Chem. Senses 36: 443–452, 2011

doi:10.1093/chemse/bjr015
Advance Access publication March 11, 2011

Butylated Hydroxytoluene Is a Ligand of Urinary Proteins Derived from Female Mice

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Accepted February 2, 2011

Abstract

Mice secrete substantial amounts of protein, particularly proteins called the major urinary proteins (MUPs), in urine. One function of MUPs is to sequester volatile pheromone ligands, thereby delaying their release and providing a stable long-lasting signal. Previously, only MUPs isolated from male mice have been used to identify ligands. Here, we tested the hypothesis that MUPs derived from females may also sequester volatile organic compounds. We identified butylated hydroxytoluene (BHT), a synthetic antioxidant present in the laboratory rodent diet, as a major ligand bound to urinary proteins derived from C57BL/6J female urine. BHT was also bound to the male-derived proteins, but the binding was less prominent than that in female urine, even though males express approximately 4 times more proteins than females. We confirmed that the majority of BHT in female urine was associated with the high molecular weight fraction (>10 kDa) and the majority of the proteins that sequestered BHT were MUPs as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The sequestration of BHT by MUPs was further confirmed by employing the recombinant MUP8 whose natural analogue has been reported in both sexes. Therefore, our data indicate that MUPs expressed in both sexes can bind, transport, and excrete xenobiotics into urine and raise the possibility that in addition to the known role in chemical communication, MUPs function as a defense mechanism against exogenous toxins.

Key words: gas chromatography/mass spectrometry (GC/MS), major urinary proteins (MUPs), solid phase microextraction (SPME), volatile ligands, xenobiotics

Introduction

Mice as well as other rodents secrete substantial amounts of protein in their urine, which is not generally observed in humans or other mammals (Miyazaki et al. 2006). Urinary proteins are detected in both male and female mice, although the total concentration in female urine is 4 times lower than that in male urine (Cheetham et al. 2009). Male mice may excrete 20–40 mg of protein in urine per day (Beynon and Hurst 2003). The major urinary proteins (MUPs) are the predominant proteins in mouse urine (Hurst and Beynon 2004). MUPs are a family of heterogeneous proteins ranging from 18 to 20 kDa and belong to the lipocalin family, which can transport lipids in blood and other hydrophilic body fluids (Flower 1996). Mudge et al. (2008) conducted thorough genomic and phenotypic analyses of MUPs in several inbred laboratory strains. No female-specific MUPs have been identified. All the identified MUPs expressed in females were expressed in males, while males additionally expressed several male-specific MUPs (Mudge et al. 2008).

Functionally, MUPs are involved in sexual communication such as puberty acceleration (Mucignat-Caretta et al. 1995), intermale aggression (Chamero et al. 2007), and female attraction (Roberts et al. 2010). The polymorphic variations in MUPs are extensive in wild populations where they are involved in individual recognition (Hurst et al. 2001; Cheetham et al. 2007), inbreeding avoidance (Sherborne et al. 2007), and evaluation of genetic heterozygosity of

potential mates (Thom et al. 2008). In addition, recent studies suggested that MUP-1 is involved in regulation of glucose and lipid metabolism (Hui et al. 2009; Zhou et al. 2009).

Some of the behavioral studies mentioned above employed recombinant MUPs that excluded the presence of volatile pheromones, demonstrating that MUPs alone, without binding to pheromones, may function as chemical signals in mice (Hurst et al. 2001; Chamero et al. 2007; Roberts et al. 2010). However, under natural conditions, it is not clear whether the behavioral consequences are due to MUPs only or MUP–ligand complexes. MUPs sequester volatile pheromone ligands, delaying their release and providing signal stability and persistence as well as structural stability for pheromone ligands (Hurst et al. 1998; Timm et al. 2001). MUPs have a hydrophobic pocket that retains volatile organic ligands (Böcskei et al. 1992). There are indications that the binding affinity between ligands and MUPs is very strong. Even after an extensive purging of male urine with helium for up to 48 h, urine samples retained an odor reminiscent of mouse urine (Novotny et al. 1980). Moreover, purified MUPs possessed a characteristic mousy odor identified as 2-*sec*-butyl-4,5-dihydrothiazole (SBT) (Lehman-McKeeman et al. 1998). Additional volatile ligands such as 3,4-dehydro-*exo*-brevicomin (DHB), 4-ethylphenol, *p*-toluidine, and 6-hydroxy-6-methyl-3-heptanone (HMH) have been identified from male MUPs (Bacchini et al. 1992; Robertson et al. 1993; Novotny et al. 1999).

Despite the fact that MUPs are expressed in both male and female mice, only ligands of MUPs isolated from male urine have been characterized. No examination of volatile organic compounds sequestered by MUPs derived from female urine has been reported. Given that MUPs derived from female mice are also expressed in males, we hypothesized that MUPs in female urine may also sequester volatile compounds. Because the ligands identified from male MUPs have been predominantly male-derived pheromones, we wondered what ligands might be bound to MUPs from female mice and their identity was the focus of this study.

Materials and methods

Mice

Six of each C57BL/6J (B6) male and female mice (3–12 months old) were born and raised in our mouse colony and used as urine donors. The mice were kept in a temperature-controlled room at 23 °C on a 12:12 h light:dark cycle. Males and females were housed separately and 2–3 mice were placed in each polypropylene cage (10 × 28.5 × 12.5 cm). All animals were maintained under uniform conditions and cared for in accordance with the Guide for the Care and Use of Laboratory Animals, and the experimental protocols were approved by the Institutional Animal Care and Use Committee in the Monell Chemical Senses Center (Approval number: 900p).

Mouse diet

All mice were fed the laboratory rodent diet 5001 as purchased from Purina Mills. The information regarding the ingredients of the rodent diet is available from the company's website (http://labdiet.com/pdf/5001.pdf).

Urine and serum collection

Urine samples were collected individually by gentle abdominal pressure (Monahan and Yamazaki 1993). Serum donors, 3 of each B6 male and female mice, were lightly anesthetized with halothane and bled from their tails. Blood was collected in a glass test tube and allowed to clot for 2 h at room temperature. The serum samples were then spun at 4000 rpm to remove any remaining blood cells. The urine and serum samples were stored at –20 °C until needed for the analysis.

Collection of volatile compounds in urine and serum by solid phase microextraction

Each urine and serum sample was extracted 2 times: first without addition of guanidine hydrochloride (GHCl) and then with GHCl. Two hundred and fifty microliters of urine, or 100 μL of serum, were placed in a 4 mL glass vial and a 2 cm three-component solid phase microextraction (SPME) fiber (30 μm carboxen, 50 μm divinyl benzene, polydimethyl siloxane, Supelco Corp.) was inserted into the vial for collection of the headspace volatile compounds released from urine or serum. The vial was submerged in a water bath at 37 °C and was equilibrated for 10 min. Then, the headspace volatile organic compounds were extracted by the SPME fiber for 30 min. The sample in the vial was agitated using a magnetic stirrer during the entire extraction period. The SPME fiber containing the adsorbed volatile compounds was then inserted into the injection port of a gas chromatograph/mass spectrometer (GC/MS) and the volatile compounds were desorbed for 5 min at 230 °C. Six urine samples and 3 serum samples each collected from different individual male and female mice were analyzed before and after protein denaturation.

Denaturation of proteins in urine and serum and monitoring ligands

We added GHCl, a protein denaturant, to intact urine and serum samples and identified any volatile compounds whose headspace concentrations increased upon denaturation as a volatile ligand released from urinary proteins. GHCl has been widely used as a denaturation agent. It binds, unfolds, and denatures proteins. Its denaturation mechanism has been described in detail elsewhere (Timasheff 1993). In this study, the denaturation was accomplished by adding 0.191 g of GHCl into a vial containing 250 μL of intact urine. For the serum samples, 0.075 g of GHCl was added for denaturation. The total concentration of GHCl in urine as well as in serum was 8 M. Each sample was allowed to denature

for an hour at room temperature prior to collection and analysis of the headspace volatile compounds in the sample.

Gas chromatography/mass spectrometry

A Thermo-Finnigan Trace GC/MS (Thermo Electron) system was used. The Trace GC/MS was equipped with a Stabilwax column (30 M × 0.32 mm with 1.0 μ coating; Restek) which was used for separation and analysis of the desorbed volatile organic compounds. We employed the following chromatographic protocol for separation before MS analyses: 60 °C for 4 min, then programmed at 6 °C/min to 210 °C with a 20-min hold at this final temperature. Column flow was constant at 1.5 mL/min. The injection port was held at 230 °C. Operating parameters for the mass spectrometer were as follows: ion source temperature at 200 °C, ionizing energy at 70 eV; scanning frequency was 4/s from m/z 41 to m/z 300.

Compound identification

About 50 volatile organic compounds were identified and are listed in Supplementary Table S1. Compound identification was accomplished through manual interpretation of spectra as well as matching against the NIST 2002 library and comparison with standard samples that were either purchased or synthesized. HMH, DHB, SBT, and 2-isopropyl-4,5-dihydrothiazole were synthesized and kindly provided by Dr Kenji Mori and their synthetic procedure is described in detail elsewhere (Tashiro and Mori 1999; Tashiro et al. 2008). Texanol (a paint-derived compound; Gallagher et al. 2008) was supplied by the Eastman Chemical Company. Benzyl methyl ketone, 2-methyl-1-butyl acetate, 6-methyl-3-heptanone, *cis*-2-penten-1-yl acetate, *trans*-5-hepten-2-one, *exo*-brevicomin, cedr-8-ene, *o*-toluidine, cedrol, and formanilide were tentatively identified using the library as well as published literature (Novotny et al. 1986; Schwende et al. 1986; Jemiolo et al. 1991, 1994). All other compounds were purchased from Sigma-Aldrich.

Data analysis

A total of 24 total ion chromatograms that constituted 6 of each intact and denatured urine samples collected from different individual male and female mice were obtained. We selected 7 previously reported MUP ligands and butylated hydroxytoluene (BHT) from the chromatograms and compared the areas calculated from their base peak (m/z) before and after protein denaturation. The selected compounds were HMH, 2-heptanone, *exo*-brevicomin, DHB, SBT, α- and β-farnesenes, and BHT.

Extraction of BHT from mouse diet

One gram of the ground mouse diet was placed in a 7 mL glass vial and 4 mL deionized water was added to the vial. The headspace BHT released from the diet was extracted by SPME and analyzed by GC/MS. The analytical conditions were identical to those of the urine analyses.

Quantification of headspace concentrations of BHT

We prepared a series of BHT standard solutions dissolved in hexane across the range of concentrations estimated to be in the headspace above urine and serum samples (50 ng/mL, 250 ng/mL, 1 μg/mL, 5 μg/mL, and 25 μg/mL). One microliter of each solution was injected into the GC/MS 3 times, and a BHT standard curve (concentration vs. area) was created. Then, the areas of BHT taken from the analyses of urine and serum were converted into the concentrations using the standard curve.

Fractionation of urine by centrifugal filtration

Five hundred microliters of each male and female urine sample were placed on a Microcon YM-10 centrifugal filter with 10 kDa molecular mass cut-off (Millipore) and spun at 14 000 g for 30 min at room temperature. We obtained 2 fractions: MW < 10 kDa and MW > 10 kDa. Each fraction was extracted by SPME and analyzed by GC/MS without addition of GHCl and then with GHCl.

Monitoring BHT binding to recombinant MUP8

The recombinant MUP8 (rMUP8) (MGI: 3709619) was provided by Dr Lisa Stowers. The cDNA for the MUP8 was amplified by polymerase chain reaction from male B6 mouse liver cDNA using oligonucleotide primers. The recombinant MUP was expressed as a fusion protein with maltose-binding protein. The detailed procedure has been described elsewhere (Logan et al. 2008; Papes et al. 2010). Five hundred microliters of a BHT solution dissolved in water (40 ng/500 μL) placed in a 4 mL glass vial were repeatedly extracted and analyzed in the absence or presence of 250 μg of the rMUP8. The headspace concentrations of BHT in both conditions were then compared.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Electrophoresis was performed on 10–20% Tris–Tricine polyacrylamide gels according to the manufacturer's instructions (BioRad). Each urine sample was diluted with phosphate-buffered saline (1:10) and Tris–Tricine sample buffer was then added to the diluted sample (1:1). The sample was heated for 5 min at 95 °C and then loaded onto the gel. A 40 mA current was applied to the gel. Protein bands were visualized with LabSafe GEL Blue stain (G-Biosciences).

Results

Figure 1 shows the total ion chromatograms of the volatile organic compounds extracted from intact male urine (**a**), protein-denatured male urine (**b**), intact female urine (**c**),

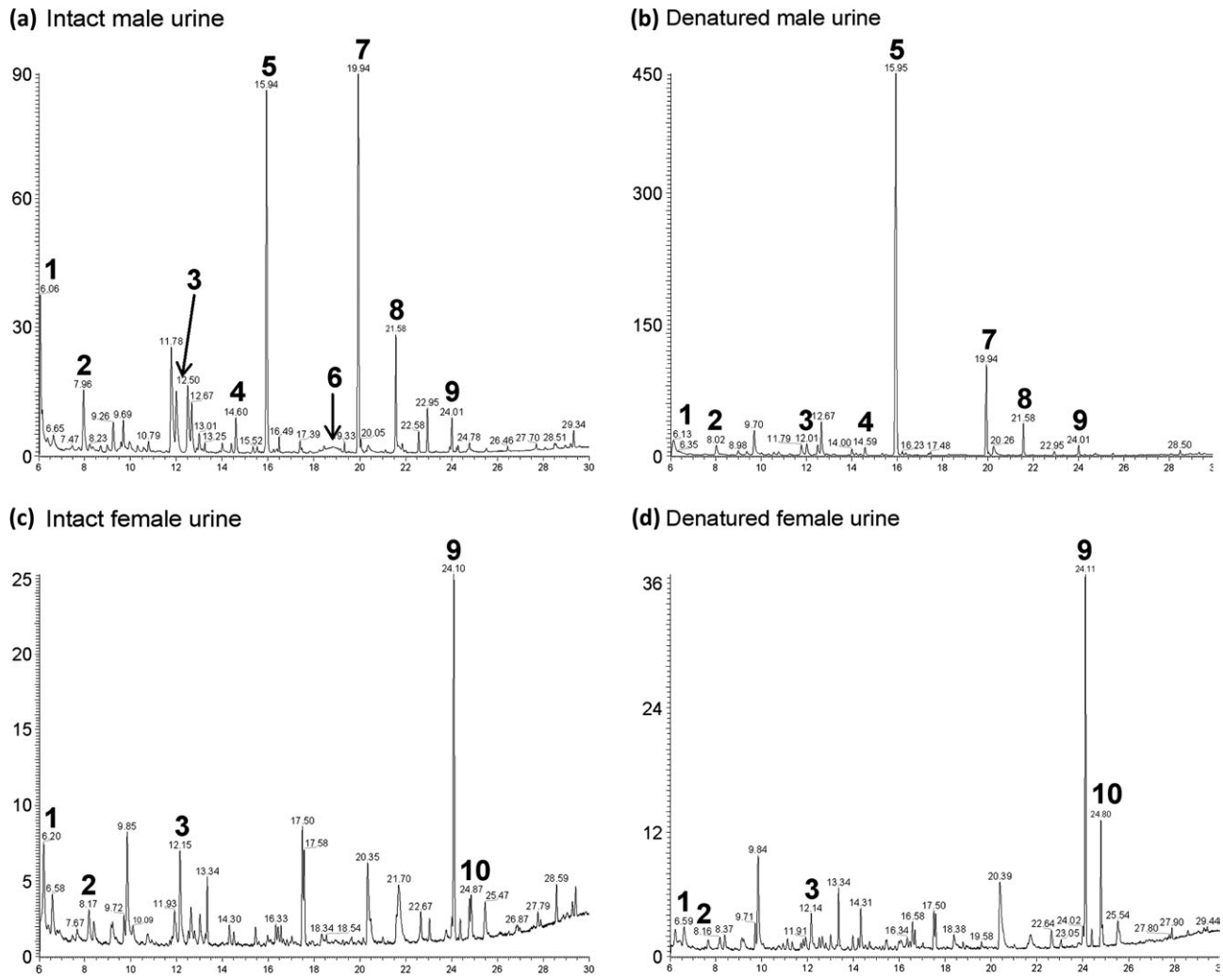

Figure 1 The total ion chromatograms of the volatile organic compounds extracted from intact male urine **(a)**, denatured male urine **(b)**, intact female urine **(c)**, and denatured female urine **(d)**. The *y* axis indicates the absolute (raw) intensity [value × 10^6] and the *x* axis indicates retention time in minutes. Prominent compounds shown in the chromatograms include cyclic dehydrated products of HMH (1 and 2), *exo*-brevicomin (3), DHB (4), SBT (5), intact HMH (6), β-farnesene (7), α-farnesene (8), Texanol (a paint-derived compound) (9), and BHT (10). Upon denaturation, the intensity of SBT released from male urine increased about 5 times and the intensity of BHT released from female urine increased more than 3 times. A comprehensive compound list is given in Supplementary Table S1.

and protein-denatured female urine (**d**). Many of volatile compounds were identified and are listed in Supplementary Table S1. Distinct volatile profiles between male and female urine were observed. For example, SBT and β-farnesene were the predominant compounds detected in intact male urine, but these compounds were absent or present at trace levels in intact female urine (Figure 1 and Supplementary Table S1).

Upon protein denaturation by the addition of GHCl, the volatile profiles in the urine samples changed dramatically. For example, a large amount of SBT was released from male urine after protein denaturation (Figure 1b). Its area increased more than 5 times (Figure 2d) and accounted for about 60% of the combined areas of the detected peaks in the chromatograms of the denatured male urine samples (Figure 1b), indicating that SBT was preferentially bound to urinary proteins from males. The amounts of previously reported MUP ligands, 2-heptanone, DHB, and α- and β-farnesenes also increased in male urine samples upon denaturation, although their increases were smaller than that of SBT (Figure 2). As observed by Zhang et al. (2007), both intact and dehydrated forms of HMH were detected in the chromatograms of male and female urine samples (Figures 1 and 2 and Supplementary Table S1). We did not see any increase in the levels of either the intact or dehydrated forms of HMH from both male and female urine following denaturation (Figure 2). In male urine, the level of DHB (an unsaturated brevicomin) increased upon denaturation, whereas that of *exo*-brevicomin (a saturated brevicomin) was unchanged (Figure 2).

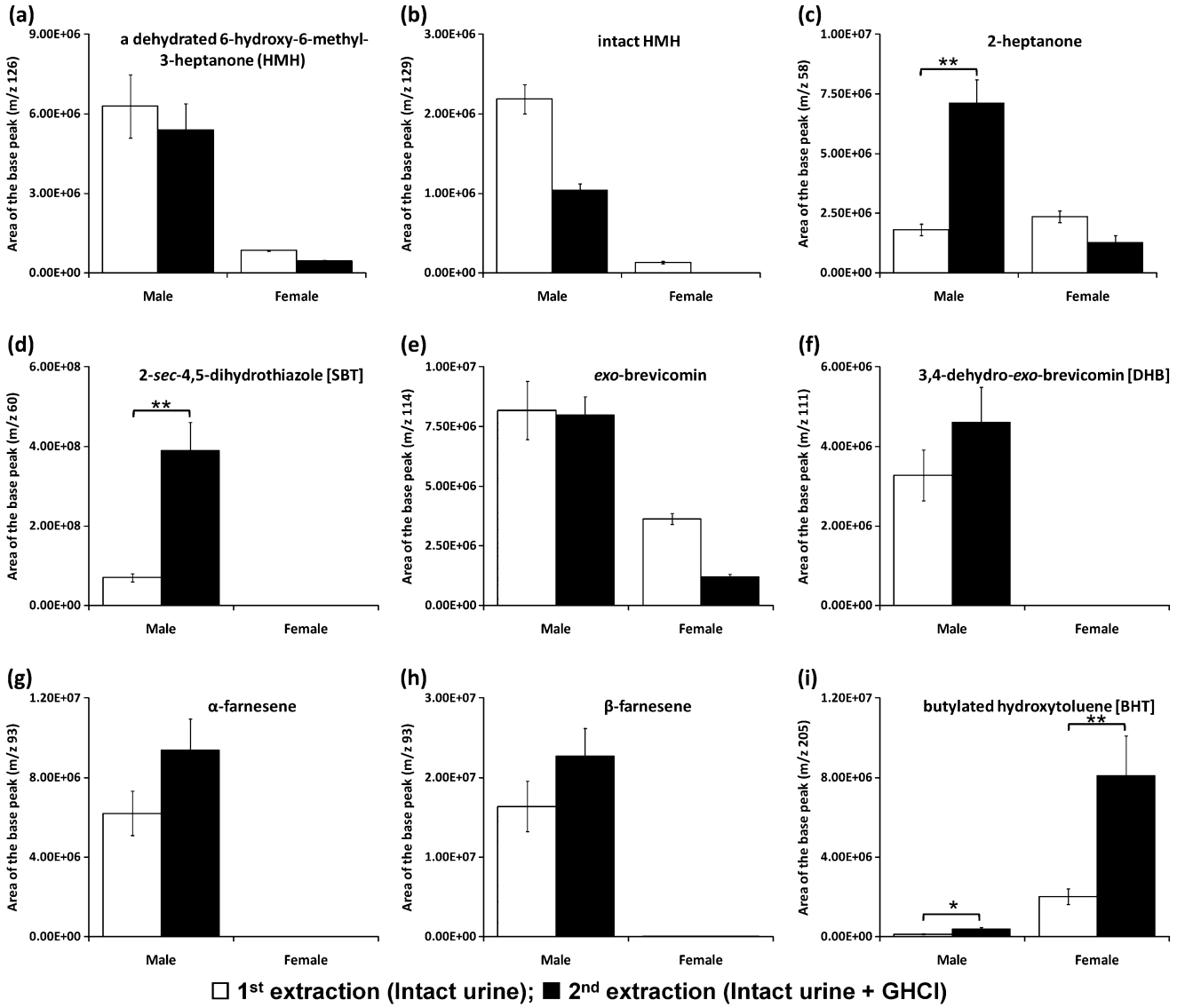

Figure 2 The area changes of several prominent compounds in male and female urine before and after protein denaturation by addition of GHCl. $N = 6$ each male and female urine collected from different individual mice. Each urine sample was extracted 2 times: first without addition of GHCl (intact) and then with GHCl (denatured). $*P < 0.05$; $**P < 0.01$ (Mann–Whitney test).

More than 40 endogenous compounds including reported mouse pheromones (e.g., HMH, 2-heptanone and 2,5-dimethylpyrazine [reviewed in Novotny 2003]) were detected in the female urine samples (Supplementary Table S1). DHB was absent or present at trace levels in our female samples (Supplementary Table S1). Instead, substantial amounts of its saturated form (*exo*-brevicomin) were observed (Figure 1c). We did not see any noticeable increase in the levels of these female mouse-derived compounds upon denaturation. Unexpectedly, we observed a substantial increase of BHT, a synthetic antioxidant present in the laboratory rodent diet (Supplementary Figure S1), released from female urine upon denaturation (Figures 1d and 2i).

To confirm whether the increased release of these volatile organic compounds upon addition of GHCl is due to the denaturation of urinary proteins or due to a "salting out" (decrease in the solubility of organic volatile molecules in urine) effect of GHCl, we prepared and extracted 2 equivalent BHT solutions (40 ng/250 μL) with and without GHCl, respectively. No difference in the BHT level was observed whether GHCl was added to the solution or not (Supplementary Figure S2a), suggesting that the release of volatile ligands in urine was due to the protein denaturation not due to a salting out effect of GHCl. In order to exclude any potential salting out effect of GHCl, we extracted each urine and serum sample sequentially without GHCl and then with GHCl, ensuring that any volatile compounds whose headspace concentrations increase upon protein denaturation by the addition of GHCl at the second extraction can be regarded as ligands released from proteins.

As shown in Figures (Figure 2i and Supplementary Figure S2b), we also observed a small but significant increase of BHT in male urine upon protein denaturation (from 0.10 to 0.29 ng; $P < 0.05$, Mann–Whitney test); however, the headspace concentration of BHT observed above female urine was far greater (from 1.29 to 4.63 ng upon denaturation; $P < 0.01$, Mann–Whitney test). Because BHT is a ligand of urinary proteins in female urine and females have no MUPs that differ from males, we presumed that BHT is also likely to be a ligand of male urinary proteins. To investigate this, we spiked BHT (250 μg) into male urine samples (250 μL) and monitored changes in the volatile profile. After addition of BHT, the level of SBT increased substantially just as was seen upon protein denaturation (Figure 3). These data suggest that the spiked BHT binds to male urinary proteins, thereby displacing SBT, the principal bound ligand, from the proteins.

In order to measure the distribution of unbound and bound BHT to proteins in urine, we fractionated male and female urine samples using centrifugal filtration and obtained 2 fractions: MW < 10 kDa and MW > 10 kDa. As shown in Figure 4, the majority of BHT in female urine was associated with the high molecular weight fraction and released upon denaturation, indicating that a large portion of BHT was bound to urinary proteins. Upon denaturation, the BHT level in the low molecular weight fraction (MW < 10 kDa) increased, although it was smaller than that in high molecular weight fraction, indicating that the filters were not totally efficient in cutting off proteins at 10 kDa.

We separated the urinary proteins through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in order to confirm that the proteins responsible for the ligand binding are MUPs. As shown in Figure 5, the majority of proteins in B6 male and female urine samples eluted around 20 kDa, which are presumably MUP bands. The binding and release of BHT by MUPs was further confirmed employing rMUP8. When rMUP8 was added to a BHT solution dissolved in water (40 ng/500 μL), SPME extraction slowly depleted BHT from the solution, whereas it was depleted rapidly in the absence of rMUP8 (Figure 6). Once GHCl was added to the sample containing rMUP8 plus BHT, a large portion of BHT was released and quickly depleted, demonstrating that the proteins responsible for the BHT binding are MUPs.

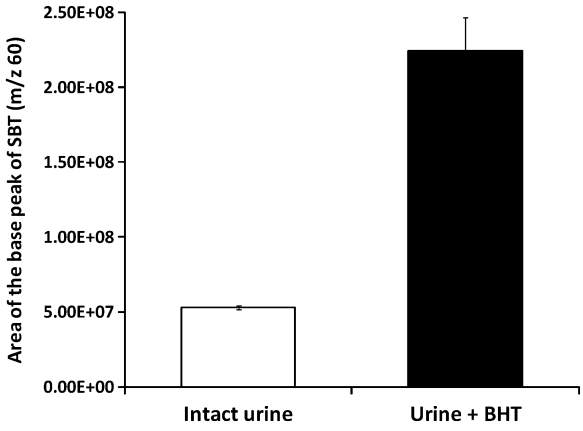

Figure 3 The area change of SBT in male urine upon addition of BHT.

Previously, MUPs have been identified in mouse serum (Finlayson et al. 1965). To examine this further, we collected serum samples from both male and female mice and monitored the release of BHT from the samples before and after protein denaturation. BHT was not detected in the intact serum samples but was released upon denaturation of serum proteins derived from both male and female mice (Figure 7), demonstrating that BHT was bound to serum proteins as it was to urinary proteins. Overall, these data suggest that MUPs expressed in both sexes can bind and excrete BHT into urine.

Discussion

To identify volatile ligands associated with MUPs, previous studies have involved extensive purification steps of MUPs prior to extraction of ligands (Bacchini et al. 1992; Robertson et al. 1993; Lehman-McKeeman et al. 1998; Novotny et al. 1999). Our approach was different. We used intact urine samples and monitored the change of volatile profiles before and after denaturation of proteins by addition of GHCl. SPME followed by GC/MS analysis was employed to isolate and identify ligands. Any volatile compounds whose headspace concentrations increased upon denaturation were likely released from urinary proteins. This method is simple and does not require extensive purification steps for MUPs prior to extraction of volatile ligands. Because each sample was sequentially extracted without and then with GHCl, this approach may not be appropriate for determining ligands whose binding affinities to proteins are weak. Those ligands may have been bound to proteins but released rapidly, and substantial portions of them may be removed at the first extraction (e.g., Supplementary Figure S2a). In other words, due to the sequential nature of extraction, no change or a decrease in the level of volatile compounds upon addition of GHCl does not necessarily mean that the volatile compounds are not bound to proteins. However, we observed substantial increases of several previously reported pheromone ligands from urine upon denaturation (Figure 2), demonstrating that this approach is a valid method for determining some of the ligands of MUPs. It is noteworthy that we observed the continuous release (slow depletion) of SBT from a denatured male urine sample (250 μL) as we performed repeated SPME extractions (Supplementary Figure S3). Because this was not due to the saturation of the ligand in the SPME fiber or in the GC/MS, we speculate that the slow depletion may be due to an incomplete denaturation of urinary proteins by GHCl.

SBT was the predominant ligand of urinary proteins in our B6 male urine samples. This is probably due to the

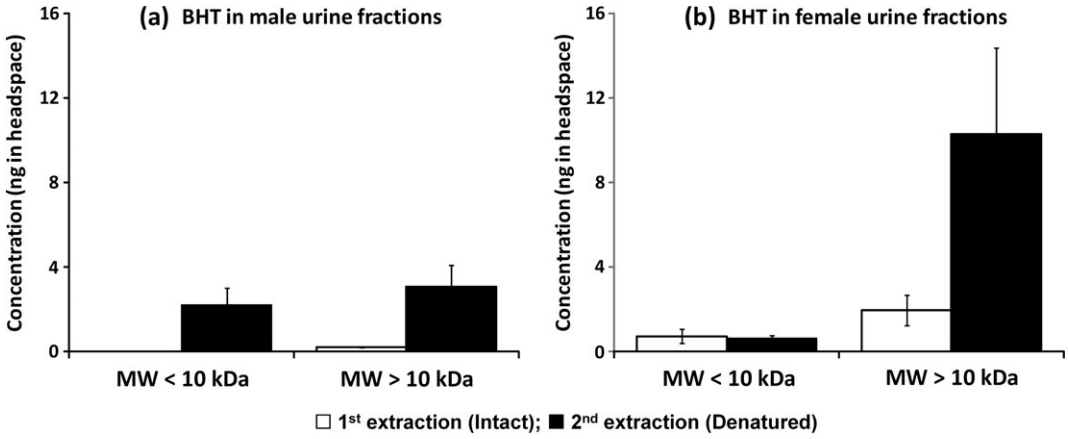

Figure 4 The distribution of BHT in the MW < 10 kDa and the MW > 10 kDa fractions obtained from male and female urine before and after protein denaturation.

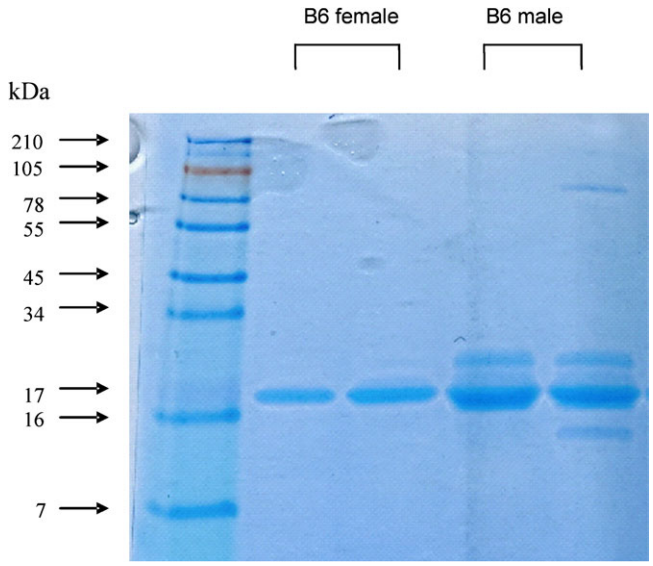

Figure 5 Intact B6 male and female urine samples resolved by SDS–PAGE. Each lane contained a urine sample collected from different individuals. MUPs were the major protein bands observed. This figure appears in color in the online version of *Chemical Senses*.

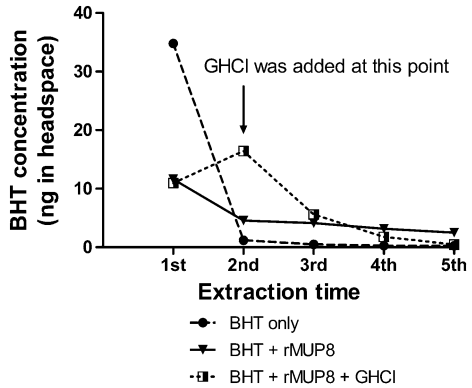

Figure 6 Differential depletion patterns of BHT dissolved in water (40 ng/500 μL) in the absence or presence of rMUP8.

presence of darcin, an atypical MUP isoform, in B6 urine that preferentially binds this compound (Armstrong et al. 2005; Roberts et al. 2010). Darcin is exclusively expressed in male mice and present in the male urines of wild mice and the inbred laboratory mice derived from the C57 lineage but not in most of those derived from the Castle and Swiss strains (Armstrong et al. 2005; Cheetham et al. 2009). Whether this dramatic increase of SBT would be observed in the denatured urines of mice with different genetic backgrounds remains to be determined.

BHT was the major ligand of urinary proteins in our B6 female urine samples. The majority of BHT in female urine appears to be associated with the high molecular weight materials in urine and is released upon denaturation (Figure 4), indicating that a large portion of BHT was bound to urinary proteins. Although the method used here does not provide the information about which specific proteins are responsible for the BHT binding, the urinary proteins that bind and release BHT are almost certainly MUPs for several reasons. First, they account for 99% of urinary proteins (Hurst and Beynon 2004). Second, our SDS-PAGE experiments demonstrated that the majority of urinary proteins in both male and female urine samples were MUPs (Figure 5), excluding the presence of other proteins that could conceivably be responsible for the binding. Third, the sequestration of BHT by MUPs was further confirmed employing the rMUP8 whose natural analogue has been demonstrated in both male and female urine (Clark et al. 1985; Logan et al. 2008 and references therein; Figure 6).

Our data suggest that the sequestration of BHT by MUPs is more prominent in female than in male (Figure 2i and Supplementary Figure S2b). This occurs despite the fact that all MUPs present in female mice are detected in male mice and the protein concentrations in female urine are about 4 times lower than those in male urine (Mudge et al. 2008; Cheetham et al. 2009). As shown in Supplementary Figure S2b, the headspace concentration of BHT was more than 10 times

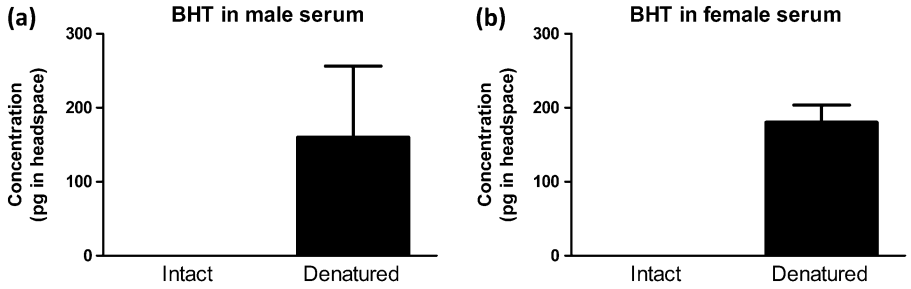

Figure 7 The changes of the headspace concentration of BHT in male and female serum before and after protein denaturation. $N = 3$ each male and female serum samples collected from different individual mice. No BHT was detected in intact serum.

higher in the intact female urine samples than in the intact male samples. This pattern was also true in the denatured urine samples. Sex differences in the excretion of BHT into urine have been reported in rats and mice, although the differences were smaller than those observed in our study. Higher levels of BHT metabolites have been detected in female urine than in male urine. When a single oral dose of either 1 or 2.4 mg [^{14}C]BHT was administered to Wistar rats, approximately 40% of the radioactivity was recovered in female urine, whereas about 25% of the radioactivity was found in male urine (Daniel and Gage 1965). (The remaining radioactivity was found mainly in feces.) The same pattern was observed in a different study that used multiple doses (44 mg/kg body weight; up to 5 times over a period of 10 days; Tye et al. 1965). When DDY/Slc mice were given a single oral dose of either 20 or 500 mg [^{14}C]BHT per kg body weight, the excretion of BHT tended to be larger in female mouse urine than in male urine during the first several days after the treatment (Matsuo et al. 1984). One possible explanation for these sex differences in the excretion of BHT is that there may be binding competitions between ligands to MUPs. Besides BHT, other ligands whose concentrations are much higher than that of BHT are present in male mice. Some of them (e.g., SBT) are preferentially bound to MUPs, preventing BHT from binding to the proteins. On the other hand, these ligands are undetected or present at lower concentrations in female mice.

When BHT was added to male urine, the pheromone SBT was displaced (Figure 3), resulting in an increase in volatile SBT. This phenomenon was previously reported by Robertson et al. (1998) who observed that whereas MUPs from fresh urine of wild mice contained SBT and DHB, MUPs derived from urine posts did not have these ligands but had menadione, a precursor of vitamin K. The authors then demonstrated that these pheromone ligands were displaced by menadione (possibly derived from the environment), suggesting that this could be a mechanism by which pheromonal communication could be disrupted in the face of environmental pollutants. For example, one function of MUPs has been proposed to ensure a slow release of pheromones, thereby prolonging the signal. In the face of high concentrations of BHT-like compounds, this delayed release may be reduced or eliminated. In addition, the uptake and retention of environmental ligands may disrupt normal mating processes by displacing chemical signals used for attraction and reproductive endocrine control. This novel effect of environmentally derived compounds on animal behavior needs to be further evaluated.

We confirmed the presence of BHT in the commercial diet fed our mice (Supplementary Figure S1), suggesting that this is the source of BHT in urine. BHT is not listed as an additive added to the diet. We suspect that BHT may be a contaminant in the manufacturing plant where other animal feeds containing this antioxidant may be processed or in one of the ingredients purchased by Purina Mills.

BHT is metabolized primarily in the liver and its oxidation products as well as their glucuronide conjugates are the major metabolites in rats, mice, rabbits, and humans (reviewed in Babich 1982; Madhavi and Salunkhe 1996). Because the presence of MUPs in mouse serum has been demonstrated (Finlayson et al. 1965) and our results showed that BHT was bound to serum proteins (Figure 7), we speculate that a small portion of BHT may be bound to MUPs in blood before BHT goes through the oxidation process, although we cannot exclude the possibility that other proteins in serum may be responsible for the binding. The sequestration of BHT by proteins in serum as well as in urine is consistent with the proposition that BHT binds to MUPs in blood and the BHT–MUP complex is filtered and secreted in urine. A further study is required to identify the serum proteins responsible for the binding and to compare the amount of BHT released from urine and serum upon denaturation, matched to the amount of MUP in each.

The sequestration of numerous xenobiotics by urinary MUPs in male rats, also known as α_{2u}-globulin proteins, has been extensively investigated since the accumulation of the α_{2u}-globulin–xenobiotic complex in rat kidney induces hyaline droplet nephropathy (reviewed in Hard et al. 1993). This is a male-specific renal carcinogenesis because female rats do not express the proteins (Hard et al. 1993 and references therein). Strikingly, it is not seen in mice mainly because MUPs are not reabsorbed by mouse kidney, whereas about 60% of α_{2u}-globulin proteins are reabsorbed by rat kidney (Lehman-McKeeman and Caudill 1992).

It has also been suggested that male-derived MUPs retain and excrete exogenous compounds in vivo. When a radiolabeled industrial chemical, a polychlorinated biphenyl derivative, was administered to male mice intraperitoneally, significant radioactivity was found to be incorporated with urinary MUPs (Larsen et al. 1990). In addition, Robertson et al. (1998) reported that menadione, when injected subcutaneously, was associated with the excreted male urinary MUPs. Our current data provide new evidence that BHT, a dietary-derived xonobiotic, can bind to the MUPs that are expressed in both sexes. Although the detoxification of BHT through MUPs is not a normal process in mammals, it is noteworthy that BHT is captured by another protein family, fatty acid–binding proteins in rat intestine (Kanda et al. 1990). It is known that MUP genes are expressed in various secretory glands (e.g., mammary, parotid, sublingual, submaxillary, lachrymal glands) (Shaw et al. 1983; Shahan et al. 1987). Whether MUPs in these glands sequester xenobiotics remains to be determined. Prior observations on binding of exogenous compounds to male MUPs combined with our current results with MUPs derived from females raise the possibility that MUPs might be involved in a general defense mechanism by binding and removing potentially toxic substances ingested or inhaled from the mouse's environment.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/.

Acknowledgements

We are grateful to Drs Kenji Mori and Lisa Stowers for providing synthesized chemicals and the recombinant MUP, respectively, and to Drs Bruce A. Kimball and Joseph Brand for useful suggestions on earlier versions of this manuscript.

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