoxic, mutagenic and/or carcinogenic properties on the one hand, and their possible adverse health effects on the other. For example, DNA adduct formation is believed to be an intermediate step in hepatocarcinogenesis due to chronic aflatoxin B1 exposure. Aflatoxin B1 is a known human carcinogen that is formed as a secondary metabolite by food and feed contaminating fungi. Its uptake results in the formation of different types of DNA adducts and also leads to a correlated increase in liver cancer risk (Marroquin-Cardona, Johnson, Phillips, & Hayes, 2014). Accordingly, DNA adduct analysis can be very useful to investigate the underlying pathways of several non-hereditary cancers, which comprise the vast majority of cancer cases (Stewart & Wild, 2014).

One of the most prevalent cancer types that mainly occurs due to environmental factors (e.g. diet and lifestyle) is colorectal cancer (CRC). CRC is the third and second most common cancer type in men and women worldwide, respectively, and important influencing factors include adoption of the Western dietary pattern with the excessive consumption of fat, and red and processed meat (Stewart & Wild, 2014). With regard to the observed increase in

Abbreviations: O^6-CM-G, O^6-carboxymethylguanine; O^6-Me-G, O^6-methylguanine; A, adenine; C, cytosine; CRO, crotonaldehyde; Cro-G, Methylhydroxypropanoguanine, the main CRO adduct with G; DNA, DeoxyriboNucleic Acid; G, guanine; Hep-G, heptenal-G; HNE-C, Hydroxynonenal-C; HESI, Heated ElectroSpray Ionisation; HRAM, High Resolution Accurate Mass; HRMS, high resolution mass spectrometry; LPO(s), lipid peroxidation product(s); M_1-acetaldehyde-A, adduct of 1 malondialdehyde and 1 acetaldehyde molecule with A; M_1-G, Pyrimidopurinone, the main malondialdehyde-guanine DNA adduct; M_2-acetaldehyde-A, adduct of 2 malondialdehyde and 1 acetaldehyde molecule(s) with A; M_2-acetaldehyde-G, adduct of 2 malondialdehyde and 1 acetaldehyde molecule(s) with G; M_2-G, adduct of 2 malondialdehyde molecules with G; M_3-C, adduct of 3 malondialdehyde molecules with C; MDA, malondialdehyde; MS, mass spectrometry; MS/MS, Tandem MS; NOC(s), *N*-nitroso compound(s); Oct-G, Octenal-G; OHE-C, oxohexenal-C; OPLS-DA, Orthogonal Partial Least Squares-Discriminant Analysis; PCA, Principal Component Analysis; RT, retention time; T, thymine; U, Uracil; UHPLC, ultrahigh performance liquid chromatography; VIP, Variable Importance in Projection.

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CRC risk due to red and processed meat consumption, different research groups have investigated the proposed underlying pathways. Currently, there are several intertwined hypotheses that are still under investigation. A prominent hypothesis states that heme stimulates the formation of both lipid peroxidation products (LPOs) and *N*-nitroso compounds (NOCs) in the gut besides its own direct (cyto)toxicity (Oostindjer et al., 2014). The heme molecule is intrinsically more present in red (e.g. beef) than white (e.g. chicken) meat in the form of myoglobin, which renders this molecule a very potent candidate to help explain the toxicity of red but not white meat. Both exo- and endogenous NOCs may contribute to red and processed meat toxicity. Several types of NOCs (e.g. nitrosamines and nitrosamides) have known carcinogenic properties (Lijinsky, 1999), and the most common route of exposure to NOCs indeed occurs via Western type foodstuffs (Hotchkiss, 1989). However, certain NOCs, i.e. nitrosamines and nitrosamides, can also be formed in the gut during digestion of food. What further supports the NOC hypothesis is the fact that exposure to NOCs has already been linked to an increase in tumor development (Lijinsky, 1999). The same reasoning applies for LPOs; LPOs can originate from both exo- and endogenous processes, and possess known cyto- and genotoxic effects that have been linked to carcinogenesis (De Bont & van Larebeke, 2004; Marnett, 1999).

In previous studies, we were able to link red meat digestion to the increased formation of LPOs (e.g. malondialdehyde), as well as LPO- and NOC-related DNA adducts (e.g. O^6-carboxymethylguanine), (Hemeryck et al., 2016; Van Hecke et al., 2016). The current study aimed to further explore the possible genotoxic effects of red meat consumption *in vivo* since (a) both NOCs and LPOs are prone to DNA adduct formation (De Bont & van Larebeke, 2004) and (b) a shift in DNA adduct profile after beef digestion has been demonstrated previously *in vitro* (Hemeryck et al., 2016).

A state-of-the-art DNA adductomics methodology (Hemeryck, Decloedt, Vanden Bussche, Geboes, & Vanhaecke, 2015), based on accurate mass measurements (HRMS), was employed to map the diet-related DNA adduct profile in tissue from rats on a meat diet. The use of an in-house DNA adduct database and specialized omics software further enabled a focused investigation of possibly relevant diet-related DNA adducts (Hemeryck et al., 2015).

2. Material and methods

2.1. Rat feeding trial

2.1.1. Meat based diets

Four different diets, based on lean chicken (LFCh), fat chicken (lean chicken with added lard; HFCh), lean beef (LFBe) or fat beef (lean beef with added lard; HFBe), were prepared in advance. To this purpose, the *m. pectoralis profundus* of chicken, as a model for white meat, and the *m. pectoralis profundus* of beef, as a model for red meat, were purchased, chopped, minced and ground. Then, the meat (and added lard) was cooked at 70 °C for 70 min, in a hot water bath (cooked to the core, but not overcooked to avoid interference from the formation of genotoxic heterocyclic amines and polycyclic aromatic hydrocarbons), followed by homogenization in a food processor. After this, the 4 different meat based diets were manufactured as is documented in Table S1, vacuum packed and stored at −20 °C.

2.1.2. Rat experiment

For this rat trial (ECD 14/58 (Ghent, Belgium)), 24 male Sprague-Dawley rats (±150 g) were purchased from Janvier laboratories (France). The rats were housed in groups of 4 upon arrival and given a standard laboratory diet (Ssniff R/M-H pellets from Ssniff, Soest, Germany) and water *ad libitum* during the first 10 days. After this adaptation period, all rats were divided at random into 4 groups and housed individually. Then, during 14 consecutive days, each group received a different diet (provided *ad libitum* and refreshed daily), i.e. a diet based on lean chicken (='low fat chicken diet' or 'LFCh'), chicken with added lard (='high fat chicken diet' or 'HFCh'), lean beef (='low fat beef diet' or 'LFBe') or beef with added lard (='high fat beef diet' or 'HFBe'). Following 14/15 days on the experimental diets, all rats were anesthetized with 5% isoflurane gas and euthanized by terminal blood collection from the abdominal aorta, after which the different organs were harvested. Rats were euthanized on 6 consecutive days; one rat of each dietary treatment was sacrificed in a random order each day (a more detailed account of this experiment is provided by Van Hecke et al. (2016)). For this particular study, the liver, duodenal mucosae and colonic mucosae were sampled from each individual rat. Tissues were rinsed with a 0.9% saline solution and stored in 95% of ethanol at −80 °C until further sample processing.

2.2. DNA extraction, DNA hydrolysis and DNA adduct extraction

DNA from liver tissue, duodenal mucosae and colonic mucosae was extracted by means of the NucleoSpin Tissue Machery Nagel DNA extraction kit (Machery Nagel GmbH & Co., Düren, Germany), according to the protocol described by the manufacturer. DNA concentration and purity in each sample were determined with a Nanodrop ND-1000 spectrophotometer (Isogen Lifescience, Ijsselstein, The Netherlands).

The DNA obtained in each individual sample was then subjected to a previously reported and validated DNA adduct extraction protocol (Vanden Bussche, Moore, Pasmans, Kuhnle, & Vanhaecke, 2012). In brief, all DNA samples were hydrolyzed in 0.1 M formic acid at 80 °C during 30 min. After this, sample purification and cleanup was performed with solid-phase extraction (SPE) (Oasis® HLB cartridges (1 cc, 30 mg) Waters (Milford, USA), after which all eluates were evaporated to dryness under vacuum at room temperature. In the final step, all samples were suspended in 100 μl of 0.05% of acetic acid in water and stored at −20 °C until analysis.

2.3. DNA adduct analysis

2.3.1. Reagents and chemicals

Analytical standards for M_1-G, Cro-dG (α-methyl-γ-hydroxy-1, N_2-propano-2′-deoxyguanosine) and their respective internal standards; M_1-G-$^{13}C_3$ and CrodG-^{13}C,$^{15}N_2$, were purchased from Toronto Research Chemicals (Toronto, Canada). O^6-Me-dG (O^6-methyl-2′-deoxyguanosine) and O^6-d_3-Me-dG (internal standard for both O^6-Me-dG and O^6-CM-dG) were obtained from Sigma-Aldrich (St. Louis, USA). O^6-CM-dG (O^6-carboxymethyl-2′-deoxyguanosine) was kindly provided by Prof. S. Moore from Liverpool John Moores University (UK).

O^6-CM-dG, O^6-Me-dG, O^6-d_3-Me-dG, Cro-dG and Cro-dG-^{13}C,$^{15}N_2$ were hydrolyzed to their nucleobase form in 0.1 M formic acid at 80 °C for 30 min. All standards were diluted in methanol and stored at −20 °C in stock and working solutions of 500 ng/ml and 5 ng/ml, respectively.

2.3.2. UHPLC-HRMS analysis

A robust, validated ultrahigh performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS) method (Hemeryck et al., 2015) enabled targeted and untargeted DNA adduct analysis. Analysis was performed on a hybrid Quadrupole-Orbitrap High Resolution Accurate Mass Spectrometer (HRAM, Q-Exactive™, Thermo Fisher Scientific, San José, USA) coupled to a heated electrospray ionization (HESI-II) source as described by Hemeryck et al. (2015). Internal calibration of