Toxicology Letters 248 (2016) 34–38

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

DNA adducts induced by food mutagen PhIP in a mouse model expressing human sulfotransferases 1A1 and 1A2

Anja Hortemo Høie[a], Bernhard Hans Monien[b,c], Hansruedi Glatt[b,c], Hege Hjertholm[a], Trine Husøy[a,*]

[a] Department of Food, Water and Cosmetics, Division of Environmental Medicine, Norwegian Institute of Public Health, 0456 Oslo, Norway
[b] Research Group Genotoxic Food Contaminants, Department of Nutritional Toxicology, German Institute of Human Nutrition Potsdam-Rehbrücke, 14558 Nuthetal, Germany
[c] Department of Food Safety, Federal Institute for Risk Assessment, 10589 Berlin, Germany

HIGHLIGHTS

- C8-PhIP-dG adducts were quantified in hSULT mice by the sensitive method UPLC–MS/MS.
- hSULT mice had a 3-14-fold increase in adducts of liver, kidney and intestine.
- UPLC–MS/MS proves to be a reproducible and sensitive detection method for adducts.

ARTICLE INFO

Article history:
Received 5 January 2016
Received in revised form 25 February 2016
Accepted 26 February 2016
Available online 3 March 2016

Keywords:
Food processing contaminant
Genotoxicity
Sulfotransferase
DNA adduct
Liquid chromatography
Mass spectrometry

ABSTRACT

Food processing contaminant 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) has previously been shown to induce formation of DNA adducts *in vivo*. In a previous study the adduct levels were found to increase in a mouse model expressing human (h) sulfotransferases (SULTs) 1A1 and 1A2 after PhIP exposure, detected by ^{32}P-postlabelling. Isotope dilution ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS/MS) is emerging as the method of choice for selective and reproducible detection of known DNA adducts. In the present study we investigated the level and distribution of PhIP induced DNA adducts in male FVB mice 9–11 weeks of age with hSULT mice or wild-type mice (wt) using UPLC–MS/MS. Mice received a single administration of 75 mg/kg bw PhIP by oral gavage, and DNA was analysed 3 h after exposure. *C*8-(2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine- N^2-yl)-2′-deoxyguanosine (C8-PhIP-dG) adduct levels are significantly higher in PhIP exposed hSULT mice compared with PhIP exposed wt mice. The liver was the least affected organ in wild-type mice, whereas it was the most affected organ in hSULT mice with a 14-fold higher adduct level.

1. Introduction

Heat treatment of food leads to the generation of numerous compounds that were not present in the raw material; among these is the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). This food processing contaminant was discovered in 1986 and found to be mutagenic in the Ames test in the presence of a metabolic activation system (Felton et al., 1986). It also demonstrated carcinogenic activity in rodents (Ito et al., 1997 Sugimura, 1997) and was classified as "possibly carcinogenic to humans" by the International Agency for Research on Cancer (IARC, 1993). PhIP is one of the most abundant heterocyclic amines in foods, reaching levels of ng/g in heat treated meat, fish and poultry (Knize et al., 2002; Skog and Solyakov, 2002).

PhIP in its original form is not mutagenic, and bioactivation requires a two-step process. The first step is hydroxylation of the exocyclic amine group by cytochrome P450 (CYP), especially CYP1A2, which produces *N*-OH-PhIP (Snyderwine et al., 2002).

Abbreviations: CYP, cytochrome P450; 2′-deoxyguanosine-PhIP, *C*8-(2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-*N*^{2}-yl)-2′-deoxyguanosine; h, human; hSULT mice, FVB mice expressing human sulfotransferases 1A1 and 1A2; NAT, *N*-acetyltransferase; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; SULT, sulfotransferase; UPLC–MS/MS, ultra performance liquid chromatography coupled with tandem mass spectrometry.

* Corresponding author.
E-mail address: trine.husoy@fhi.no (T. Husøy).

http://dx.doi.org/10.1016/j.toxlet.2016.02.017

N-OH-PhIP can undergo further transformation by sulfotransferases (SULTs) or *N*-acetyltransferases (NATs) to become esters that go through heterolytic cleavage to produce a reactive nitrenium ion, which may bind to DNA (Frandsen et al., 1992). In humans, SULT1As are enzymes with high capacity to transform *N*-OH-PhIP into reactive compounds. The importance of human (h) SULT1A1 was demonstrated when the mutagenicity of *N*-OH-PhIP was strongly increased in a recombinant *Salmonella typhimurium* strain expressing hSULT1A1 compared with the parental strain, but also compared with recombinant strains expressing NATs (Muckel et al., 2002). Homozygous FVB/N mice expressing multiple copies of the human SULT1A1–1A2 gene cluster was generated. The level of hSULT1A1 protein in the cytosolic tissue fraction was found to be 10–100 times higher than in human tissue. Furthermore, the sulphating activities of the cytosolic tissue preparation were roughly ten times higher in the transgenic mice compared to wt mice (Dobbernack et al., 2011). Previously, in transgenic mice hemizygous for the expression of hSULT1A1 and 1A2, oral PhIP administration substantially elevated the adduct level compared to wt mice (Dobbernack et al., 2011). Adducts were measured by ^{32}P-postlabelling method. However, this method has limitations regarding specificity and there is a risk of underestimating the adduct levels. For this reason, highly specific liquid chromatography and mass spectrometry based approaches using internal reference compounds have become the method of choice for the accurate and reproducible detection of chemically defined adducts (Farmer and Singh, 2008; Monien, 2014). Such methods have previously successfully quantitated PhIP induced DNA adducts *in vivo* (Goodenough et al., 2007; Singh et al., 1988).

In the present study, we aimed at quantifying *C*8-(2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine- N^2-yl)-2′-deoxyguanosine (C8-PhIP-dG) adducts in organs of mice expressing hSULT1A1 and 1A2 after oral administration of PhIP, using the more reproducible and sensitive method isotope-dilution ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS/MS).

2. Methods

2.1. Chemicals

PhIP-HCl (CAS no. 105650-23-5) of 99% purity (Wako Pure Chemical Industries Ltd., Osaka, Japan) was dissolved in saline and adjusted to pH 3.5. Proteinase K and ribonuclease A were purchased from Qiagen (Hilden, Germany). Calf intestine alkaline phosphatase, micrococcal nuclease (from *Staphylococcus aureus*) and calf spleen phosphodiesterase were purchased from Sigma-Aldrich (Steinheim, Germany). HPLC-grade methanol, formic acid and acetic acid were from Carl Roth GmbH (Karlsruhe, Germany). The synthesis of the isotope-labelled reference standard [$^{15}N_5$,$^{13}C_{10}$]C8-PhIP-dG was described previously (Lin et al., 1992).

2.2. Animals and housing

FVB mice expressing human sulfotransferases 1A1 and 1A2 (hSULT mice, termed tg1 in the original publication) have been described previously (Dobbernack et al., 2011). Homozygous hSULT males of 9–11 weeks of age were included in the experiment along with male FVB wild-type mice purchased from the Jackson Laboratories (Bar Harbour, ME, USA). Littermates were co-housed in plastic cages on Nestpak Aspen 4HK bedding (Datesand Ltd., Manchester, UK) with a 12 h light/dark cycle and free access to food (SDS RM1 maintenance diet, Special Diet Services Ltd., Witham, UK) and tap water. The experiments were carried out in conformity with the laws and regulations for experiments with living animals in Norway, and were approved by the Norwegian Animal Research Authority.

2.3. Treatment and harvest of organs

Mice were placed in individual cages the day before the experiment. A single dose of 75 mg/kg bw PhIP or 0.9% saline was administered by oral gavage (n = 5 for each treatment group and strain) and mice were sacrificed 3 h after exposure. Liver, kidneys, small intestine and colon were collected and rinsed in ice cold PBS. The small intestine was divided in proximal and distal parts of equal length. Organs were immediately snap frozen in liquid nitrogen and stored at −80 °C.

2.4. DNA isolation

A Qiagen Blood and Cell Culture DNA kit was used to isolate DNA. G2 lysis buffer supplemented with 0.2 mg/ml ribonuclease A was added to the frozen tissue, which was cut roughly and then mechanically homogenated using a T10 Ultra Turrax (IKA, Staufen, Germany). Aliquots (250–300 μl) of homogenate (equivalent to ∼100 mg tissue) were frozen at −80 °C. Samples were thawed and stirred gently before addition of G2 lysis buffer with 0.5 mg/ml Proteinase K and 0.2 mg/ml ribonuclease A, to a total volume of 9.75 ml. The homogenate was incubated in a water bath for 2 h at 37 °C for digestion. Samples were centrifuged at 4000*g* for 10 min and DNA was isolated from the supernatant using Genomic-tip 100/G columns (Qiagen), according to the manufacturer's protocol. Finally, DNA was dissolved in 400 μl of distilled H_2O, the concentration was measured with a ND-1000 Nandodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and samples were frozen at −80 °C until further analysis.

2.5. Enzymatic digestion of DNA

Samples containing 100 μg DNA were dried together with 5413 fmol of the reference standard [$^{15}N_5$,$^{13}C_{10}$]C^8-PhIP-dG. The residues were dissolved in 56 μl water and 16 μl of 100 mM sodium succinate (pH 6.0) containing 50 mM $CaCl_2$. Aliquots of 12 μl calf spleen phosphodiesterase (4.0 mU/μl) and 12 μl micrococcal nuclease (200 mU/μl) were added and the samples were incubated for 8 h at 37 °C. A volume of 38 μl 0.5 M Tris (pH 10.9) and 3 of μl shrimp alkaline phosphatase (1 U/μl) were added and the incubation was resumed at 37 °C for 14 h. The DNA digests were diluted with 300 μl methanol. The mixture was centrifuged and the pellet was extracted with another 400 μl of methanol. After evaporation of the solvents the residuals were taken up in 50 μl methanol and centrifuged at 15 000*g* for 15 min. The supernatant was used for mass spectrometric analysis.

2.6. Isotope dilution UPLC–MS/MS for quantification of C8-PhIP-dG

An Acquity UPLC System (Waters, Eschborn, Germany) with an HSS T3 column (1.8 μm, 2.1 × 100 mm, Waters) was used for sample separation. Aliquots of 8 μl were injected and eluted with a gradient of water (solvent A) and acetonitrile (solvent B). Both eluents were acidified with 0.25% acetic acid and 0.25% formic acid. Following a washing interval of 1 min, the content of eluent A was decreased from at 90% to 20% within 5 min. The flow rate was 0.35 ml/min. The UPLC was connected to a Quattro Premier XE mass spectrometer (Waters) with an electrospray interface operated in the positive ion mode. The adduct quantification was based on a characteristic neutral loss of the 2′-deoxyribose in the analyte and the isotope-labeled reference standard (C_8-PhIP-dG, $m/z = 490.0 \rightarrow 374.0$; [$^{15}N_5$,$^{13}C_{10}$]$C_8$-PhIP-dG, $m/z = 505.0 \rightarrow 384.0$) (Monien, 2014). The tune parameters were as

follows: temperature of the electrospray source: 110 °C; desolvation temperature: 485 °C; desolvation gas: nitrogen (950 l/h); cone gas: nitrogen (50 l/h); collision gas: argon (indicated cell pressure $\sim 5 \cdot 10^{-3}$ mbar). For the fragmentation of C8-PhIP-dG the collision energy was set to 35 eV. The dwell time was 100 ms and the capillary voltage was 1.0 kV. The cone and RF1 lens voltages were 25 V and 0.1 V, respectively. Data acquisition and handling were performed with MassLynx 4.1 software (Waters).

2.7. Statistical analysis

Differences between the groups were evaluated using a two way analysis of variance on ranks, applying the Holm-Šídák method for pairwise multiple comparison procedures, in SigmaPlot version 12.0 (Systat Software GmbH, Erkrath, Germany). A p-value of ≤ 0.05 was considered significant.

3. Results

The UPLC–MS/MS chromatograms of a DNA sample from the liver of a PhIP exposed hSULT mouse is shown in Fig. 1.

The levels of C8-PhIP-dG adducts were significantly higher in PhIP exposed hSULT mice compared with exposed wt mice for all organs tested ($p < 0.001$, Fig. 2). The detected amount of C8-PhIP-dG adducts in wt mice was lowest in the liver and kidney, and somewhat higher in the intestinal and colonic DNA ($p < 0.001$ – distal small intestine versus liver and kidney, $P < 0.05$ – intestine versus liver, kidney and colon). In the exposed hSULT mice, however, the level of adducts was clearly highest in the liver ($p < 0.001$—liver versus kidney, intestine and colon), followed by intestinal and colonic levels ($p < 0.05$–intestine versus kidney), and the number of adducts in the kidney was lowest. Comparing the exposed hSULT mice with the wt mice, there was a 14-fold increase in adducts in the liver, while the kidney and proximal small intestine had a 3.0 and 3.5-fold increase, respectively, and in the distal small intestine and colon the levels increased by just above 2.5-fold. No C8-PhIP-dG adducts were found in unexposed animals.

4. Discussion

The low level of PhIP adducts detected in the liver compared to other tissues of the wt mice after oral exposure has also been reported by others (Dobbernack et al., 2011; Snyderwine et al., 2002), and similar results are found in rats (Kaderlik et al., 1994). The pronounced increase in adduct formation in the hSULT mouse compared with the wt mouse, between 2.5 and 14-fold, confirms previous findings that human SULT1As have the ability to transform PhIP into a reactive metabolite that contribute to genotoxicity *in vivo* (Dobbernack et al., 2011). The ratio between adduct levels in the extra hepatic tissues in the hSULT mouse was similar to those in the wt mouse, but the number of adducts was always roughly threefold higher in the hSULT mouse. For the liver, however, hSULT1A1 and 1A2 expression changed the adduct formation dramatically. In the wt mice the liver was the organ with the lowest level of adducts, whereas it was the organ with the highest number of adducts in the hSULT mouse (14-fold increase). The observed change in tissue distribution matches the findings of

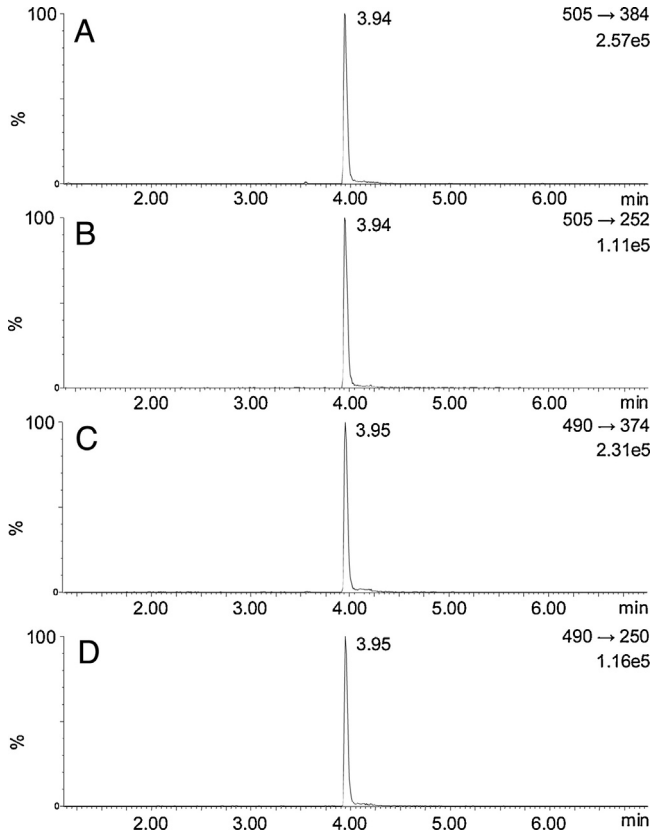

Fig. 1. UPLC–MS/MS chromatograms of digested DNA sample from a hSULT1A1/1A2 expressing FVB/N mouse treated with PhIP. The chromatograms of C8-PhIP-dG with the fragmentations $m/z = 490.1 \rightarrow 374.1$ (panel C) and $m/z = 490.1 \rightarrow 250.1$ (panel D) are shown together with the parallel recording of [$^{15}N_5$,$^{13}C_{10}$]C8-PhIP-dG fragmentational chromatograms, $m/z = 505.1 \rightarrow 384.1$ (panel A) and $m/z = 505.1 \rightarrow 252.1$ (panel B).

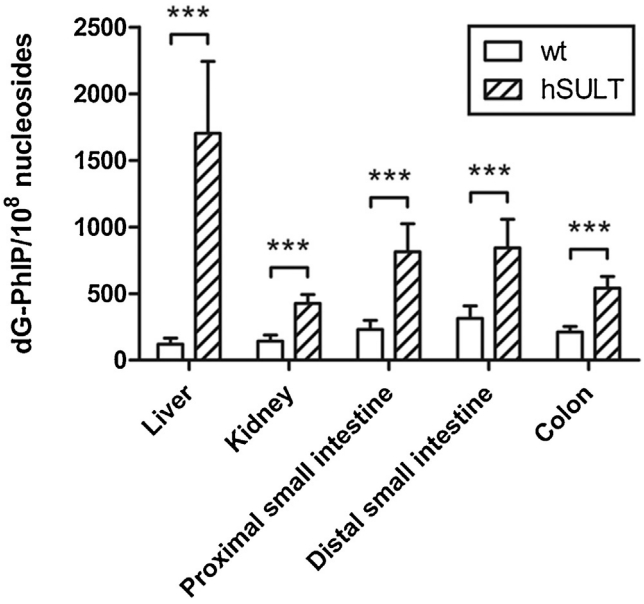

Fig. 2. Adduct levels detected in DNA isolated from organs of FVB males with (hSULT mice) or without (wt) hSULT1A2 and 1A2 exposed to one oral dose of 75 mg/kg PhIP. Figure shows mean and SD of 5 mice. (*** $p < 0.001$). No C8-PhIP-dG adducts were found in unexposed animals.

Dobbernack and colleagues (Dobbernack et al., 2011). The levels of adducts also relates well to the concentration of expressed hSULT1A1 enzyme in the different tissues of the hSULT mouse reported in the same paper, even though that study used hemizygous animals, in contrast to the homozygous animals in the present work.

Quantitation of C8-PhIP-dG in wt and hSULT mice after oral exposure of PhIP using UPLC–MS/MS yielded comparable results as those obtained using traditional ^{32}P-postlabelling methodology (Dobbernack et al., 2011). One noticeable difference between the results, however, is that the adduct levels in the present work are approximately 10 times higher than the levels found in the previous study, even though the administered dose applied in the present experiment is lower (75 mg/kg bw instead of 90 mg/kg bw). This discrepancy can be explained by three factors, namely (i) different time point for adduct analyses (3 versus 8 h) after PhIP exposure (ii) the fact that the present study used homozygous animals compared to hemizygous in the previous, and (iii) the tendency of underestimation by the ^{32}P-postlabelling method used in the previous study. The different time point of adduct analysis between the present study and the study by Dobbernack et al. (2011) is not likely to be an explanation for the difference in adduct levels as considerable repair of PhIP adducts has been shown to take several days (Steffensen et al., 2005). If homozygosity was the sole explanation, one should only expect a doubling of the number of adducts, consequently methodological aspects are likely to be important as well. The risk of underestimation using the ^{32}P-postlabelling method is based on the possibility of incomplete digestion of DNA and subsequent insufficient incorporating of labelled [^{32}P]phosphoryl groups. One also risks partial phosphorylation of the modified nucleotides, which will then escape detection (Monien, 2014). However, using the heterozygous hSULT mice might be preferable when using the ^{32}P-postlabelling method. Overall, the UPLC–MS/MS is the preferred method for determining exact concentrations of chemically defined adducts, and strengthens the credibility of the present work.

In conclusion, the results obtained from this study confirm that human enzymes hSULT1A1 and 1A2 efficiently convert PhIP into a reactive metabolite and confirms the altered tissue distribution of DNA adducts in the presence of hSULTs. UPLC–MS/MS technology proves to be a reproducible and well suited method for sensitive detection of C8-PhIP-dG adducts.

Funding

This work was supported by the Research Council of Norway (project no. 204487).

Acknowledgements

The authors thank Tone Rasmussen, Trude Karin Olsen and Victor Labay Ong at the Norwegian Institute of Public Health and Martina Scholtyssek at the German Institute of Human Nutrition for their excellent technical assistance and animal care taking.

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