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Isothiocyanates may chemically detoxify mutagenic amines formed in heat processed meat

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ABSTRACT

Meat consumption represents a dietary risk factor increasing the incidence of common cancers, probably due to carcinogenic amines (HAAs) formed upon meat heating. Interestingly, cancers whose incidence is increased by meat consumption, are decreased in populations consuming brassica vegetables regularly. This inverse correlation is attributed to brassica anticarcinogenic components, especially isothiocyanates (ITCs) that stimulate detoxification of food carcinogens. However, ITC reactivity towards amines generating stable thioureas, may also decrease mutagenicity of processed meat. We confirmed here that combining meat with cabbage (fresh or lyophilized), in proportions found in culinary recipes, limited by 17–20% formation of HAAs and significantly lowered mutagenic activity of fried burgers. Moreover, MeIQx mutagenicity was lowered in the presence of ITCs, as well as for synthetic ITC-MeIQx conjugates. This suggests that formation of thioureas could lead to chemical detoxification of food carcinogens, reducing the cancer risk associated with meat consumption.

1. Introduction

Diet is a major risk factor in human cancer and meat is the main dietary ingredient behind this increased incidence of carcinogenesis. This observation was brought to public attention for the first time by the results of classic analysis by Doll and Peto (1981) and has been confirmed by a number of meta-analyses (Genkinger & Koushik, 2007; WCRF/AICR, 2007; Parkin, 2011) and also by recently published results of network case–control studies (Di Maso et al., 2013). Although initially disputable, the growing body of evidence points to the association between the intake of heterocyclic aromatic amines (HAAs) formed upon heat processing of meat and the risk of common cancers, such as colorectal (Fu et al., 2011), bladder (Lin et al., 2012), prostate (Major et al., 2011), breast, colon, pancreatic (Zheng & Lee, 2009) or lung (Lam, Cross et al., 2009; Lam, Gallicchio et al., 2009). These compounds are formed during heat processing in ng per g of meat amounts, but are highly mutagenic, and their carcinogenicity is dozens of times higher than that of other genotoxic food carcinogens, such as aflatoxin B_1 or nitrosoamines, and also much higher than that of benzo[α]pyrene (Püssa, 2013). Interestingly, consumption of brassica vegetables (cabbage, broccoli and cauliflower) decreases the incidence of the same types of cancers – colorectal (Wu et al., 2013), prostate (Kristal & Lampe, 2002), breast (Terry, Wolk, Persson, & Magnusson, 2001), bladder, colon, pancreatic (Bosetti et al., 2012) and lung (Lam, Cross et al., 2009; Lam, Gallicchio et al., 2009).

The most important bioactive phytochemicals produced by brassica plants are glucosinolates, that are degraded by the endogenous enzyme myrosinase and modifier proteins to a number of products, among which isothiocyanates (ITCs) exhibit the strongest anticarcinogenic potential (Dinkova-Kostova & Kostov, 2012). These compounds trigger an array of cytoprotective mechanisms and were shown to affect HAA metabolism (Murray et al., 2001; Walters, 2004) and detoxification (Steck & Hebert, 2009) in humans, that may diminish the carcinogenic effects of processed meats. However, the ability to stimulate human organism's own defense against xenobiotic insult is not the only possible way

Abbreviations: AITC, allyl isothiocyanate; HAA, heterocyclic aromatic amine; ITC, isothiocyanate; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PEITC, phenethyl isothiocyanate; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; SFN, sulforaphane.

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attributable to ITCs by which they may reduce cancer risk associated with thermally processed meat consumption. Chemical properties of ITCs make them conducive to the reaction with amines. Such a reaction with the amino group of HAAs leading to the formation of thioureas could prevent the formation of genotoxic N^2-hydroxy derivatives. Since heat processed meat is often either combined during preparation or served with brassica vegetables, there is possibility for such chemical processes to occur before food absorption from alimentary tract takes place. In this study, we tested whether the proposed above *ex vivo* chemical detoxification of carcinogenic food amines could diminish their mutagenic potential.

2. Materials and methods

2.1. Preparation of pork burgers

Raw pork (22% fat) lard and French bread loaf were purchased in a local supermarket (Poland). White cabbage (*Brassica oleracea* var. *capitata* f. *alba*), Kamienna Glowa variety, originated from a nearby organic farm and contained about 10 μmoles of glucosinolates per g of dried weight. From the bread loaf, the crust was removed and the flesh was dried then grated. The meat was minced and manually mixed with either 20% [w/w] of minced frozen cabbage or 4% [w/w] of the same cabbage lyophilisate or 4% of dry breadcrumbs. To the two latter samples, 16% [v/w] of water was added to maintain the proportion 20 g of additives per 100 g of meat mixture. The amount of water in the frozen cabbage was established based on the 80% weight loss upon cabbage lyophilisation. The 60 g portions of meat mixtures were formed into burgers and fried in lard for 5 min on each side, which ensured that they become very well-done. Fried burgers were stored in freezer bags at −18 °C until extraction of HAAs.

2.2. Extraction of MeIQx and PhIP from the outer and inner layers of burger samples

The HAAs were extracted and purified using a solid-phase extraction method adopted from Gross and Grüter (1992) with some modifications. One extract was prepared from one burger. Fried meat (6 g), outer and inner layers separately, was homogenised in 18 ml of 1 M NaOH, mixed with 20 g of diatomaceous earth and transferred into blank Extrelut NT 20 columns (Merck, Germany). The amines were extracted with 80 ml of eluent consisting of dichloromethane:toluene 95:5 (v/v) directly to propanesulfonic acid (PRS) cationic exchanger cartridges (J.T. Baker, Germany), containing 0.5 g of sorbent preconditioned with 4 ml of the eluent. The sorbent was dried and rinsed with the following sequence of eluents: 6 ml of 0.1 M HCl, 15 ml of MeOH:0.1 M HCl 6:4 (v/v) and 2 ml of water. In this way, less polar compounds (e.g. PhIP) were eluted. The combined acidic solutions were mixed with 25 ml of water, then neutralised with 0.5 ml of aqueous ammonia (25% v/v). These less polar HAAs were subsequently adsorbed on C_{18} (0.5 g) cartridges (J.T. Baker, Germany) preconditioned with a mixture of 5 ml methanol and 5 ml water. This fraction of amines was eluted with 2 ml of mixture composed of methanol:25% aqueous ammonia 9:1 (v/v). To recover the more polar HAAs (e.g. MeIQx and residues of PhIP), the PRS cartridges were coupled with octadecylsilane C_{18} (0.1 g of sorbent) cartridges (J.T. Baker, Germany) preconditioned with a mixture of 5 ml methanol and 5 ml water. The adsorbed polar HAAs were eluted with 25 ml of 0.5 M ammonium acetate, pH 8.5. After washing C_{18} cartridges with ultrapure water (5 ml) and drying, the polar fraction of amines was recovered from the C_{18} cartridges with 2 ml of a mixture composed of methanol:25% aqueous ammonia 9:1 (v/v). Both fractions of HAAs, after drying in nitrogen flow in an evaporator, were redissolved in 0.5 ml of MeOH each and 0.25 ml samples were submitted to chromatographic analysis. The remaining portions of these solutions were again dried in nitrogen flow in an evaporator, dissolved in 0.25 ml of DMSO and subjected to Ames assay.

2.3. Chromatographic analysis of HAAs in burger extracts

The content of MeIQx and PhIP in the extracts from fried burgers was assessed by the modified method described by Gorlewska-Roberts, Teitel, Lay, Roberts, and Kadlubar (2004). An Agilent Technologies 1200 Series HPLC–DAD system connected to API–ESI–MS Agilent 6130 Quadrupole LC/MS (*Agilent Technologies*, USA) was used throughout the study. Chromatographic separations were performed on an *Agilent Zorbax RX-*C_{18} column (150 × 4.6 mm, 5 μm). The mobile phase was a mixture of water with 0.01% formic acid (A) and 95% acetonitryle with 0.01% formic acid (B). The HPLC program was 100% A for 4 min followed by a linear gradient to 10% A till 30 min and 5 min post-run delay. The flow rate was set at 0.7 ml/min and the injection volume was 50 μl. MS parameters were as follows: capillary voltage, 3000 V; fragmentor, 180 V; drying gas temperature, 350 °C; gas flow (N_2), 12 l/min; nebulizer pressure, 35 psig. The instrument was operated in positive ion mode and selected ion monitoring (SIM) was used to detect m/z 214.1 (for MeIQx), and m/z 225.1 (for PhIP). Instrument control data acquisition and data analyses were carried out with ChemStation (*Agilent Technologies*, USA). Calibration standards were prepared by diluting the stock solutions of MeIQx and PhIP (*Toronto Research Chemicals Inc.*, Canada) with methanol in a range from 0.1 to 5 ng/μl and from 0.01 to 0.5 ng/μl, respectively. Calibration curves were obtained by injecting 1 μl of standard solutions to the HPLC–DAD–MS analysis.

2.4. Mutation assay using microplate Ames test (MPF)

Mutagenicity assessments were carried out for MeIQx, reaction mixtures consisting of MeIQx and ITCs (allyl isothiocyanate, AITC, or phenethyl isothiocyanate, PEITC, both from *Sigma–Aldrich,* UK, and sulforaphane, SFN, synthesised at the Gdansk University of Technology, Poland), corresponding synthetic ITC-MeIQx thioureas (synthesis and characterisation presented in Supplementary), as well as the extracts of HAAs obtained from outer and inner layers of pan-fried burgers. The induction of mutations was examined by a microplate version of the Ames assay (*Xenometrix*, Switzerland) using two tester strains: *Salmonella typhimurium* TA98 (*Xenometrix*, Switzerland) or *S. typhimurium* YG1024 overexpressing N-hydroxylamine O-acetyltransferase (OAT) kindly provided by Dr. T. Nohmi from the National Institute of Health Sciences, Japan. All tests were carried out without and with metabolic activation by Aroclor-induced rat liver microsomal fraction S9 (*Xenometrix*, Switzerland). The methodology followed strictly that recommended by the producer (http://www.xenometrix.ch/index.php?id=61).

2.5. Stability of thioureas in the presence of S9-mix

In order to investigate the stability of three studied ITC-MeIQx thioureas under Ames test conditions, a 0.64 ml portion of thiourea solution (final concentration in the mixture 2 μM) was mixed with the growth (6 ml) and exposure (0.8 ml) media, as well as 0.6 ml of S9-mix (S9 fraction with cofactors). The mixtures were stirred at 37 °C and the samples (0.4 ml) were collected every 30 min for 7 h and once after 24 h. To precipitate proteins, the collected samples were immediately mixed with 0.4 ml of mixture consisting of phenol:chloroform:isoamyl alcohol (25:24:1) supplemented with 1 mM EDTA and buffered with 10 mM Tris, pH 8. The mixtures