TOXICOLOGICAL SCIENCES **135(1)**, 37–47 2013
doi:10.1093/toxsci/kft143
Advance Access publication June 28, 2013

Identification of 2-Piperidone as a Biomarker of CYP2E1 Activity Through Metabolomic Phenotyping

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Received April 15, 2013; accepted June 17, 2013

Cytochrome P450 2E1 (CYP2E1) is a key enzyme in the metabolic activation of many low molecular weight toxicants and also an important contributor to oxidative stress. A noninvasive method to monitor CYP2E1 activity *in vivo* would be of great value for studying the role of CYP2E1 in chemical-induced toxicities and stress-related diseases. In this study, a mass spectrometry–based metabolomic approach was used to identify a metabolite biomarker of CYP2E1 through comparing the urine metabolomes of wild-type (WT), *Cyp2e1*-null, and *CYP2E1*-humanized mice. Metabolomic analysis with multivariate models of urine metabolites revealed a clear separation of *Cyp2e1*-null mice from WT and *CYP2E1*-humanized mice in the multivariate models of urine metabolomes. Subsequently, 2-piperidone was identified as a urinary metabolite that inversely correlated to the CYP2E1 activity in the three mouse lines. Backcrossing of WT and *Cyp2e1*-null mice, together with targeted analysis of 2-piperidone in mouse serum, confirmed the genotype dependency of 2-piperidone. The accumulation of 2-piperidone in the *Cyp2e1*-null mice was mainly caused by the changes in the biosynthesis and degradation of 2-piperidone because compared with the WT mice, the conversion of cadaverine to 2-piperidone was higher, whereas the metabolism of 2-piperidone to 6-hydroxy-2-piperidone was lower in the *Cyp2e1*-null mice. Overall, untargeted metabolomic analysis identified a correlation between 2-piperidone concentrations in urine and the expression and activity of CYP2E1, thus providing a noninvasive metabolite biomarker that can be potentially used in to monitor CYP2E1 activity.

Key Words: **CYP2E1; biomarker; metabolomics; 2-piperidone; cadaverine.**

Cytochromes P450 (P450) are the most important enzymes in xenobiotic metabolism (Guengerich and Cheng, 2011). CYP2E1 is involved in the oxidative metabolism of numerous therapeutic agents and environmentally important chemicals such as alcohol, aliphatic and aromatic hydrocarbons, and solvents and industrial monomers. Many CYP2E1-catalyzed reactions not only generate reactive metabolites but also produce reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (Trafalis *et al*., 2010). The involvement of CYP2E1 in metabolic bioactivation of oxidative stress indicates that this P450 plays an important role in many toxic events, particularly in the liver where it is constitutively expressed (Caro and Cederbaum, 2004). It was shown that CYP2E1-catalyzed biotransformation might be responsible for some chemical-induced carcinogenesis (Leon-Buitimea *et al*., 2012; Trafalis *et al*., 2010) and tissue injury (Chen *et al*., 2008), whereas CYP2E1-mediated ROS production and lipid peroxidation contribute to the pathogenesis of diabetes (Schafer *et al*., 2010) and nonalcoholic steatohepatitis (Abdelmegeed *et al*., 2012; Aubert *et al*., 2011). The increased risk of malignancy and metabolic diseases has been associated with elevated CYP2E1 expression and activity, which can be caused by chronic exposure to acetone, alcohol, isoniazid, and other compounds (Gonzalez, 2005) or genetic polymorphisms (Catanzaro *et al*., 2012; Lu *et al*., 2011; Sotsuka *et al*., 2011).

Determination of the role of CYP2E1 in disease would be greatly facilitated by real-time evaluation of CYP2E1 expression and activity. Chlorzoxazone (CNZ), a muscle relaxant, has been used as a selective probe substrate for measuring CYP2E1 activity *in vivo* (Ernstgård *et al*., 2007). CNZ assays require the intake of a pharmacologically active compound, multiple blood samplings, and the bioanalysis of CNZ and its metabolite (6-hydroxy-CNZ) (Emery *et al*., 1999; Vesell and Korsunsky, 1998), which makes it invasive and inconvenient. In addition, the accuracy of this method is affected by factors such as the dose and the involvement of other P450s (Ernstgård *et al*., 2004). A preferred solution is to

identify endogenous substrates that can function as noninvasive biomarkers of CYP2E1 activity. *Cyp2e1*-null mice (Lee *et al.*, 1996) and *CYP2E1*-humanized mice (Cheung *et al.*, 2005) could provide the ideal experimental platform for screening and characterizing endogenous biomarkers of CYP2E1 activity because the metabolic consequences of the loss of mouse CYP2E1 activity in the *Cyp2e1*-null mice and the introduction of human CYP2E1 activity in the *CYP2E1*-humanized mice can be directly examined in these models.

Metabolomics, as a high-throughput systems biology tool, possesses the analytical power to comprehensively examine the metabolism of both exogenous or endogenous substances and to determine the metabolizing properties of enzymes and their physiological functions in the biological system (Johnson *et al.*, 2012). In this study, a mass spectrometry–based metabolomics approach was applied to examine the metabolic differences among the wild-type (WT), *Cyp2e1*-null, and *CYP2E1*-humanized mice. 2-Piperidone was identified and characterized as a potential biomarker of CYP2E1 activity.

MATERIALS AND METHODS

Animals and chemicals. The WT, *Cyp2e1*-null, and *CYP2E1*-humanized mice used in this study were on the 129/Sv background. All mice were housed in temperature- and light-controlled rooms and were given water and pelleted chow *ad libitum*. Animal experiments were carried out in accordance with the Institute of Laboratory Animal Resources guidelines and approved by the National Cancer Institute Animal Care and Use Committee. 2-Piperidone, cadaverine, glutarimide, disulfiram, acetone, and the solvents for liquid chromatography and organic synthesis were obtained from Sigma-Aldrich (St Louis, MO). 3-Hydroxy-2-piperidone, 4-hydroxy-2-piperidone, and 5-hydroxy-2-piperidone were obtained from Ark Pharm (Libertyville, IL).

Sample collection and preparation. Pathogen-free mice (2- to 3-month-old) were housed individually in Nalgene metabolic cages (Tecniplast, Exton, PA), and urine was collected for 24 h to avoid the effects of diurnal variation. Blood was collected by retro-orbital bleeding and processed to serum, and liver samples were harvested after killing. Liver microsomes were prepared by ultracentrifugation. All samples were stored at −80°C.

Diets and chemical treatments. NIH-31 diet was used as the maintenance chow for WT, *Cyp2e1*-null, and *CYP2E1*-humanized mice but was replaced by AIN-93G purified diet 6 days prior to chemical treatments. To determine the biotransformation of 2-piperidone or cadaverine *in vivo*, male WT mice were treated with 2-piperidone (ranging from 0.1 to 40 mg/kg) or cadaverine (20 mg/kg), respectively, by ip injection, and urine was collected for 24 h. The inhibition of CYP2E1 in WT mice was achieved by ip injection of disulfiram (20 mg/kg), whereas the induction of CYP2E1 in WT mice was achieved by ip injection of acetone (2 g/kg) for 3 days.

Primary hepatocyte extraction and culture. Primary hepatocytes were isolated from the WT and *Cyp2e1*-null mice using a two-step perfusion technique, which involves the sequential perfusion of the liver with a solution containing EDTA and collagenase (Seglen, 1976). Cells were treated with 100μM of 2-piperidone or 100μM of cadaverine dissolved in DMSO. After 12- and 24-h treatment periods at the prescribed time points, the cells were harvested and subjected to metabolite analysis.

Metabolomic analyses of urine and serum samples. Urine samples were prepared by mixing 40 μl of urine with 160 μl of 50% aqueous acetonitrile and centrifugation at 18,000 × g for 10 min to remove protein and particulates. Serum samples were denatured by the addition of 40-fold dilution of acetonitrile (66% vol:vol water). The supernatants were injected into the UPLC (Waters Corporation, Milford, MA) and metabolites separated by a gradient ranging from water to 95% aqueous acetonitrile containing 0.1% formic acid over a 10-min run. An Acquity UPLC BEH C18 column (Waters) was used to separate chemical components at 35°C. The mobile phase flow rate was 0.5 ml/min with an aqueous acetonitrile gradient containing 0.1% formic acid over a 10-min run. The QTOF Premier mass spectrometer (Waters) was operated in the positive electrospray ionization mode. Capillary voltage and cone voltage were maintained at 3 kV and 20 V, respectively. Source temperature and desolvation temperature were set at 120°C and 350°C, respectively. Nitrogen was used as both cone gas (50 l/h) and desolvation gas (600 l/h), and argon was used as collision gas. For accurate mass measurement, the time-of-flight mass spectrometry was calibrated with sodium formate solution (ranging 100–1000 *m/z*) and monitored by the intermittent injection of the lock mass sulfadimethoxine ($[M + H]^+ = 311.0814$ *m/z*) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx software (Waters) in centroid format. Metabolites were quantitated via peak areas that were normalized using an internal standard.

Gas chromatography-mass spectrometry analysis of urine samples. Diluted urine samples and standard solutions containing 5μM 4-nitrobenzoic acid as the internal standard were applied to Waters Oasis HLB SPE columns, washed with water, and eluted with methanol. The eluate was dried under nitrogen, resuspended in acetonitrile, and derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide/10% trimethylchlorosilane. Samples were heated at 60°C for 30 min and then transferred to autoinjector vials. Gas chromatography-mass spectrometry (GC-MS) analysis was conducted using an Agilent GC 6890N/MS 5973N system. The initial oven temperature of 100°C was held for 2 min, then ramped at 10°C/min, and held for 2 min at 220°C. The injector temperature was 200°C, and the interface temperature was 220°C. One microliter of sample was injected onto the column using helium as the carrier gas, held at a constant flow of 1 ml/min. The mass spectrometer was operated in scan mode (50–550 amu) with a solvent delay of 7 min.

Multivariate analysis of metabolomic data. Chromatographic and spectral data were deconvoluted by MarkerLynx (Waters) software. A multivariate data matrix containing information on sample identity, ion identity (retention time and *m/z*), and ion abundance was generated through centroiding, deisotoping, filtering, peak recognition, and integration. The intensity of each ion was calculated by normalizing the single ion counts versus the total ion counts in the whole chromatogram. The data matrix was exported into SIMCA-P software (Umetrics, Kinnelon, NJ) and transformed by mean-centering and *Pareto* scaling. Principal components of urine were generated by principal components analysis (PCA) or partial least squares discriminant analysis (PLS-DA) and were depicted in a scores scatter plot. The ions contributing to the separation of sample groups were identified in the respective loadings plot (Chen *et al.*, 2007).

Synthesis of 6-hydroxy-2-piperidone. An oven-dried 100 ml round-bottomed flask was equipped with a stir bar and charged with glutarimide (0.20 g, 1.8 mmol, 1.0 eq.). The system was placed under an atmosphere of nitrogen, and 20 ml of dichloromethane was added. After cooling to 0°C, a solution of diisobutylaluminium hydride (DIBAL) in toluene (1.5M, 1.2 ml, 1.8 mmol, 1.0 eq.) was injected over the course of 5 min. When effervescence had ceased, the reaction was stirred for another 10 min. Then, an additional portion of DIBAL (1.5M, 1.3 ml, 1.9 mmol, 1.05 eq.) was injected over the course of 5 min. The reaction was monitored by thin-layer chromatography and stirred to completion on ice.

Analysis on 2-piperidone and cadaverine in mouse chows. After drying, 100 mg of NIH-31 diet and 100 mg of AIN-93G diet were homogenized in acetonitrile. The supernatants were obtained through centrifuging thrice at 10,000 × g for 10 min and diluted 10 times by acetonitrile. Five microliters of supernatant were injected into the liquid chromatography-mass spectrometry (LC-MS) or GC-MS systems. Target molecules were quantitated via peak areas that were normalized using an internal standard compared with standard curves of 2-piperidone and cadaverine.

Statistical analysis. Experimental values are expressed as mean ± SD. Statistical analysis was performed using two-tailed Student's *t*-test, except for the *in vitro* metabolism of 2-piperidone that was analyzed by one-way ANOVA. A value of $p < 0.05$ is considered statistically significant.

RESULTS

Urinary Metabolomics of WT and Cyp2e1-null Mice

Following LC-MS analysis of urine samples from male WT and *Cyp2e1*-null mice, a clear separation of metabolites between the two groups was revealed in a PLS-DA model (Fig. 1A). A similar separation was observed between female WT and *Cyp2e1*-null mice (Fig. 1B). More importantly, the loading plots of both male and female urinary metabolomes (Figs. 1C and D) showed a metabolite with $[M + H]^+ = 100.077^+$ *m/z* as a major contributor to the distinctive separation of WT and *Cyp2e1*-null mice in the PLS-DA models. The elemental composition and chemical structure of this metabolite were proposed based on the accurate mass measurements and MS/MS fragmentography. This compound was finally determined to be 2-piperidone by a comparison with authentic standard (Fig. 2).

Distribution of 2-Piperidone in the Urine of WT, Cyp2e1-*null, and* CYP2E1-*humanized Mice*

Quantitative analysis confirmed that 2-piperidone was present at high levels in the urine of *Cyp2e1*-null mice but virtually undetectable in the WT mice ($p < 0.001$) (Fig. 3A). A similar distribution pattern of 2-piperidone was also observed in the urine samples of *CYP2E1*-humanized mice [hCYP2E1(+/+), mCyp2e1(−/−)], and their littermate controls [hCYP2E1(−/−), mCyp2e1(−/−)] ($p < 0.001$) (Fig. 3B) and further confirmed by examining its levels in serum samples from both male and female WT and *Cyp2e1*-null mice (Fig. 3C). These results strongly suggested that the level of 2-piperidone in mouse biofluids was highly correlated with the presence of both mouse and human CYP2E1.

Backcrossing of WT and Cyp2e1-*null Mice to Confirm That 2-Piperidone Is Related to CYP2E1 Expression*

The correlation between 2-piperidone and *Cyp2e1* genotypes was further examined by backcrossing of WT and *Cyp2e1*-null mice. The heterozygous m*Cyp2e1*(+/−) male and female mice were generated as the F1 generation. Subsequently, three

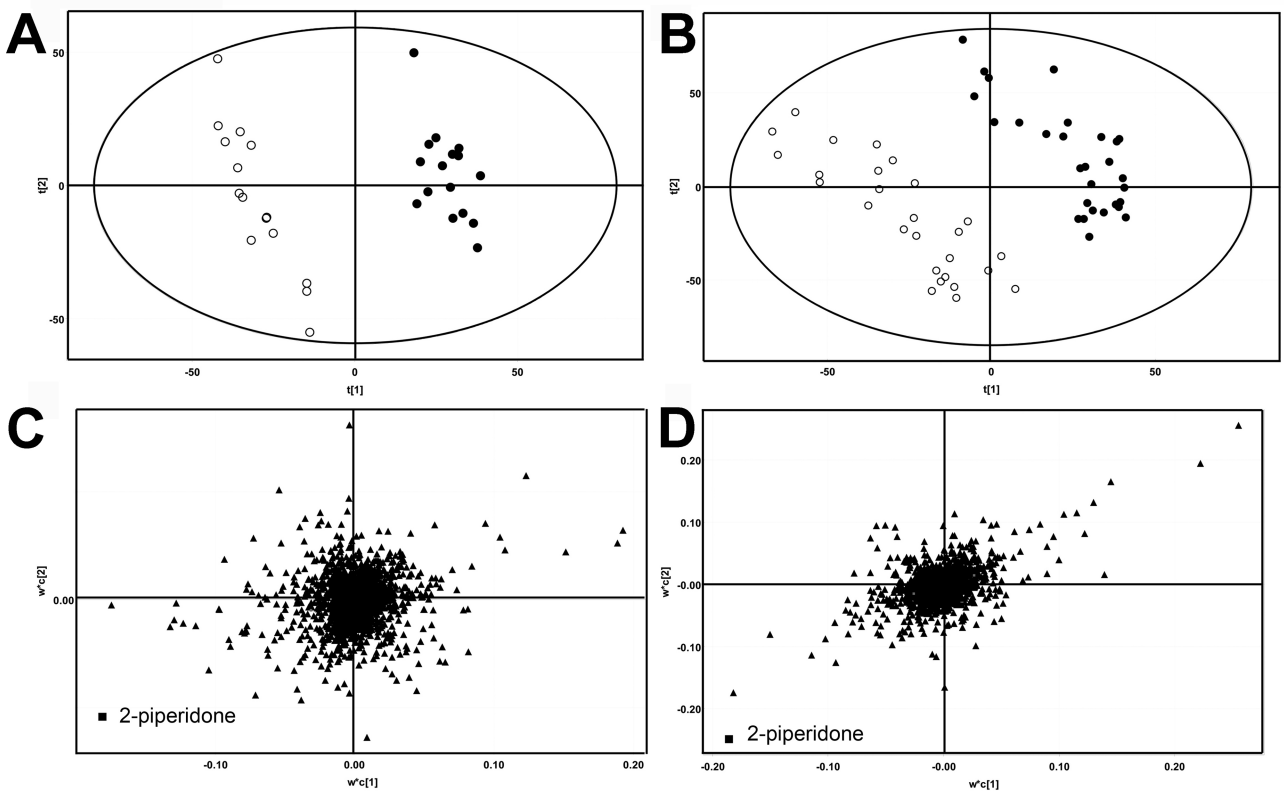

FIG. 1. Metabolomic analysis of urine samples from the WT (●) and *Cyp2e1*-null mice (○). Urine samples were analyzed in positive mode in a LC-MS system. Data were processed using Markerlynx and SIMCA-P$^+$ software. (A) Scores plot of the PLS-DA model on male WT and *Cyp2e1*-null mice. The fitness (R^2 value) and prediction power (Q^2 value) of the model were 0.315 and 0.196, respectively. (B) Ion ($[M + H]^+ = 100.077^+$ *m/z*) is shown as a box which is located in the lower left corner of the loading plot. (C) Scores plot of the PLS-DA model on female WT and *Cyp2e1*-null mice. R^2 value and Q^2 value of the model were 0.424 and 0.289, respectively. Loading plot of the PLS-DA model on male WT and *Cyp2e1*-null mice. (D) Loading plot of the PLS-DA model on female WT and *Cyp2e1*-null mice. Ion ($[M + H]^+ = 100.077^+$ *m/z*) is shown as a box that is located in the lower left corner of the loading plot.

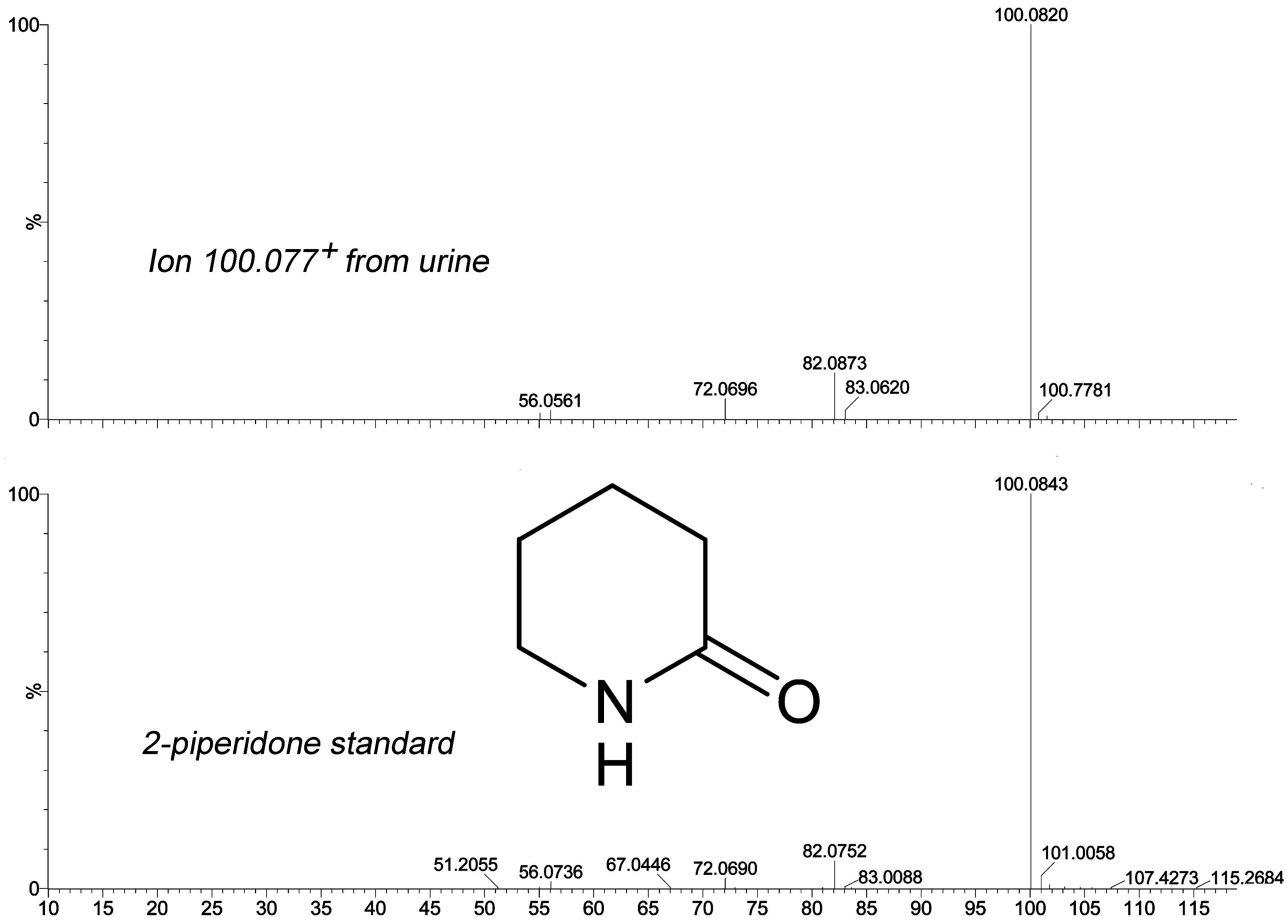

FIG. 2. Identification of 2-piperidone through a comparison of MS/MS spectra. MS/MS fragmentography is shown for the fragmentograms of urinary ion ([M + H]$^+$ = 100.077 *m/z*) (top) and 2-piperidone (bottom).

genotypes, m*Cyp2e1*(+/+), m*Cyp2e1*(+/−), and m*Cyp2e1*(−/−) mice, were produced at the expected Mendelian ratios in the F2 generation after crossing the F1 mice. Similar to the parent WT and m*Cyp2e1*-null mice, the distribution of 2-piperidone in the urine of F2 mice was consistent with the genotypes in both genders, i.e., 2-piperidone was abundant in backcrossed m*Cyp2e1*(−/−) mice but deficient in backcrossed m*Cyp2e1*(+/+) and m*Cyp2e1*(+/−) mice (Figs. 4A and B). This backcrossing experiment further confirmed that 2-piperidone is inversely correlated to CYP2E1 expression and therefore a potential biomarker for CYP2E1 activity. A higher variability in 2-piperidone levels was noted in the females compared with males (Figs. 3 and 4). The mechanism of this gender variability is not known, although it could be due to differences in estrous cycle and hormones in the female mice under study.

In Vivo *Metabolism of 2-Piperidone*

The contrast between the high abundance of 2-piperidone in *Cyp2e1*-null mice and its low abundance in WT mice suggested 2-piperidone as a potential CYP2E1 substrate. To investigate this possibility, two doses of 2-piperidone (20 and 40 mg/kg) were administered to WT mice. The resulting metabolomic profiles showed separation of 2-piperidone-treated mice from control mice in a dose-dependent manner (Fig. 5A). 2-Piperidone (I) and a metabolite with [M + H]$^+$ = 116.0709$^+$ *m/z* (II) were identified as the major contributors to the separation of sample groups in the loadings plot (Fig. 5B). Metabolite II was assumed to be a hydroxylated 2-piperidone, with the possible sites of hydroxylation at the carbon-3, -4, -5, or -6 position of 2-piperidone. The chemical identity of metabolite II was finally determined to be 6-hydroxy-2-piperidone after comparing the chromatograph and MS/MS fragmentography of this metabolite with a synthesized standard of 6-hydroxy-2-piperidone and other hydroxylated 2-piperidone standards (Fig. 5C; Supplementary fig. 1). The retention times of 3-hydroxy-2-piperidone, 4-hydroxy-2-piperidone, and 5-hydroxy-2-piperidone were 0.48, 0.61, and 0.39 min, respectively. 6-Hydroxy-2-piperidone in urine and the 6-hydroxy-2-piperidone standard have a retention time of 0.53 min. The MSMS fragmentation of 6-hydroxy-2-piperidone in urine only matched the MSMS pattern of the 6-hydroxy-2-piperidone standard.

To further confirm the role of CYP2E1 in the conversion of 2-piperidone to 6-hydroxy-2-piperidone, WT mice were treated

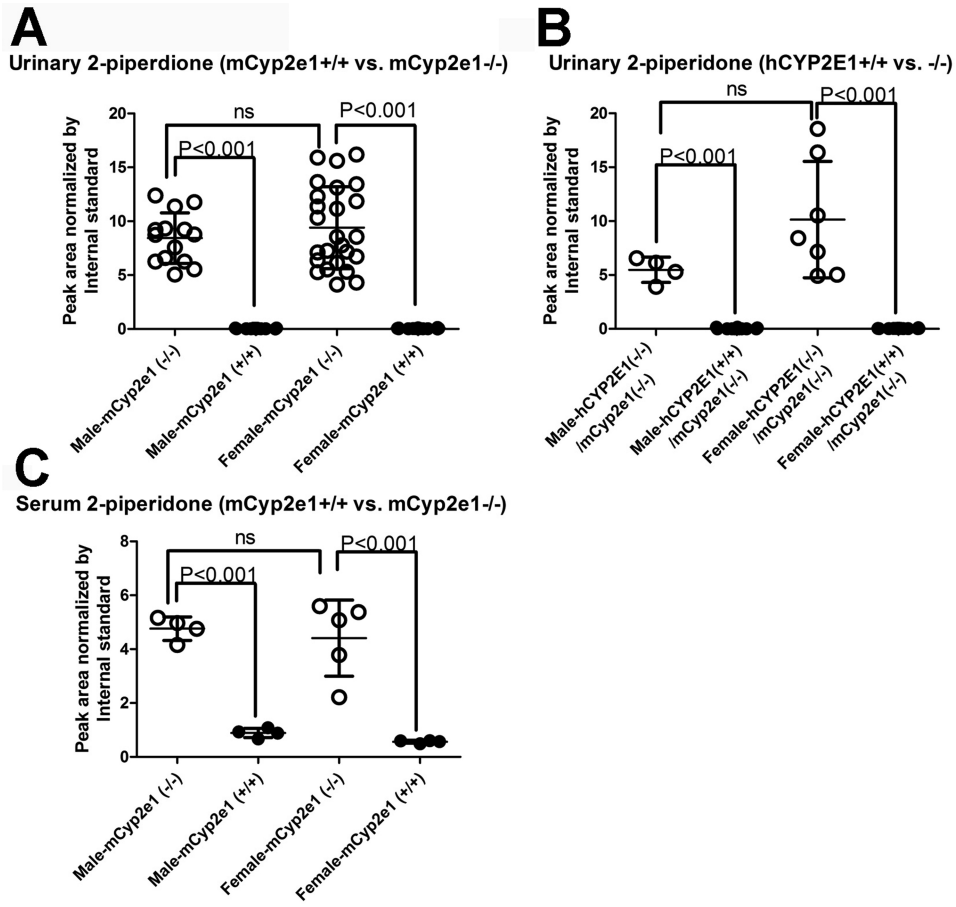

FIG. 3. Genotype-dependent distribution of 2-piperidone. Relative abundance of 2-piperidone was expressed by the peak area normalized by internal standard. (A) Distribution of 2-piperidone in the urine of male and female WT (mCyp2e1+/+) and *Cyp2e1*-null (mCyp2e1−/−) mice. (B) Distribution of 2-piperidone in the urine of male and female *CYP2E1*-humanized mice (hCYP2E1+/+, mCyp2e1−/−) and their littermates (hCYP2E1−/−, mCyp2e1−/−). (C) Distribution of 2-piperidone in the serum of male and female WT and *Cyp2e1*-null mice. ns, no significant difference.

with six doses of 2-piperidone (0.1, 0.2, 1, 2, 10, and 20 mg/kg). In addition, disulfiram, a CYP2E1 inhibitor (Emery *et al.*, 1999), was coadministered with the 20 mg/kg 2-piperidone dose. The level of 6-hydroxy-2-piperidone in urine increased dose dependently after treating 2-piperidone ranging from 0.1 to 10 mg/kg (Fig. 6A), suggesting that 2-piperidone is the direct precursor of 6-hydroxy-2-piperidone. Interestingly, the production of 6-hydroxy-2-piperidone from the 20 mg/kg 2-piperidone treatment was lower than that from the 10 mg/kg 2-piperidone treatment, which could be due to substrate inhibition of CYP2E1 (Jones *et al.*, 2011; Lee and Kim, 2013). Cotreatment of disulfiram led to the decrease of 6-hydroxy-2-piperidone in WT mice, thus further suggesting a catalytic role for CYP2E1 in the biotransformation of this compound (Fig. 6A). Moreover, when WT mice were treated with acetone, a CYP2E1 inducer (Bondoc *et al.*, 1999; Sinclair *et al.*, 2000), incubation of 2-piperidone with liver microsomes from control and acetone-treated mice showed that 2-piperidone 6-hydroxylase activity in mouse liver was significantly induced by acetone and dramatically inhibited by disulfiram (Fig. 6B). These data indicate that CYP2E1 is the major enzyme responsible for converting 2-piperidone to 6-hydroxy-2-piperidone both *in vivo* and *in vitro*.

Identifying Mouse Chow as a Source of 2-Piperidone

In Vivo

In order to identify the source of 2-piderione in urine, a diet-switching experiment was performed. The normal NIH-31 chow, which contains both seasonal plant and animal materials, was replaced with AIN-93G chow that comprised purified nutritional components. After feeding the AIN-93G purified diet, PCA analysis of urinary metabolomics did not show a clear separation between WT and *Cyp2e1*-null mice (Supplementary fig. 2A). 2-Piperidone was also not detected in the sera of AIN-93G diet-fed WT and *Cyp2e1*-null mice (Supplementary fig. 2B). Therefore, the observed contrast between the feeding of NIH chow (Fig. 1) and the feeding of AIN-93G diet (Supplementary fig. 2) suggested the dietary relevance of 2-piperidone.

To determine whether the mouse chow is the only source of 2-piperidone *in vivo*, a mass balance experiment was conducted

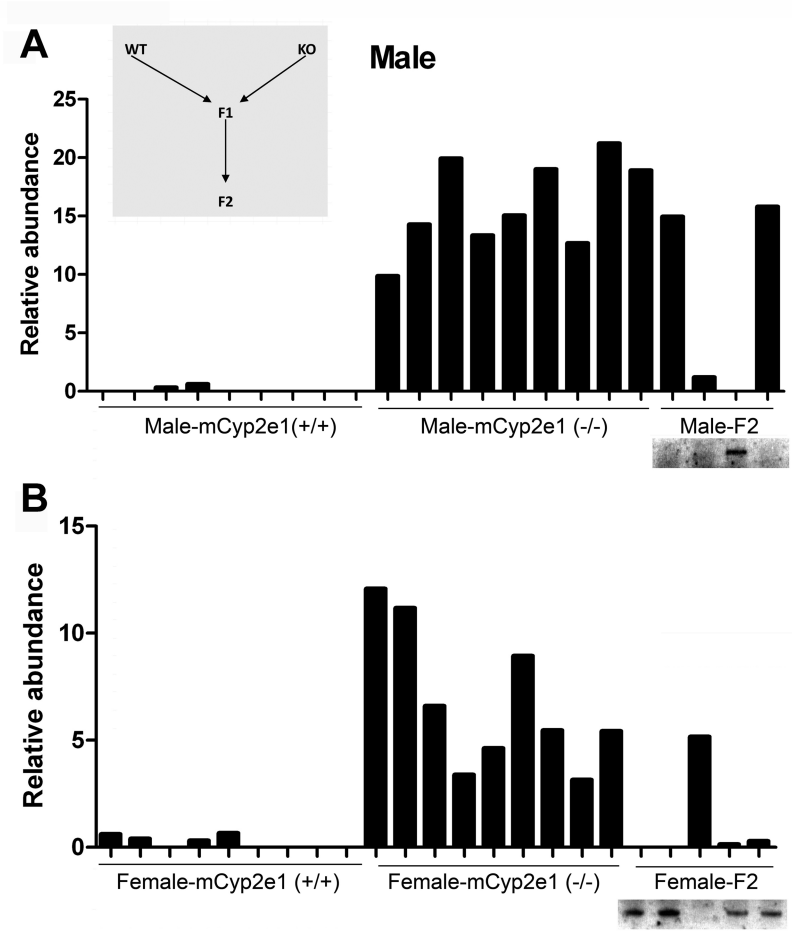

FIG. 4. The influence of backcrossing between WT and *Cyp2e1*-null mice on 2-piperidone distribution in urine. (A) Distribution of 2-piperidone in male WT, *Cyp2e1*-null, and F2 generation mice. The procedure of backcrossing to produce F1 and F2 generations is illustrated in the insert. The genotypes of male F2 generation mice are shown in an inlaid image of *Cyp2e1* genotyping. (B) Distribution of 2-piperidone in female WT, *Cyp2e1*-null, and F2 generation mice. The genotypes of female F2 generation mice are shown in an inlaid image of *Cyp2e1* genotyping.

by comparing the quantity of 2-piperidone in the NIH diet consumed by mice with the quantity of 2-piperidone excreted in the urine. The amount of 2-piperidone in the NIH chow was determined as 87 ng/kg chow, but none was detected in the AIG-93G purified diet. Dietary chow uptake was around 2–3 g per mouse per day, equivalent to 175–262 ng 2-piperidone per mouse per day. However, the average amount of 2-piperidone in urine was determined to be 507 ± 62 ng/day in a *Cyp2e1*-null mouse and 6.2 ± 4.1 ng/day in a WT mouse ($n = 7$). The quantitative difference between 2-piperidone in urine and 2-piperidone in dietary intake suggested that in addition to its dietary source, 2-piperidone in the *Cyp2e1*-null mice might also come from endogenous metabolism.

Identification of Cadaverine as a Potential Precursor Metabolite of 2-Piperidone

Cadaverine is known to be metabolized to 2-piperidone through formation of 1-piperideine (Callery and Geelhaar, 1984), which is subsequently metabolized to 2-piperidone via amine oxidase–mediated oxidation (Naila *et al.*, 2010). To examine whether cadaverine is another source of 2-piperidone *in vivo*, GC-MS analysis of urine samples was conducted. The result of GC-MS-based metabolomics revealed clear separation between WT and *Cyp2e1*-null mice in a PCA model (Fig. 7A). Among the urinary metabolites that contributed to differences between the two mouse lines, a higher level of 2-piperidone was correlated with the *Cyp2e1*-null mice, whereas a higher level of cadaverine was correlated with the WT mice (Fig. 7B).

Dietary analysis indicated that, in contrast to 2-piperidone, cadaverine is undetectable in either NIH chow or purified diet (Supplementary fig. 3). Furthermore, cadaverine was not detected in the urine from the mice fed purified diet but detected from urine of mice fed NIH diet (Supplementary fig. 3). To examine whether cadaverine metabolism contributes to the genotype-dependent distribution of 2-piperidone, cadaverine was administered to both WT and *Cyp2e1*-null mice fed the AIN-93G purified diet. Subsequent metabolite

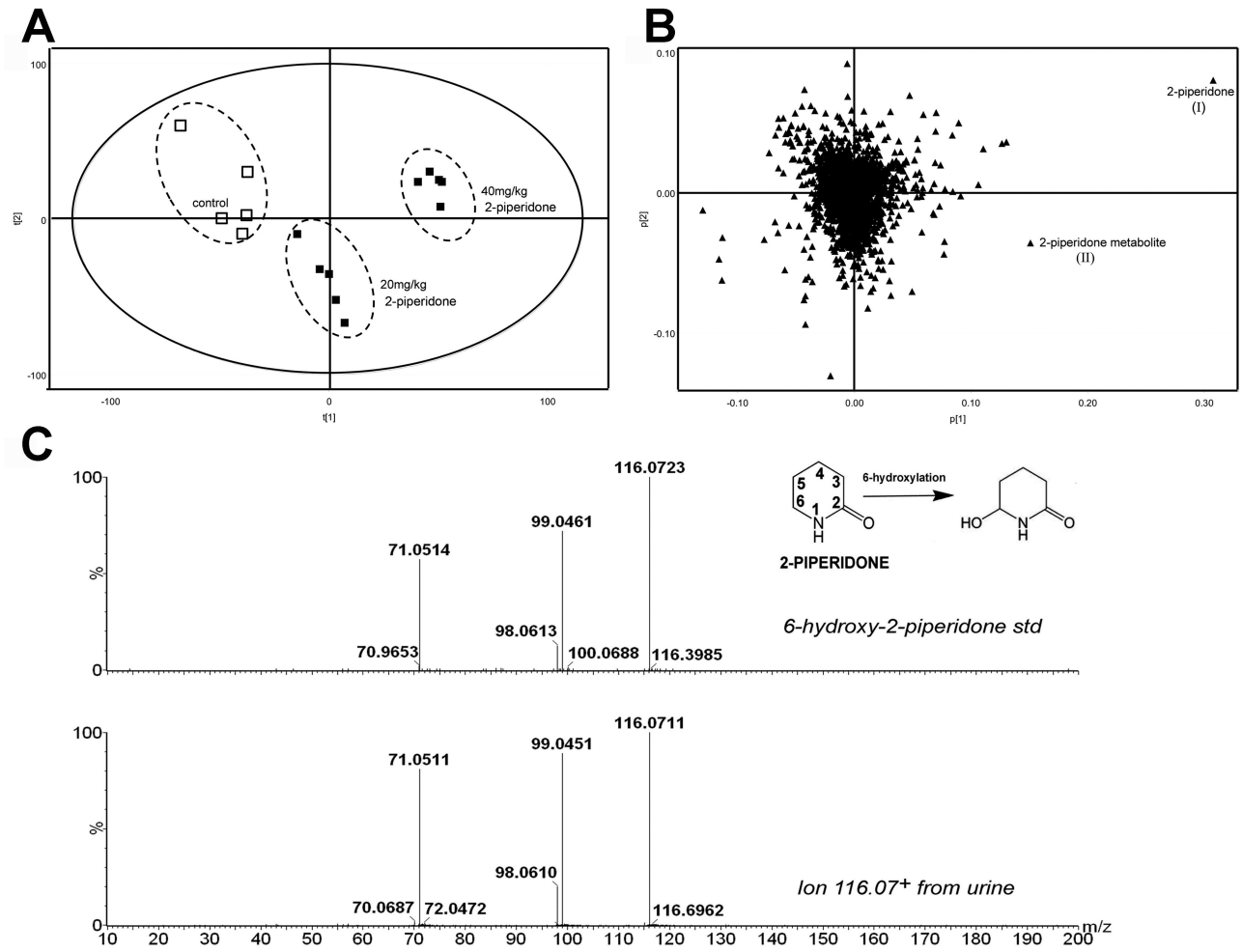

FIG. 5. Identification of 6-hydroxy-2-piperidone as a 2-piperidone metabolite. (A) Separation of control WT (□) mice from the WT mice (■) treated with 20 and 40 mg/kg of 2-piperidone in the scores plot of a PLS-DA model on the urine metabolomes. (B) Identification of 2-piperidone (I) and 2-piperidone metabolite (II) in the loadings plot. (C) A comparison of MS/MS spectra of urinary 2-piperidone metabolite ($[M + H]^+ = 116.07$ *m/z*) and synthesized 6-hydroxy-2-piperidone standard.

analysis revealed a high abundance of 2-piperidone and a low abundance of cadaverine in the urine of *Cyp2e1*-null mice, while an opposite distribution pattern was found in the urine of WT mice, suggesting that efficient conversion of cadaverine to 2-piperidone occurred in the *Cyp2e1*-null mice but not in WT mice (Fig. 7C). This observation on the *in vivo* biosynthesis of 2-piperidone from cadaverine was further confirmed by *in vitro* incubations because primary hepatocytes from the *Cyp2e1*-null mice were far more effective than their counterparts from the WT mice for converting cadaverine to 2-piperidone (Fig. 7D). The expression of Cyp2e1 was not changed in primary hepatocytes of WT and *Cyp2e1*-null mice treated with either 2-piperidone or cadaverine.

DISCUSSION

2-Piperidone, also called δ-valerolactam, is a widely used monomer for synthesizing polymers (nylon-5) in industry (Mahjoub *et al.*, 2011). However, the appearance of 2-piperidone in urine and serum has not been reported previously. Metabolomic analysis of WT, *Cyp2e1*-null, and *CYP2E1*-humanized mice demonstrated that the distribution of 2-piperidone in mice is genotype dependent. More importantly, 2-piperidone was identified as a potential biomarker of CYP2E1 activity based on genotype-dependent distribution, backcrossing of WT and *Cyp2e1*-null mice, and *in vitro* assays. 2-Piperidone bears the structural features of typical CYP2E1 substrates, such as small size, ring structure, and modest hydrophilicity (Li *et al.*, 2012). Indeed, the conversion of 2-piperidone to its main metabolite 6-hydroxy-2-piperidone was inhibited by disufiram, a CYP2E1 inhibitor, and elevated by pretreatment of mice with acetone, a CYP2E1 inducer. Another important observation was the dietary association of 2-piperidone. Two different mouse chows were used in this study. One is NIH-31 diet, an open-formula diet based on cereal and fish meal, and the other is AIN-93G diet, which contains refined ingredients including proteins, sugars,

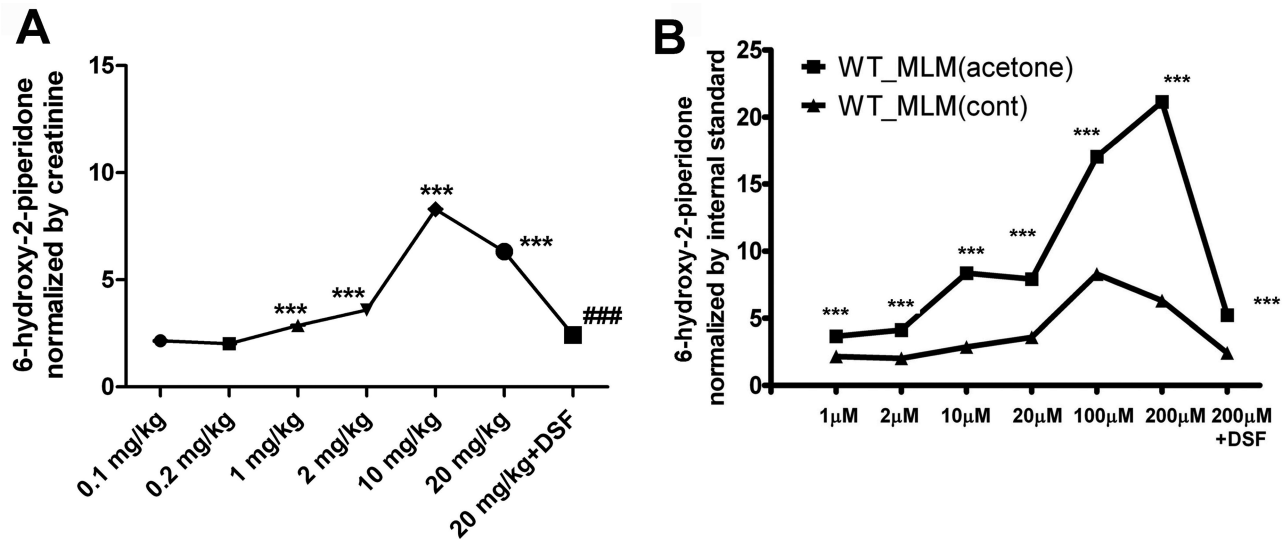

FIG. 6. The influence of 2-piperidone treatment and CYP2E1 activity on the production of 6-hydroxy-2-piperidone *in vivo* and *in vitro*. (A) WT mice were treated with 0.1, 0.2, 1, 2, 10, and 20 mg/kg of 2-piperidone by ip injection. The influence of CYP2E1 inhibitor *in vivo* was examined by cotreatment with 20 mg/kg disulfiram (DSF) coadministered by ip injection with 20 mg/kg of 2-piperidone. (***$p < 0.001$, compared with 0.1 mg/kg of 2-piperidone treatment; ###$p < 0.001$, compared with 20 mg/kg of 2-piperidone treatment). (B) 2-Piperidone ranging from 1 to 200μM was incubated with mouse liver microsomes (MLM) from control and acetone-treated WT mice. The influence of CYP2E1 inhibitor on microsomal incubation was examined by the cotreatment of 200μM disulfiram and 200μM 2-piperidone (***$p < 0.001$, compared with the incubation using control MLM).

oils, vitamins, and minerals (Reeves, 1997). 2-Piperidone was detected in urine from mice fed regular NIH-31 diet that contains 2-piperidone but not from the mice fed AIN-93G purified diet that lacks 2-piperidone. Therefore, the genotype-dependent distribution of 2-piperidone in mouse urine can be partially attributed to the presence of *CYP2E1*-mediated metabolism of 2-piperidone in the WT and *CYP2E1*-humanized mice and the absence of the same reaction in the *Cyp2e1*-null mice.

Dietary exposure of 2-piperidone could not be the sole source of urinary 2-piperidone in the *Cyp2e1*-null mice because quantitative analysis revealed that the daily dietary intake of 2-piperidone was less than the amount of 2-piperidone excretion in urine. Through GC-MS–based metabolomics, cadaverine, a common urine and semen metabolite derived from the decarboxylation of lysine (Bardocz, 1993; Seiler, 2004), was defined as another potential source of 2-piperidone because the urinary excretion of 2-piperidone was correlated to the cadaverine intake in the *Cyp2e1*-null mice in a dose-dependent manner. Therefore, besides its proven role in the catabolism of 2-piperidone, CYP2E1 can also negatively affect the biogenesis of 2-piperidone, in which cadaverine functions as a precursor of 2-piperidone. Further investigations on the source and metabolism of cadaverine in this study provided some mechanistic insights on how cadaverine can contribute to CYP2E1-dependent levels of 2-piperidone.

Treatment of WT and *Cyp2e1*-null mice with cadaverine resulted in more 2-piperidone in the *Cyp2e1*-null mice. In addition, a higher conversion rate from cadaverine to 2-piperidone was observed in primary hepatocytes from *Cyp2e1*-null mice compared with hepatocytes from WT mice. Similar to 2-piperidone, the distribution of cadaverine is also diet dependent. Cadaverine was present in the urine from mice fed the NIH-31 diet but absent in the urine from the mice fed the AIN-93G diet. Because cadaverine was not detected in both NIH-31 and AIN-93G diets used in this study, the observed correlation between NIH-31 diet with urinary cadaverine suggest that NIH-31 diet may stimulate the production of cadaverine *in vivo*, subsequently leading to the increase of urinary 2-piperidone, especially in the *Cyp2e1*-null mice. Thus, 2-piperidone metabolism is not only related to CYP2E1 and other enzymes in the WT and *Cyp2e1*-null mice but can also be altered in a diet-dependent manner. In fact, it was reported that consuming food rich in polyphenols, such as cocoa, tea, and fruit, can promote the production of valerolactone, an analogue of 2-piperidone in human and rat urine (Jurikova *et al*., 2011; Schantz *et al*., 2010; Urpi-Sarda *et al*., 2009). Overall, the results from animal and *in vitro* incubation experiments in this study indicated that the observed inverse correlation between 2-piperidone and CYP2E1 can be attributed to the changes in the biosynthesis and degradation of 2-piperidone. Compared with the 2-piperidone metabolism in the WT mice, the conversion of cadaverine to 2-piperidone was higher, whereas the metabolism of 2-piperidone to 6-hydroxy-2-piperidone was lower in the *Cyp2e1*-null mice, leading to the accumulation of 2-piperidone in the urine of *Cyp2e1*-null mice. The putative metabolic events contributing to the inverse correlation between urinary 2-piperidone and CYP2E1 are outlined in Figure 8.

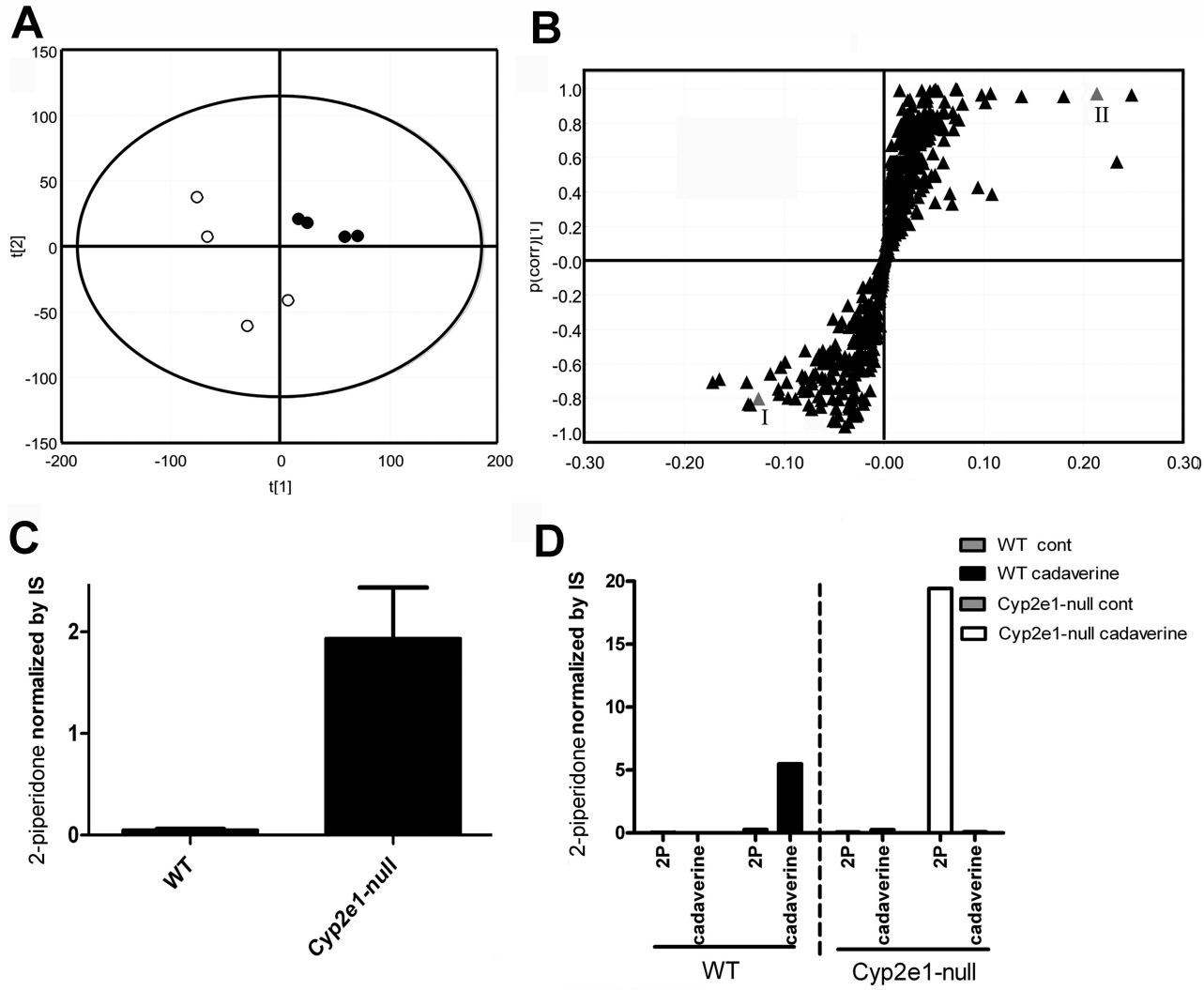

FIG. 7. Identification of cadaverine as a source of 2-piperidone *in vivo*. (A) Separation of WT (●) and *Cyp2e1*-null (○) mice in a PCA model on GC-MS analysis of urine samples. (B) Contribution of cadaverine and 2-piperidone to the separation of WT and *Cyp2e1*-null mice in the S-plot of urinary metabolites. (C) Levels of 2-piperidone in the urine of WT and *Cyp2e1*-null mice after feeding cadaverine together with AIN-93G purified diet (***$p < 0.001$, compared with WT mice). (D) The production of 2-piperidone after incubating cadaverine with primary hepatocytes from the WT and *Cyp2e1*-null mice. No cadaverine was added in the control.

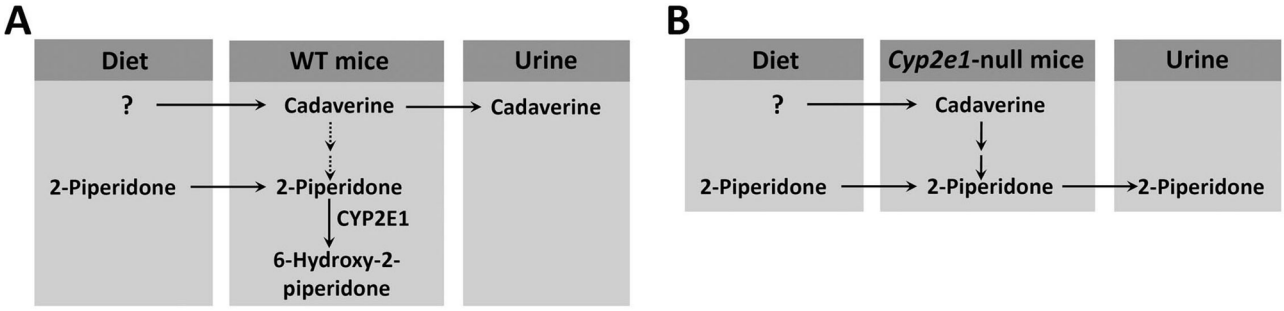

FIG. 8. Metabolic events contributing to the inverse correlation between urinary 2-piperidone and CYP2E1. Dietary 2-piperidone and endogenous cadaverine are two sources of 2-piperidone *in vivo* and in the urine. (A) Biogenesis and metabolic fate of 2-piperidone in the WT mice. (B) Biogenesis and metabolic fate of 2-piperidone in the *Cyp2e1*-null mice.

The applicability of 2-piperidone as a noninvasive and clinical biomarker of CYP2E1 activity requires further studies to determine the correlation between CYP2E1 activity and the content of urinary 2-piperidone in humans. Data from this mouse-based study suggest that besides the factors that can affect human CYP2E1 activities, such as genetic polymorphism and physiological status, the influence of human diet on the biogenesis of 2-piperidone should also be considered. Proper control of these experimental factors will likely reduce the chance of identifying false positive or false negative correlations between CYP2E1 and 2-piperidone.

In summary, this is the first study to investigate an endogenous biomarker of CYP2E1 expression and activity through metabolomics phenotyping of WT, *Cyp2e1*-null, and *CYP2E1*-humanized mice. The identification of a correlation between the levels of 2-piperidone in urine and the activity of CYP2E1 provides an opportunity to establish a clinically applicable biomarker that can be used to monitor CYP2E1 activity through noninvasive measurements.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

National Cancer Institute Intramural Research Program.

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