Abnormalities in Uridine Homeostatic Regulation and Pyrimidine Nucleotide Metabolism as a Consequence of the Deletion of the Uridine Phosphorylase Gene*

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We report in the present study the critical role of uridine phosphorylase (UPase) in uridine homeostatic regulation and pyrimidine nucleotide metabolism, employing newly developed UPase−/− mice. Our data demonstrate that the abrogation of UPase activity led to greater than a 6-fold increase in uridine concentrations in plasma, a 5–6-fold increase in lung and gut, and a 2–3-fold increase in liver and kidney, as compared with wild type mice. Urine uridine levels increased 24-fold normal in UPase−/− mice. Uridine half-life and the plasma retention of pharmacological doses of uridine were significantly prolonged. Further, in these UPase−/− mice, abnormal uridine metabolism led to disorders of various nucleotide metabolisms. In the liver, gut, kidney, and lung of UPase−/− mice, total uridine ribonucleotide concentrations increased 2–3 times as compared with control mice. Cytidine ribonucleotides and adenosine and guanosine ribonucleotides also increased, although to a lesser extent, in these organs. Most significant deoxiribonucleotide changes were present in the gut and lung of UPase−/− mice. In these tissues, dTTP concentration increased more than 4-fold normal, and dCTP, dGTP, and dATP concentrations rose 1–2 times normal. In kidney, dTTP concentration increased 2-fold normal, and dCTP and dGTP concentrations rose less than 1-fold normal. In addition, the accumulated uridine in plasma and tissues efficiently reduced 5-fluorouracil host toxicity and altered the anesthetic effect of pentobarbital. These data indicate that UPase is a critical enzyme in the regulation of uridine homeostasis and pyrimidine nucleotide metabolism, and 5-fluorouracil activity.

Uridine phosphorylase (UPase)1 is an enzyme that catalyzes the phosphorylation of uridine into uracil. In the presence of ribose-1-phosphate, UPase can also catalyze the reverse reaction, forming uridine from uracil, which in turn can be salvaged into uracil nucleotides by uridine kinase (1–5). Clinically, this anabolic reaction represents one of the two main pathways of activation of 5-fluorouracil (5-FU) in both normal and neoplastic tissues (1, 6).

Uridine is an important nucleoside precursor in the pyrimidine salvage pathway (4, 5, 7). Except for erythrocytes and the liver and kidney, which maintain de novo pyrimidine biosynthesis and supply other tissues with uridine for salvage, most normal tissues in adults rely on the salvage of uridine from plasma (5). For instance, uridine incorporation has been shown to increase almost exponentially in human peripheral blood lymphocytes when stimulated by phytohemagglutinin as compared with their resting status (up to 30-fold in 24 h). However, de novo pyrimidine biosynthesis only increases 2–3-fold when stimulated by phytohemagglutinin (8). In cultured L1210 cells, the plasma concentration of uridine is sufficient to meet their pyrimidine requirements. The work of Karle’s laboratory (9) has indicated that 3–5 μM uridine in the cell culture medium led to a 50% increase in the UTP pool of that culture, coupled with a 50% inhibition of de novo pyrimidine biosynthesis from that cell culture. At 12 μM uridine, the UTP pool doubled, and more than 95% of the de novo pyrimidine biosynthesis was inhibited, indicating the importance of plasma uridine in nucleic acid metabolism.

Uridine also participates in the regulation of several physiological and pathological processes (10–14). In the absence of sugar, uridine can serve as an essential precursor for both carbohydrate metabolism and nucleic acid synthesis. Besides the direct salvage to pyrimidine nucleotides, the ribose moiety produced by the phosphorolysis of uridine can either enter the pentose cycle to meet cellular energy requirements or form phosphoribosyl pyrophosphate, which is used in the biosynthesis of purine nucleotides (11). In the nervous system, uridine acts as a physiological regulator of sleep function and demonstrates the ability to maintain brain metabolism during ischemia and severe hypoglycemia (3, 13). In the clinical setting, uridine is utilized in the treatment of autism with seizures (14) and pyrimidine-deficient genetic diseases such as orotic aciduria (12).

In addition, the levels of uridine in plasma and tissues are also critical for the success of anticancer therapies based on pyrimidine antimetabolites. Many preclinical and clinical studies have indicated that RNA, damaged by fluoropyrimidine incorporation, can be rescued by the administration of exogenous uridine or conservation of endogenous uridine by UPase inhibitors, such as benzylacyclouridine, thus leading to chemotherapeutic dose escalation and improvement of therapeutic index (15–17).
otide metabolism, and evaluated the toxicity of 5-fluorouracil. The effects of uridine homeostasis disruption on pyrimidine nucleotide metabolism, examined the knock-out mouse model that lacks the UPase activity. In this study, we have generated a UPase knock-out mouse model that lacks the UPase locus. In this study, we have generated a UPase knock-out mouse model that lacks the UPase locus. However, the regulatory mechanisms of this strict homeostasis remain to be elucidated. As an enzyme catalyzing uridine phosphorylation, UPase may play an important part in the regulation of uridine homeostasis. In this study, we have generated a knock-out mouse model that lacks the UPase gene, quantified the subsequent changes in uridine metabolism, examined the effects of uridine homeostasis disruption on pyrimidine nucleotide metabolism, and evaluated the toxicity of 5-fluorouracil.

MATERIALS AND METHODS

Generation of UPase−/− Mice—Selected UPase+/− embryonic stem cells (Ref. 1) with normal karyotypes were introduced into C57BL/6J blastocysts that then completed their development in pseudopregnant C57BL/6J hosts. Chimeric offspring were used to generate the F1 UPase+/−, wild littermates obtained from inbreedings of the UPase+/+ mice were used for the experiments. The genotypes of the mice were determined by Southern blot analysis, and the abbreviation of the UPase gene was confirmed by RT-PCR and uridine phosphorylatic activity assays. All experiments were conducted according to guidelines for the humane treatment of animals.

Southern Blot Analysis—Genomic DNA was extracted from murine tails, digested with BamHI, and separated in 0.8% agarose gel, and then transferred onto a nylon filter (Amersham Biosciences). A 600-bp PCR fragment located immediately outside of the XhoI cloning site was transferred onto a nylon filter (Amersham Biosciences). A 600-bp PCR fragment located immediately outside of the XhoI cloning site was transferred onto a nylon filter (Amersham Biosciences). A 600-bp PCR fragment located immediately outside of the XhoI cloning site was transferred onto a nylon filter (Amersham Biosciences). A 600-bp PCR fragment located immediately outside of the XhoI cloning site was transferred onto a nylon filter (Amersham Biosciences). A 600-bp PCR fragment located immediately outside of the XhoI cloning site was transferred onto a nylon filter (Amersham Biosciences). A 600-bp PCR fragment located immediately outside of the XhoI cloning site was transferred onto a nylon filter (Amersham Biosciences). A 600-bp PCR fragment located immediately outside of the XhoI cloning site was transferred onto a nylon filter (Amersham Biosciences). A 600-bp PCR fragment located immediately outside of the XhoI cloning site was transferred onto a nylon filter (Amersham Biosciences). A 600-bp PCR fragment located immediately outside of the XhoI cloning site was transferred onto a nylon filter (Amersham Biosciences). A 600-bp PCR fragment located immediately outside of the XhoI cloning site was transferred onto a nylon filter (Amersham Biosciences). A 600-bp PCR fragment located immediately outside of the XhoI cloning site was transferred onto a nylon filter (Amersham Biosciences).

Enzyme Activity Assays—UPase activity was measured by the conversion of [3H]uridine to [3H]uracil, and uridine kinase levels were determined by measuring the formation of [3H]UMP from [3H]uridine as described previously (1).

Measurement of Uridine and Ribonucleotides—Amounts of uridine and ribonucleotides were determined by high-performance liquid chromatography (HPLC) analysis (16). Briefly, collected samples (plasma, urine, and tissues) were extracted with 2 volumes of 15% trichloroacetic acid at 14,000 rpm and 4 °C for 10 min and neutralized with an equal volume of triethylamine-Freon (45:55). Uridine was separated from aqueous phase using a C18 Microsorb column (250 × 4.6 mm, Varian) at 10 °C, with 50 mM potassium phosphate (KH2PO4, pH 3.0) as a mobile phase at 1.0 ml/min. Total ribonucleotide pools were determined by boiling the tissue extracts for 15 min and separating the samples on a Whatman Partisil-10 SAX anion exchange column using a 5 mM sodium phosphate mobile phase (pH 3.3) at 1.4 ml/min. Eluted uridine/nucleotides were monitored at 260 nm and quantitated by integration of the peak areas (16).

DNA Polymerase Analysis of Deoxyribonucleotides—Deoxyribonucleotide pools were determined by a DNA polymerase assay (20). Tissues were extracted with 0.4 n perchloric acid and neutralized to triethylamine-Freon (55:45). DNA polymerase assays were conducted in a 20-µl mixture containing 1 unit of Klenow fragment of Escherichia coli DNA polymerase I, 100 mM HEPEs (pH 7.3), 10 mM MgCl2, 0.25 µM synthetic oligonucleotide template and primer, 2.5 µM [3H]dATP or [3H]dTTP (for dATP determination), and appropriate dNTP standards or tissue extracts. The reactions were carried out at room temperature for 1 h, and then appropriate aliquots were spotted on filter disks (Whatman DE81). After the free isotopes were eluted with 5% Na2HPO4, the radioactivity of the reaction products left on the filters was determined by scintillation counting. Primer templates (Ref. 20 for the sequences) were prepared by annealing appropriate oligonucleotides, i.e. equimolar quantities of complementary strands were mixed together in a capped tube, heated at 70 °C for 5 min, and then cooled down at room temperature.

Uridine Disposition—To detect the metabolic kinetics of uridine in mice, 500 mg/kg of [3H]uridine was injected (intraperitoneally) into a mouse, and blood samples were collected at indicated time points by eye bleeding. Both [3H]uracil and [3H]uridine in plasma were determined by HPLC and scintillation counting. For pharmacokinetic analysis of pharmacological doses of uridine, 500 mg/kg of uridine was administered (intraperitoneally), and blood samples were collected by eye bleeding at indicated time points. After extraction with 15% trichloroacetic acid, uridine concentrations were determined as above.

With all of these clinical roles in mind, it is no surprise that under physiologic conditions, the uridine plasma concentration is strictly regulated at 3–5 µM among different species (2, 18). However, the regulatory mechanisms of this strict homeostasis remain to be elucidated. As an enzyme catalyzing uridine phosphorylation, UPase may play an important part in the regulation of uridine homeostasis. In this study, we have generated a knock-out mouse model that lacks the UPase gene, and the results described previously (1).
Pentobarbital Anesthesia—The anesthetic action of pentobarbital was assessed using the loss of righting reflex (LORR). Wild type and UPase−/− mice (2–3 months old) were injected (intraperitoneally) with 40 mg/kg of pentobarbital. After administration of the anesthetic, the duration of LORR in pentobarbital anesthesia was the time between the onset of and the emergence from LORR (21).

5-Fluorouracil Toxicity—Six wild type and UPase−/− mice (split equally for gender) were administered (intraperitoneally) weekly at doses of 5-FU ranging from 85 up to 250 mg/kg. Mouse weights were measured every other day as a proxy monitor for host toxicity. The doses of 5-FU ranging from 85 up to 250 mg/kg. Mouse weights were equally for gender) were administered (intraperitoneally) weekly at doses of 5-FU ranging from 85 up to 250 mg/kg. Mouse weights were measured every other day as a proxy monitor for host toxicity. The doses leading to 15–20% weight loss were defined as the maximal tolerated dose. Observations ceased for each group after the first mouse died.

RESULTS

Targeted Disruption of the Murine UPase Locus—The mouse UPase gene was cloned, and the UPase locus-disrupted murine embryonic stem cells were generated as reported in our previous publications (1, 22). UPase+/− mice were produced using the UPase+/− embryonic stem cell clones UPA503 and UPA532, both of which demonstrated normal karyotypes (data not shown). The genotypes of the mice were determined by Southern blot analysis. The wild type UPase allele shows up on a 4.0-kb band due to the introduction of a BamHI cut site. Therefore, UPase mutant heterozygous mice show both a 4.0-kb and an 18.0-kb band, whereas homozygous mice demonstrate a 4.0-kb band alone (Fig. 1A). RT-PCR and UPase activity assays both indicate that UPase gene products were completely abrogated in UPase−/− mice except for a residual uridine phosphorolytic activity present in liver tissues (Fig. 1, B and C). To understand the downstream effects of potential uridine accumulation secondary to UPase gene disruption, we also investigated uridine kinase activity. An increase in uridine kinase activity was detected in liver (77.8% increase, p < 0.05, Student’s t test) and kidney (75.0% increase, p < 0.05, Student’s t test) of UPase−/− mice, but no uridine kinase activity change was detected in the lung and gut (Fig. 1C).

UPase−/− mice at the age of 3–5 months showed no changes in growth, development, sexual maturation, and fertility as compared with wild type littermates. The mutant UPase allele displayed a Mendelian distribution (1:2:1), indicating no lethal phenotype in UPase null mice. There were also no significant histological alterations present in the major organs surveyed, including brain, lung, heart, liver, spleen, gut, and kidney (data not shown).

Alterations of Uridine Metabolism in UPase−/− Mice—To understand the effects of UPase gene disruption on uridine metabolism, we have measured the concentrations of uridine in the plasma and some major organs of UPase−/− mice. As compared with wild type mice, the uridine concentration increased by more than 6-fold in the plasma of UPase−/− mice, and in tissues, the uridine concentration was elevated 5–6 times normal in lung and gut and 2–3 times normal in liver and kidney (Table I), indicating a critical role for UPase in the regulation of uridine concentrations in both plasma and tissues. In addition, a detection of urinary uridine concentration revealed a 24× normal elevation in UPase−/− mice, indicating
the compensatory role of the kidney in uridine homeostatic regulation of UPase gene-deficient mice.

The metabolic kinetics of uridine in UPase−/− mice were also evaluated by tracing the fate of radiolabeled uridine. In wild type mice, tracer doses of [3H]uridine (25 μCi, intraperitoneally) were rapidly degraded with a t1/2 < 2 min. On the contrary, the [3H]uridine t1/2 in UPase−/− mice was ~10–15 min, and [3H]uridine was still detectable 60 min after administration (Fig. 2A). Five minutes after administration, an obvious [3H]uracil peak was present in wild type mouse plasma but was notably absent in that of UPase−/− mice in which [3H]-uridine was still the major peak (Fig. 2B), indicating that [3H]-uridine was rapidly cleared via the phosphorolysis by UPase in wild type mice. Similarly, the disruption of UPase gene greatly increased the plasma retention of pharmacological doses of uridine. In UPase−/− mice, a pharmacological dose of 500 mg/kg of uridine (intraperitoneally) produced plasma uridine concentration above 1 mM for more than 1 h, whereas the plasma uridine declined in 1 h to ~100 μM in wild type mice when administered with same doses of uridine (Fig. 2C).

Abnormalities of Nucleotide Metabolism in UPase−/− Mice—Accumulation of uridine in plasma and tissues, as well as the induction of uridine kinase activity in some organs, may lead to alterations in purine nucleotide metabolism in UPase−/− mice because uridine plays an important role in the pyrimidine salvage pathway. Therefore, we have examined the quantity of total ribonucleotides and deoxyribonucleotides in the liver, gut, lung, and kidneys of UPase−/− mice. As shown in Fig. 3A, total uridine ribonucleotide (UXP) concentrations increased 2–3 times normal in all organs surveyed. Cytidine, adenosine, and guanosine ribonucleotides (CXP, AXP, and GXP) also increased in these organs but generally to a lesser extent. Deoxyribonucleotides on the whole increased in these UPase−/− mouse tissues but with greater variations among different nucleotides and organs (Fig. 4A). As compared with wild type animals, dTTP concentrations in UPase−/− mice were elevated more than 4-fold in gut and lung and ~2-fold in kidney; dCTP increased 1–2-fold in the gut, lung, and kidney of UPase−/− mice. Alterations in purine deoxyribonucleotides (dGTP and dATP) were also detected in gut, lung, and kidney, but they were generally elevated less than 1-fold. Interestingly, the increase of each deoxyribonucleotide in UPase−/− mouse livers was less than 5%, indicating that the liver, a major metabolic organ in vertebrate animals, has a strong self regulatory ability. Further, due to the unequal increases of each nucleotide pool in various tissues, the abnormalities of nucleotide metabolism were also characterized with the loss of the normal ratios among nucleotides (Figs. 3B and 4B).

Reduced 5-FU Toxicity in UPase−/− Mice—To understand 5-FU-induced host toxicity in UPase−/− mice, weekly boluses of 5-FU were administered (intraperitoneally) at various doses, and the resulting toxicity to the mice was estimated by tracking weight changes. As shown in Fig. 5, 85 mg/kg of 5-FU represents the maximal tolerated dose in wild type mice, causing ~15% weight loss within 4 weeks. Higher doses of 5-FU (100 mg/kg) resulted in animal deaths after the second dose (data not shown). In UPase−/− mice, however, 85 mg/kg of 5-FU caused no toxicity, and at 150 mg/kg, 5-FU-induced toxicity in UPase−/− mice was comparable with that observed at 85 mg/kg in wild type mice. A dose up to 200 mg/kg caused no animal deaths for 3 weeks.

Reduced Sensitivity of UPase−/− Mice to Pentobarbital Anesthesia—To understand the influence of the elevated uridine levels on neuronal activity, we observed behavioral circadian rhythms and pentobarbital sensitivity in UPase−/− mice. There was no circadian cycle change observed in these mice; however, pentobarbital had a significantly reduced anesthetic effect on the UPase−/− mice. Although 40 mg/kg pentobarbital (intraperitoneally) induced a LORR of 1 h in wild type mice, the same dose of pentobarbital caused only 25 min of LORR in UPase−/− mice (Fig. 6). Also, the onset of LORR in UPase−/− mice was relatively longer (7 min versus 4 min) compared with wild type mice.

**DISCUSSION**

UPase is an enzyme that facilitates the reversible conversion of uridine to uracil. Our group and several other investigators have also established the ability of UPase to catalyze the conversion of thymidine into thymine, albeit with a lower efficiency (23, 24). In this study, we have generated UPase−/− mice, determined the role of UPase in the regulation of uridine homeostasis in vivo, and evaluated the effect of uridine homeostatic disruption on nucleotide metabolism, 5-FU, toxicity, and pentobarbital anesthesia. These UPase−/− mice had no apparent abnormalities in growth, development, sexual maturation, or reproductive ability. The mutant UPase allele displayed a typical Mendelian disemination, indicating that UPase is not an essential gene for murine development or survival. However, lack of the UPase gene in mice indeed resulted in
significant alterations in uridine and pyrimidine nucleotide metabolism.

In UPase−/− mice, RT-PCR and UPase activity assays both confirmed a complete abrogation of UPase gene products in all tissues surveyed. The analysis of uridine phosphorolytic activity was important to exclude the possibility of residual activity from disrupted UPase gene products and to understand the presence of isoforms in UPase−/− mice. Indeed, Johansson (25) reported the presence of an mRNA species (designated UPase-2) identical to the UPase gene in both humans and mice as we were conducting this study. In humans, UPase-2 is predominantly expressed in the kidney, whereas the mouse UPase-2 gene is mainly expressed in the liver. Consistent with this report, our results showed that a residual uridine phosphorolytic activity (1.3 nmoles/mg of protein/h) was present in the liver of UPase−/− mice, which is insensitive to benzylacycloclouridine (data not shown). In addition, the accumulation of uridine in the tissues of UPase−/− mice stimulated the induction of uridine kinase in the liver and kidney but not in the gut and lung.

In UPase−/− mice, the severe abnormalities in uridine and pyrimidine nucleotide metabolism were reflected in the accumulation of uridine in plasma and tissues and in the expansion of various nucleotide pools with the loss of the normal ratios between pyrimidine and purine nucleotides, and the kidney became a major regulator of plasma uridine through uridine excretion. UPase abrogation also greatly changed the metabolic properties of uridine, as demonstrated by tracer doses of [3H]uridine, leading to a 5–6-fold increase in its half-life and the limited production of uracil, otherwise present in wild type
UPase not recognize thymidine metabolism alterations in these essential role for UPase in uridine clearance. However, we did
tion declined rapidly in wild type mice. These data indicate an
differences from UPase weekly (intraperitoneally), and mouse weights were tracked every other
mM for more than 1 h, whereas the plasma uridine concentra-
dine. In UPase−/− mice with high plasma and tissue uridine concentrations displayed a significant tolerance to 5-FU, indicating the role that uridine plays in protecting host tissues. However, it is noteworthy that UPase itself can catalyze the anabolic
reaction of 5-FU, forming fluoropyrimidine nucleotides via the pyrimidine salvage pathway (1, 6). This may also contribute to the explanation of the reduced 5-FU toxicity in UPase−/− mice. Nevertheless, no matter the cause, i.e. UPase-catalyzed
abolism or “uridine rescue,” that is ascribed to this protection, UPase can serve as a target to modulate fluoropyrimidine activity due to its central role.

Urudenine is a sleep-promoting substance (29, 30). Although we did not see significant behavioral abnormalities in UPase−/− mice, the high plasma levels of uridine in these mice indeed antagonized the anesthetic effect of pentobarbital, leading to a lighter and shorter sleep as compared with wild type mice. This may be attributed to the inhibition of barbiturate binding to y-aminobutyric acid receptor-ionosphere complexes by the high intracellular uridine due to the base-related structural similarity (30, 31).

In summary, we have defined the critical role of UPase in the regulation of uridine homeostasis and pyrimidine nucleotide metabolism using UPase−/− mice. The abnormalities of pyrimidine metabolism caused by UPase abrogation greatly changed the metabolic kinetics and pharmacokinetics of uridine, increased mouse tolerance to 5-FU, and reduced mouse sensitivity to pentobarbital. Therefore, this study provided valuable information for the clinical uses of uridine, 5-FU, and pentobarbital and for the development of strategies modulating the actions of these compounds

FIG. 5. 5-Fluorouracil toxicity. 5-Fluorouracil was administered weekly (intraperitoneally), and mouse weights were tracked every other
day to monitor for 5-fluorouracil toxicity. Values represent mean ± S.D.
for three individual experiments. WT, wild type; KO, knock-out.

FIG. 6. Pentobarbital anesthetic effect. 40 mg/kg pentobarbital was administered (intraperitoneally), and LORR time was recorded for
each mouse. Values represent mean ± S.D. from five mice. * statistical differences from UPase−/− mice (p < 0.05, Student’s t test). WT, wild type; KO, knock-out.

mice. This change in uridine metabolism was further confirmed by a pharmacokinetic analysis of pharmacological doses of uridine. In UPase−/− mice, 500 mg/kg of uridine (intraperitone-
ally) brought the plasma uridine concentration to more than 1 mM for more than 1 h, whereas the plasma uridine concentration declined rapidly in wild type mice. These data indicate an essential role for UPase in uridine clearance. However, we did not recognize thymidine metabolism alterations in these UPase−/− mice (data not shown), indicating that UPase is not essential for thymidine metabolism in mice.

Uridine is an important precursor of pyrimidine nucleotides (4, 5, 7). Through the uridine salvage pathway, uridine can be converted to UMP and then to other pyrimidine nucleotides, including CMP, dCMP, and TMP (4, 10, 18). In UPase−/− mice, therefore, we have detected significant elevations in the concentrations of various pyrimidine nucleotides, including to-
tal uridine ribonucleotide, total cytidine ribonucleotide, dCTP, and dTTP. These changes were present in all tissues we sur-
vived. Interestingly, we have also found, although to a lesser extent, an increase in the concentrations of purine nucleotides, including total guanosine ribonucleotide, total adenosine ribo-
nucleotide, dGTP, and dATP, in the surveyed organs. Cur-
rently, we do not have a direct explanation for the changes of the purine nucleotides. We speculate that it could possibly be due to ribose-1-phosphate- and phosphoribosyl pyrophosphate-
mediated regulatory mechanisms employed to maintain the

Uridine Phosphorylase Knock-out and Pyrimidine Metabolism

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Uridine Phosphorylase Knock-out and Pyrimidine Metabolism

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