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Formation of DNA adducts in wild-type and transgenic mice expressing human sulfotransferases 1A1 and 1A2 after oral exposure to furfuryl alcohol

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Abstract

Furfuryl alcohol (FFA) is present in many heat-treated foods as a result of its formation via dehydration of pentoses. It is also used legally as a flavouring agent. In an inhalation study conducted in the National Toxicology Program, FFA showed some evidence of carcinogenic activity in rats and mice. FFA was generally negative in conventional genotoxicity assays, which suggests that it may be a non-genotoxic carcinogen. However, it was recently found that FFA is mutagenic in *Salmonella* strains expressing appropriate sulfotransferases (SULTs), such as human or mouse SULT1A1. The same DNA adducts that were formed by FFA in these strains, mainly N^2-((furan-2-yl)methyl)-2′-deoxyguanosine (N^2-MF-dG), were also detected in tissues of FFA-exposed mice and even in human lung specimens. In the present study, a single oral dose of FFA (250 mg/kg body weight) or saline was administered to FVB/N mice and transgenic mice expressing human SULT1A1/1A2 on the FVB/N background. The transgenic mice were used, since human and mouse SULT1A1 substantially differ in substrate specificity and tissue distribution. DNA adducts were studied in liver, kidney, proximal and distal small intestine as well as colon, using isotope-dilution ultra performance liquid chromatography (UPLC–MS/MS). Surprisingly, low levels of adducts that may represent N^2-MF-dG were detected even in tissues of untreated mice. FFA exposure enhanced the adduct levels in colon and liver, but not in the remaining investigated tissues of wild-type (wt) mice. The situation was similar in transgenic mice, except that N^2-MF-dG levels were also strongly enhanced in the proximal small intestine. These different results between wt and transgenic mice may be attributed to the fact that human SULT1A1, but not the orthologous mouse enzyme, is strongly expressed in the small intestine.

Introduction

Numerous substances are formed during heat treatment of food. Furfuryl alcohol (FFA) is a substituted furan formed during thermal and acid-catalysed dehydration of pentoses (1). It is also permitted to use FFA as a flavouring substance in foods (2).

FFA has been detected in considerable amounts in instant coffee (267 µg/g) and roasted coffee (564 µg/g) in a recent study (3),

although the level of FFA in coffee beans will depend on roasting time and temperature (4). Other foodstuffs, like red wine (5), beer (6), deep fried coated fish (7,8), rice cakes (9), stored non-fat dry milk (10), roasted hazelnuts (11), honey (12) and whey powder (13) have also been reported to contain FFA. In addition, the European Food Safety Authority has estimated the exposure to FFA as a flavouring agent to be 180 µg/capita/day based on production volumes (2).

The genotoxicity and carcinogenicity of FFA has been evaluated in several studies. FFA was not found to be mutagenic in a range of conventional *Salmonella typhimurium* strains (14–16), while other *in vitro* genotoxicity tests have provided equivocal results (15,17,18). Furthermore, no genotoxic effects were found in adult human lymphocytes from FFA-exposed workers (19). Overall, the indications of genotoxicity of FFA from these studies are weak. In contrast, detection of DNA adducts N^2-((furan-2-yl)methyl)-2′-deoxyguanosine (N^2-MF-dG) and N^6-((furan-2-yl)methyl)-2′-deoxyadenosine (N^6-MF-dA) was reported in FVB/N mice exposed to FFA though drinking water for 28 days. Additionally, a 2-year inhalation study found some evidence of carcinogenic activity of FFA in male F344/N rats and in male B6C3F1 mice in the form of increased incidences of combined neoplasms of the nose and renal tubule neoplasms, respectively (15).

FFA is readily absorbed from the gastrointestinal tract, and as much as 87% of an oral dose of [^{14}C]FFA was excreted through urine after 72 h in rats, and only minor quantities were recovered in faeces and tissues (20). The metabolites found in urine were furoylglycine (73.2% ± 5.9), furanacrylic acid (7.9% ± 2.2) and furoic acid (5.5% ± 0.7). The major metabolite, furoylglycine, is formed from oxidation of FFA to furoic acid and further conjugation with glycine (20). It has been proposed that bioactivation of FFA by endogenous sulfotransferases (SULTs) and co-factor 3′-phosphoadenosine-5′-phosphosulfate could contribute to the genotoxicity of FFA. The resulting furfuryl sulfate (FFS) is unstable and spontaneous cleavage of the sulfate group generates an electrophilic carbonium ion, which may react with nucleophilic sites on the DNA (21). Whereas FFA has no mutagenic effect in the standard Ames tests, bacteria expressing human SULTs suffer a substantial number of reverse mutations following FFA exposure (21). Structural characterisations of the DNA adducts N^2-MF-dG and N^6-MF-dA have been published by Monien *et al.* (22), and their chemical structures are depicted along with those of FFA and FFS in Figure 1.

As SULTs are not expressed in target cells of most standard *in vitro* genotoxicity assays and there is considerable species difference in the substrate specificity and tissue distribution of many SULT forms, conventional *in vitro* and rodent genotoxicity tests may not be appropriate tools to predict the human risk. Humans express a broad spectrum of enzymes from the SULT superfamily (23), including the human (h) SULT1A1 form, which was found to have the greatest capacity of the human SULTs to increase the mutagenicity of FFA in recombinant bacteria in the Ames test (21). hSULT1A1 is highly expressed not only in the liver, colon and jejunum (24) but also in platelets, placenta, brain, endometrium, adrenal gland, kidneys and lungs (25). The murine (m) Sult1a1 also efficiently converts FFA into a metabolite with genotoxic capacity, followed by mSult1d1 with a 10-fold lower effect (21). The expression of mSult1a1 mRNA is essentially restricted to the liver, colon and lung (26). While the mSult1a1 protein is present at high levels in liver and colon, it is close to the limit of detection (LOD) in lung (W. Meinl and H. Glatt, unpublished results), leaving the liver and the colon as the major expression sites of mSult1a1 protein.

Applying new transgenic models in the testing of FFA has revealed new aspects of its reactivity. While no effect was detected in the parental strain, FFA was found mutagenic in *S.typhimurium* TA100 expressing hSULT1A1 (22). In addition, transgenic mice expressing hSULT1A1/1A2, even when knocked out for the *Sult1a1* and *Sult1d1* genes, showed high levels of DNA adducts in liver, kidney, lung and small intestine compared with wild-type (wt) mice after intraperitoneal injection of 400 mg/kg body weight (bw) FFA (27).

This previous study addressing the influence of different SULT forms on the *in vivo* genotoxicity of FFA was performed using intraperitoneal injection. The objective of the present study was to study the genotoxic effect of FFA after oral administration to mimic the route of human dietary uptake using a transgenic mouse model that expresses hSULT1A1/1A2. Male mice were used, since FFA has demonstrated carcinogenic activity in this sex, in the form of renal tubule neoplasms in an inhalation study (15).

Materials and methods

Chemicals

FFA (CAS: 98-00-0) of 98% purity (Sigma-Aldrich, Steinheim, Germany) was diluted in saline shortly before use. Proteinase K and ribonuclease (RNase) A were purchased from Qiagen (Hilden, Germany). Calf intestine alkaline phosphatase, micrococcal nuclease (from *Staphylococcus aureus*) and bovine spleen phosphodiesterase were purchased from Sigma-Aldrich. HPLC-grade methanol, 2-propanol, formic acid and acetic acid were from Carl Roth GmbH (Karlsruhe, Germany). The syntheses of the isotope-labelled reference standard [$^{15}N_5$,$^{13}C_{10}$]N^2-MF-dG has been described previously (22).

Animals

FVB/N (FVB) mice expressing human SULTs 1A1 and 1A2 (hSULT mice) were constructed at the German Institute of Human Nutrition

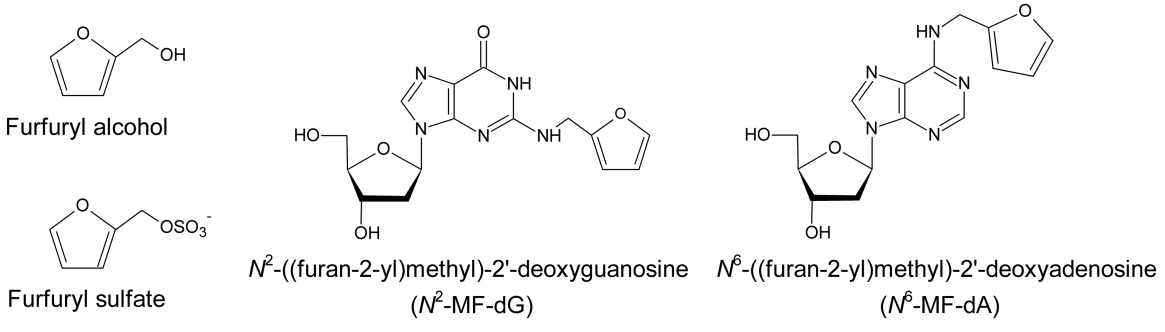

Figure 1. Structures of FFA, FFS and the two adducts, N^2-((furan-2-yl)methyl)-2′-deoxyguanosine (N^2-MF-dG) and N^6-((furan-2-yl)methyl)-2′-deoxyadenosine (N^6-MF-dA), formed after nucleophilic attack by FFS on 2′-deoxyguanosine or 2′-deoxyadenosine.

(28). A line containing multiple copies of the human SULT1A1-1A2 gene cluster, termed tg1 in the original publication (28), was transferred to and further bred at the Norwegian Institute of Public Health. Male FVB mice purchased from the Jackson Laboratories (Bar Harbour, ME, USA) and homozygous male hSULT mice of 9–11 weeks of age were used in the present study. The experiments were carried out in conformity with the laws and regulations for experiments with live animals in Norway and were approved by the Norwegian Animal Research Authority.

Housing

Animals were housed in plastic cages on Nestpak Aspen 4HK bedding (Datesand Ltd, Manchester, UK) with a 12-h light/dark cycle. All mice had free access to food (SDS RM1 maintenance diet, Special Diet Services Ltd, Witham, UK) and tap water. Male littermates were co-housed until the day before the experiment when mice were placed in individual cages.

Treatment of mice and harvest of organs

Animals were given a single administration of 250 mg/kg bw FFA or vehicle (saline) by oral gavage and sacrificed 1 h (FFA) or 3 h (saline control) after exposure. Small intestine, colon, liver and kidneys were collected and rinsed in ice-cold phosphate-buffered saline. The small intestine and colon were cut open longitudinally and rinsed again. The small intestine was divided into proximal and distal parts of equal length. Organs were immediately snap frozen on liquid nitrogen for storage at –80°C.

DNA isolation

DNA was isolated using a Qiagen Blood and Cell Culture DNA Kit. G2 lysis buffer containing 0.2 mg/ml RNase A was added to the frozen tissue, which was cut roughly using a small scissor and mechanically homogenized 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 RNase 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 sterile 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.

Enzymatic digestion of DNA samples

Samples containing 200 µg DNA were dried together with fixed amounts of [$^{13}C_{10}$,$^{15}N_5$]N^2-MF-dG (1161 fmol). The residues were dissolved in 112 µl water and 32 µl of 100 mM sodium succinate buffer (pH 6.0) containing 50 mM $CaCl_2$. Bovine spleen phosphodiesterase (0.5 mU/µg DNA) and micrococcal nuclease (27 mU/µg DNA) were added. The samples were incubated at 37°C for 8 h. A volume of 76 µl 0.5 M Tris (pH 10.9) and 30 mU/µg DNA calf intestine alkaline phosphatase were added and the incubation was resumed at 37°C for 14 h. The samples were diluted with 600 µl water and centrifuged at 15 000 × *g* for 15 min. Adducts were enriched by solid-phase extraction using Oasis HLB columns (3 cm^3, 60 mg, Waters, Eschborn, Germany), which were conditioned with 3 ml methanol and 3 ml water. The columns were loaded with the digestion mixtures, washed with 3 ml water/methanol (95:5) and dried by application of low pressure in the solid-phase extraction chamber for 30 s. Adducts were eluted with 3 ml methanol. After evaporation of the solvents, the residuals were taken up in 50 µl water/methanol (25:75). Samples were centrifuged at 15 000 × *g* for 15 min and the supernatant was used for mass spectrometric analysis.

Quantification of 2-methylfuran adducts by isotope-dilution UPLC–MS/MS

The samples containing concentrated adducts and the internal reference compounds were subjected to analytical reversed phase chromatography using an Acquity UPLC System (Waters) with a UPLC HSS T3 column (1.8 µM, 2.1 × 100 mm, Waters). Samples of 8 µl were injected and eluted with water (Solvent A) and acetonitrile (Solvent B), applying a 10-min gradient starting from 98% Solvent A to 80% Solvent A at 0.35 ml/min flow rate. Both solvents were acidified with 0.25% acetic acid and 0.25% formic acid. The ultra performance liquid chromatography (UPLC) was connected to a Quattro Premier XE tandem quadrupole mass spectrometer (Waters) with an electrospray interface operated in the positive ion mode. The fragmentations of the analytes were monitored by multiple reaction monitoring. The fragmentation of N^2-MF-dG into the aglycone cation [N^2-((furan-2-yl)methyl)-guanine-H]$^+$ (348.1 → 232.1), into [N^2-methylguanine-H]$^+$ (348.1 → 164.0) and the cleavage of the 2-methylfuranyl cation (348.1 → 81.0) were recorded together with the corresponding fragmentations of the internal standard [$^{13}C_{10}$,$^{15}N_5$]N^2-MF-dG (363.1 → 242.1, 363.1 → 174.0 and 363.1 → 81.0). The transition resulting from the neutral loss of 2′-deoxyribose from N^2-MF-dG was used as quantifier signal and the other transitions served as qualifier signals. Levels of N^2-MF-dG were calculated by the peak areas of the traces 348.1 → 232.1 (analyte) and 363.1 → 242.1 (internal standard), based on the known ratio of internal standard in relation to nucleosides in the samples. The tune parameters for N^2-MF-dG detection were as follows: temperature of the electrospray source 110°C; desolvation temperature 450°C; desolvation gas: nitrogen (850 l/h); cone gas: nitrogen (50 l/h); collision gas: argon (indicated cell pressure ~ 5×10^{-3} mbar). For the fragmentation of N^2-MF-dG, collision energies were 12, 25 and 37 eV for the transitions 348.1 → 232.1, 348.1 → 164.0 and 348.1 → 81.0, respectively. The dwell time was set to 100 ms, and capillary voltage was set to 2.5 kV. The cone and RF1 lens voltages were 17 and 0.1 V, respectively. Data acquisition and handling were performed with MassLynx 4.1 software (Waters).

The quality of the quantifier signals of N^2-MF-dG was determined from calculating the signal-to-noise ratio (S/N) of the peaks using a preceding 1-min interval of the chromatogram as background. Peaks with S/N > 4 were considered above the LOD. The LOD of the mass spectrometric method was 0.34 N^2-MF-dG, which corresponds well with the LOD = 0.1 N^2-MF-dG/10^8 2′-deoxynucleosides determined in a previous study in which 500 µg DNA was used for the analysis (22). Quantifier signals of N^2-MF-dG not accompanied by detectable signals for the 348.1 → 164.0 and 348.1 → 81.0 transitions were excluded. Two outliers were also removed from the data set.

Statistical analyses

Grubbs test was used to identify significant outliers, using GraphPad (GraphPad Software Inc., San Diego, CA, USA). Differences between the groups were evaluated with a two-way analysis of variance test, applying the Holm-Sidak method for pairwise multiple comparison

procedures, in SigmaPlot version 12.0 (Systat Software GmbH, Erkrath, Germany). Individual measurements and the means of the groups (2–5 animals as indicated) are presented. A P value of 0.05 or less was considered significant.

Results

N^2-MF-dG adducts in untreated mice

Somewhat surprisingly, we detected signals corresponding to the quantifier signal for N^2-MF-dG in most tissue specimens from untreated animals. Assuming that these signals were produced by N^2-MF-dG, their levels would be up to 0.73 adducts/10^8 nucleosides for all organs, which is close to the LOD of 0.34 adducts/10^8 nucleosides (Figure 2 and Supplementary Table S1, available at *Mutagenesis* Online). The detection of three fragmentations at the same retention time as those of the internal standard strongly indicates that the three peaks in DNA from untreated mice are indeed from N^2-MF-dG. These adducts may result from an unknown exposure, yet, we could not detect any FFA in the standard SDS RM1 diet used in the study when analysed using HPLC with UV detection (unpublished data).

N^2-MF-dG adducts in FFA-exposed wt mice

The administration of FFA led to higher adduct levels in two tissues. In the colon, the mean adduct level was 4.7-fold higher compared to that in the untreated mice ($P < 0.001$) (Figure 2 and Supplementary Table S1, available at *Mutagenesis* Online). Additionally, a borderline significant 2-fold increase in the N^2-MF-dG level was observed in hepatic DNA ($P = 0.052$). In contrast, the adduct levels in the DNA from the small intestine and kidney were similar between the exposed and the untreated wt mice.

Impact of hSULT on the formation of N^2-MF-dG adducts by FFA

Levels of N^2-MF-dG adducts in hSULT mice exposed to FFA increased by 3.8-fold in colonic DNA ($P < 0.001$) and by 2.4-fold in hepatic DNA ($P = 0.006$), compared to those in tissues of untreated hSULT mice (Figure 2 and Supplementary Table S1, available at *Mutagenesis* Online). These effects of FFA were similar to those observed in wt mice. However, the highest level of N^2-MF-dG was detected in DNA from the proximal small intestine of exposed

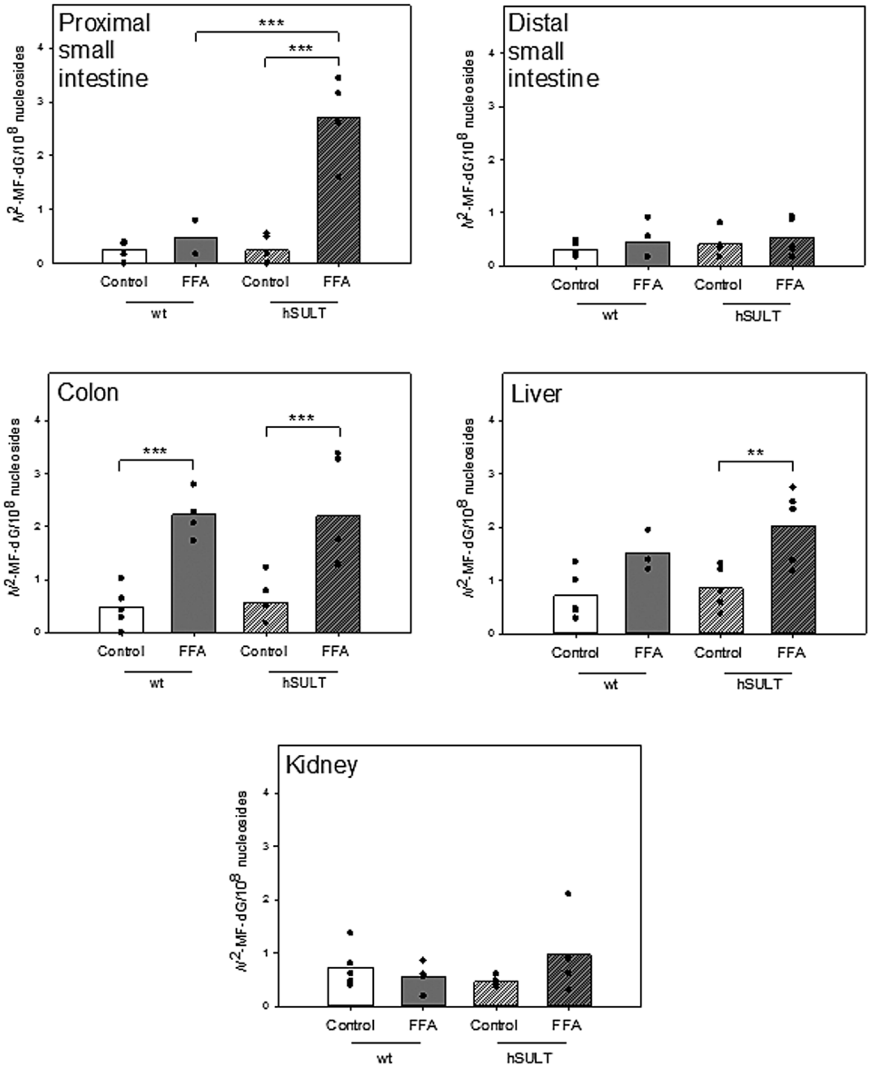

Figure 2. Influence of oral exposure to FFA (versus control) and transgenic hSULT (versus wt) on N^2-MF-dG adducts detected in various mouse organs. Bars indicate mean values and dots represent measurements from individual mice. For some intestinal samples (proximal small intestine = 3, distal small intestine = 5, colon = 2), the quantifier signal was below LOD. These measurements were replaced with LOD/2 before the means were calculated. $^{**}P < 0.01$; $^{***}P < 0.001$.

hSULT mice, and this level was 11- and 6-fold higher compared with those of untreated hSULT mice ($P < 0.001$) and FFA-exposed wt mice ($P < 0.001$), respectively. These signals obtained for the proximal small intestine of FFA-exposed hSULT mice were sufficiently strong for verification using the qualifier transitions (Figure 3). All three signals reflecting the different *m/z* transitions of the analyte matched those of the stable isotope-labelled internal standard. No N^2-MF-dG adducts beyond the background level were formed in the distal small intestine or kidneys of FFA-exposed hSULT mice.

Discussion

In this study, we demonstrate that orally administered FFA forms DNA adducts in proximal small intestine, liver and colon, but not in distal small intestine and kidney of wt and/or hSULT mice. Formation of adducts in proximal small intestine was only observed in the hSULT mice, not in the wt. This is plausible as hSULT1A1 and mSult1a1 strongly differ in their expression in the small intestine, being very high for the human enzyme, especially in the proximal part (28), but negligible for the orthologous mouse enzyme (26). Humans also have abundant SULT expression in the small intestine, including hSULT1A1 (24,29), which suggests that the observed adduct formation may also occur in humans. In the other target tissues, colon and liver, adduct levels were similar in wt and hSULT mice. It is probable that mSult1a1, which is highly expressed in these tissues (26), was involved in the activation. Somewhat surprisingly, transgenic hSULT1A1/1A2, which is also highly expressed in liver and colon (28), did not enhance the adduct formation. We suspect that the high expression of hSULT1A1 in small intestine, the site of absorption, and in many other tissues reduced the amount of FFA reaching the liver and the colon of hSULT mice.

FFA-induced DNA adduct formation in mouse tissues has been investigated in two previous studies. In the first study (22), FFA was administered to wt mice in the drinking water for 28 days. Adducts were found in all three tissues investigated (liver, lung and kidney). In the second study (27), mouse lines differing in SULT status (wt, Sult1a1-knockout, Sult1d1-knockout and transgenic hSULT1A1/1A2 on the Sult1a1/1d1-double knockout background)

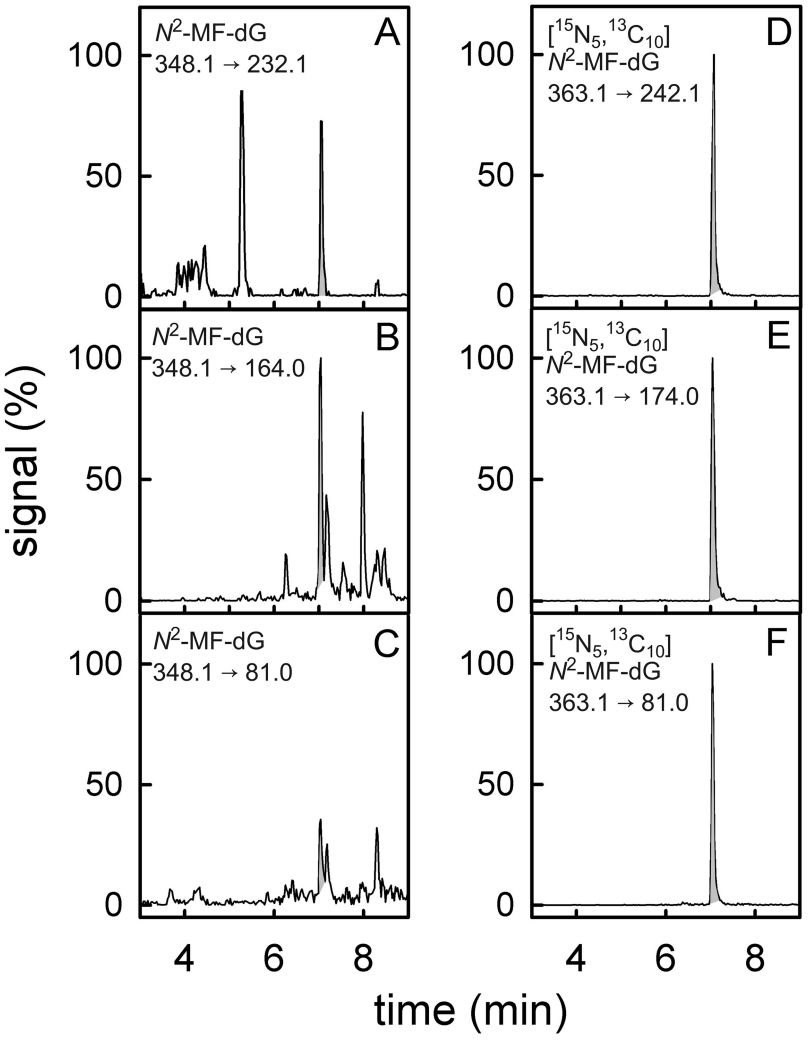

Figure 3. UPLC–MS/MS chromatograms of a DNA sample isolated from proximal small intestine of an FFA-exposed hSULT mouse. Panels **A–C** show chromatograms of the fragmentation of N^2-MF-dG into the aglycone cation [N^2-((furan-2-yl)methyl)-guanine-H]$^+$ (A: 348.1 → 232.1), into [N^2-methylguanine-H]$^+$ (B: 348.1 → 164.0) and the cleavage of the 2-methylfuranyl cation (C: 348.1 → 81.0). Simultaneously, the corresponding fragmentations of the internal standard [$^{13}C_{10}$,$^{15}N_5$]N^2-MF-dG were recorded (**D**: 363.1 → 242.1; **E**: 363.1 → 174.0; **F**: 363.1 → 81.0). The ratio of peak areas for the transition 348.1 → 232.1 (N^2-MF-dG) and for the transition 363.1 → 242.1 ([$^{13}C_{10}$,$^{15}N_5$]N^2-MF-dG) was used to calculate the N^2-MF-dG content of the DNA.

received a single intraperitoneal injection of FFA. Adduct formation was studied in five organs (liver, lung, kidney, small intestine and colon) of both sexes. Adducts were detected in all tissues of wt animals, in males following the descending order: colon > liver > kidney and small intestine > lung. Sult1a1 knockout clearly reduced adduct formation in all tissues, except male small intestine. Sult1d1 knockout diminished adduct formation in male kidney and in small intestine of both sexes. Transgenic hSULT1A1/1A2 (in the Sult1a1/1d1-double knockout background) enhanced adduct formation, compared to the wt, in all tissues except male colon, leading to the following order of adducts in the humanized males: liver > small intestine > kidney > colon > lung. Thus, there were some differences between the results of the previous study (intraperitoneal treatment with 400 mg FFA/kg bw) and those of our new study (gavage of 250 mg FFA/ kg bw): (i) adducts in renal and small intestinal tissues were detected after intraperitoneal, but not after oral treatment; the difference may be due to the higher dose used and the larger DNA samples analysed in the former study (500 rather than 200 µg); (ii) in transgenic animals, the highest adduct levels were observed in the proximal small intestine after oral exposure, in contrast to liver after intraperitoneal treatment; some reservation is required in the judgement of this difference, as proximal and distal intestine were combined in the previous study. Additionally, homozygous transgenic animals with intact endogenous Sult were used in one study, but hemizygous transgenic animals in the Sult1a1/1d1-double knockout background in the other study. Nevertheless, it is tempting to speculate that hSULT1A1 expressed at high levels in the small intestine of humans (29) and hSULT mice (28) may lead to first-pass metabolism/activation at the chief site of absorption of FFA after oral exposure, namely the proximal small intestine; (iii) expression of hSULT1A1/1A2 (even in the Sult1a1/1d1-double knockout background) enhanced hepatic adduct formation after the intraperitoneal injection compared to untreated wt animals, but not after oral exposure; as mentioned in a previous section, metabolism in small intestine may have reduced the amount of FFA reaching the liver of hSULT mice after oral (compared to intraperitoneal) exposure.

In conclusion, the data from this study indicate that various tissues in both wt and hSULT mice are susceptible to DNA adduct formation following oral FFA exposure. Murine enzymes, e.g. mSult1a1, are likely contributors to formation of the detected adducts in the liver and colon in the present experiment. The high level of adducts formed in the proximal small intestine after oral FFA exposure of mice expressing hSULT1A enzymes demonstrates that the transgenic animal model detects effects of oral FFA exposure that are missed in the wt mouse. This finding indicates that hSULT1A1, which unlike mSult1a1 is highly expressed in small intestine (28), mediated the activation of FFA in the proximal small intestine. It is probable that exposure of this tissue to orally administered FFA was particularly high, as FFA is a small, amphiphilic compound expected to be rapidly absorbed. It is important to note that hSULT1A1 is specifically expressed in differentiated enterocytes, but not in undifferentiated enterocytes and other intestinal cells in humans (29) as well as hSULT mice (S. Florian, W. Meinl and H. Glatt, unpublished results). Thus, adduct formation may be restricted to the terminally differentiated cells, destined for defoliation. Such a mechanism may actually be protective—a hypothesis to be verified. The situation may be different for other routes of exposure, such as inhalation. Indeed, inhalation of FFA produced neoplasms of the nose in male rats and renal tubule neoplasms in male mice (15). In this context, it is of interest to know that nasal tissue (only studied in the mouse) is rich in SULT activity (30). Likewise, mouse Sult1d1, a form able to activate FFA (21,27), shows its highest expression in the kidney (26). Finally, N^2-MF-dG and N^6-MF-dA adducts have been detected in human lung specimens (31), a tissue expressing hSULT1A1. For these reasons, further studies are required to estimate genotoxic and carcinogenic risks that may result from human exposure to FFA. Thereby, the route of exposure will deserve special attention.

Supplementary data

Supplementary Table S1 is available at *Mutagenesis* Online.

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