Tissue Uridine Pools: Evidence in Vivo of a Concentrative Mechanism for Uridine Uptake

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ABSTRACT

Pools of free uridine, ranging from 7.3 to 38.0 nmol/g wet weight, have been detected in a variety of freeze-clamped murine tissues. These concentrations average 10-fold greater than that detected in plasma. The kinetics of these pools after an i.v. tracer dose of [3H]uridine suggest that the initial rapid disappearance of [3H]uridine from plasma (t½ = 2 min) reflects distribution into tissues as well as catabolism by the liver. Subsequently, the tissue uridine pools turn over with half-lives of 13 to 18 h. Analyses of the activity of the proximal enzymes in uridine metabolism (uridine phosphorylase and uridine kinase) suggest that the phosphorylase correlates with the size of tissue uridine pools. Further evidence for this is seen in the sustained 5- to 15-fold increase in both tissue and plasma uridine concentrations after treatment with benzylcyclouridine, a potent uridine phosphorylase inhibitor. In contrast, a nonphysiological dose of exogenous uridine (250 mg/kg) briefly increases the plasma concentration of uridine to over 1 mM but it returns to below 10 µM within 1 h. Under these conditions, as well, tissue concentrations of uridine increase 5- to 10-fold in most tissues, 20-fold in spleen, and 70-fold in kidney. High cellular concentrations of free uridine relative to medium are also observed in dispersed murine splenocytes. Furthermore, splenocytes incubated in 5 µM [3H]uridine achieved a 2-fold higher intracellular concentration of [3H]uridine in less than 1 min independent of phosphorylation. Thymidine was not concentrated in this system nor did nitrobenzylthioinosine inhibit [3H]uridine uptake. These findings suggest that in normal tissues and explanted cells, pools of uridine are sustained by a concentrative transport mechanism and constitute a previously unrecognized reservoir of pyrimidine nucleosides in tissues.

INTRODUCTION

The nonconcentrative diffusion of uridine and other nucleosides across cell membranes has been documented and characterized in a variety of neoplastic cell lines which grow in either suspension or monolayer cultures (1-4). This translocation of nucleosides across cell membranes involves a carrier molecule with broad substrate specificity (1, 5, 6). Studies by Belt (7, 8), and others (9, 10), suggest that there may be several transport systems responsible for the equilibration of nucleosides, each probably involving a different carrier molecule and distinguished by its sensitivity to nucleoside transport inhibitors such as NBMPR, chloromercuribenzenesulfonate, and dipyridamol. It has been assumed that this very rapid facilitated diffusion mechanism would equilibrate tissue concentrations of nucleosides with the concentration in plasma. However, several of our previous observations were inconsistent with this assumption. Large doses of nonradioabeled uridine (250 mg/kg), alone or in combination with the uridine phosphorylase inhibitor BAU, only minimally decrease the incorporation of a tracer dose of [3H]uridine into the uracil nucleotide pools and nucleic acids of several murine tissues (11). Similarly, this large dose of uridine, which increases the plasma pool of uridine by 500-fold, failed to cause more than a 2-fold increase in tissue pools of uracil nucleotides (11). These data, along with recent reports by Schwenk et al. (12) and LeHir and Duback (13), suggesting that uridine is actively transported both in isolated intestinal epithelial cells and in brush border vesicles prepared from kidney epithelial cells by a Na+ dependent mechanism, encouraged the assay of the concentration of uridine in various tissues and plasma. We report that concentrations of uridine in tissues is much greater than in plasma and can expand to different degrees when the plasma pool of uridine is increased. The turnover of these tissue pools suggests that uridine in the plasma rapidly enters tissue uridine pools and then slowly reequilibrates with the plasma. We also present evidence that suggests the size of the uridine pool in tissues is inversely related to the uridine phosphorylase activity and, in isolated splenocytes, may be the result of a concentrative uptake process. Details of the in vitro evidence concerning active transport are presented elsewhere.

MATERIALS AND METHODS

Animals. All experiments utilized 3-month-old female C57BL/6 mice (hereafter called C57) obtained from the NIH. Colon tumor 38 was transplanted by s.c. inoculation with 0.3 ml of a 10% tumor brei in the right axillary region (14, 15). Tumor-bearing mice were used in experimental procedures 20-30 days after tumor transplantation when their tumors weighed approximately 1 g.

Chemicals. Uridine and dThd were purchased from Sigma Chemical Co. (St. Louis, MO). [5,6-3H]Uridine (40 Ci/mmol), [6-3H]dThd (18 Ci/mmol), and H2O (18 µCi/mmol) were purchased from New England Nuclear (Boston, MA). [2-14C]FUr (56 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA). Inulin-[14C]carboxylic acid (10 mCi/mmol) was purchased from Amersham (Arlington Heights, IL). BAU was the generous gift of Dr. S. Chu of Brown University. For injection purposes, uridine was dissolved in saline before administration. BAU was dissolved in ethanol and then diluted 1:5 in saline. All injections were i.v. (tail) and 0.1 ml injected/10 g of body weight.

Tissue uridine pools and their turnover were determined by treating C57 mice with an injection of a tracer dose of [3H]uridine (15 µg/kg, 50 µCi/mouse). At various times after [3H]uridine administration, 150 µl of whole blood were collected from the orbital sinus in a heparinized Natelson pipet and placed on ice (11). The animal was then anesthetized with ether and approximately 15 cm of intestine distal to the pyloric sphincter were removed, quickly flushed with ice-cold saline, and then frozen in liquid nitrogen. Immediately thereafter, the tumor, spleen, liver, and kidneys were quickly removed and immediately frozen in liquid nitrogen. The order of removal or organs was varied to assure that the uridine content was not affected. The frozen tissues were weighed and coarsely ground in a mortar and pestle maintained below 0°C with dry ice. These tissues, as well as plasma, were homogenized in 2 volumes of 15% TCA at 4°C. The acid-soluble fractions were neutralized by washing with ether and approximately 15 cm of intestine distal to the pyloric sphincter were removed, quickly flushed with ice-cold saline, and then frozen in liquid nitrogen. The order of removal or organs was varied to assure that the uridine content was not affected. The frozen tissues were weighed and coarsely ground in a mortar and pestle maintained below 0°C with dry ice. These tissues, as well as plasma, were homogenized in 2 volumes of 15% TCA at 4°C. The acid-soluble fractions were neutralized by extraction with 1 volume of 1 N triethylamine in 1,1,2-trichloroethane (Freon) and stored at -20°C until HPLC analysis to determine their uridine and ATP content.

The total uridine content as well as the turnover of uridine pools in plasma and tissue extracts obtained at various times after injection were

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: NBMPR, 6-(4-nitrobenzyl)thio-9-D-ribofuranosyl purine; dThd, thymidine; BAU, 5-benzylcyclouridine [5-benzyl-1-(2'-hydroxyethyl)thymidine]; FUr, 5-fluorouracil; FUr'd, 5-fluorouridine; TCA, trichloroacetic acid; HPLC, high pressure liquid chromatography.

4 J. W. Darnowski and R. E. Handschumacher, manuscript in preparation.
Tissue Uridine Pools

The uridine content of selected tissue samples was also determined by reverse-phase HPLC methods previously reported (11). The uridine content of selected tissue samples was also determined by ion-exchange HPLC methods reported previously (16, 17). Confirmation of the identity of the material which eluted with authentic uridine was achieved by collecting the column effluent in the region of the uridine peak and adjusting the pH to 7.4 with 1 N NaOH. To this solution were added 3 units of purified rat liver uridine phosphorylase (1 unit converts 1 μmol of uridine to uracil per hour) and the reaction was monitored spectrophotometrically at 266 nm. After the reaction was complete (approximately 30 min), protein was precipitated with 15% TCA, the soluble material was extracted with triethylamine in Freon to remove TCA, and rechromatographed to determine its uridine content by the reverse-phase HPLC method described above. Further confirmation was obtained by dividing tissue samples into two aliquots and treating one with 5 drops of bromine-water (18), and the other with an equal volume of water. After treatment, both samples were chromatographed to detect uridine as described above. The ATP content of the acid-soluble fraction of tissue extracts was determined by ion-exchange HPLC methods utilizing a Whatman SAX column (25 cm x 4.6 mm) eluted at 1.5 ml/min with 0.4 M sodium phosphate (pH 3.3); the column effluent was monitored at both 254 and 280 nm.

Uridine phosphorylase and uridine kinase activities of various tissues from colon tumor 38-bearing C57 mice were assayed in homogenates prepared in 10 volumes of 50 mM Tris buffer (pH 7.4) containing 2 mM sodium fluoride and 2 mM dithiothreitol at 4°C. For intestinal mucosa studies, scrapings from 15 cm of intestine, starting distal to the pyloric sphincter, were homogenized in Tris buffer as described above. All homogenates were frozen and thawed twice in a dry ice-methanol bath and centrifuged at 100,000 x g for 70 min at 4°C in a Spincoc Model L ultracentrifuge equipped with a Ti50 fixed angle rotor. Uridine phosphorylase activity was assayed by incubating 10 to 50 μl of the resulting cytosol fraction at 37°C with 50 mM Tris (pH 7.4) containing 1 mM inorganic phosphate and 200 μM [3H]Urf (3.5 μCi/ml) in a final volume of 100 μl. At various times 10 μl of the reaction mixture were removed, spotted directly onto silica gel thin layer chromatography plates (E. Merck; Darmstadt, West Germany) and dried immediately at 80°C. Uridine kinase activity was assayed by the same procedure except that the reaction mixture contained 1 mM ATP, 1 mM MgCl2, and 50 μM BAU (to inhibit Urd phosphorylase activity) with the elimination of 1 mM inorganic phosphate. Thin layer chromatography plates from both assays were developed in chloroform:methanol:acetic acid (85:15:5) and radioactivity in the Fura, FUrdf, or fluorouridine monophosphate regions was assayed by cutting the plates into sections and placing in scintillation vials containing 0.5 ml methanolic 0.1 N HCl. After 30 min, 4 ml of Liquiscint (National Diagnostics, Somerville, NJ) were added and the radioactivity in each vial was determined in a Beckman LS 7000 liquid scintillation counter. The protein content of the tissue cytosol fractions was determined by the Bradford method (19), and enzyme activity was expressed as nmol of FUrd converted per h per mg protein.

The uridine content of splenocytes obtained from normal C57 mice was determined after dispersing intact spleens into Fischer’s medium by gently abrading the spleen between two frosted microscope slides. After allowing debris to settle, the cell density was adjusted to approximately 1 x 107 cells/ml. The apparent total water volume and extracellular volume of pelleted cells was determined by incubating a 1-ml aliquot of splenocyte suspension with 2.5 μCi of 3H2O and 0.5 μCi of [3H]Julinin for 1 min. Aliquots of 50, 100, and 200 μl were placed in a 400-μl Eppendorf microfuge tube containing 120 μl of a mixture of paraffin:silicone oil (16:84) layered over 35 μl of 15% TCA. The cells were pelleted into the TCA microfuge at centrifugation at 10,000 x g for 1 min in a Beckman Model B microfuge. The internal cellular volume was then calculated as the difference between the total pellet water volume (3H-related dpm) and the extracellular water volume (4C-related dpm). In the present study, the medium trapped in the cell pellet averaged between 20 and 30% of the total cell water volume. The actual uridine content of splenocytes the remaining cell suspension (~15 ml) was then centrifuged through a mixture of paraffin:silicone oil (16:84) into 200 μl of 15% TCA. After aspirating off the media and oil, the cell-TCA mixture was homogenized and then extracted with triethylamine in Freon as described previously for the preparation of tissue extracts. The uridine content of the TCA fraction was determined by the above reverse-phase HPLC method, and expressed as nmol per ml cell water. The uridine content of HCT-8 cells was determined by harvesting cells grown as a monolayer in RPMI 1640 as previously reported (20), and determined the cell volume and uridine content as described above. The cell volume and uridine content of Sarcoma 180, L1210, LS178Y, W256, and P388 cells grown in suspension cultures in Fischer’s media were also determined as described above.

The ability of dispersed spleen cells to concentrate [3H]uridine or [3H]Tdo was tested in the presence and absence of 1 μM NBMPR by exposing these cells to 3H-nucleoside (5 μM; 1 μCi/ml) and assaying [3H] label uptake into cells at various times during the incubation by using the rapid sampling method described above (20). Additionally, the identity of the tritium-labeled material in the cell pellet-TCA fraction obtained at various times was confirmed by extracting the cell pellet-TCA fraction with triethylamine in Freon and determining the [3H] uridine content by the reverse-phase HPLC method reported above.

RESULTS

Analysis of the acid-soluble fraction of a selection of tissue homogenates indicates that the free uridine concentration in tissues is from 3- to 13-fold greater than that in plasma (Table I). To assure that the peak detected by UV absorption and radioactivity that eluted with authentic uridine in HPLC analysis was indeed uridine, it was treated with uridine phosphorylase. This treatment resulted in the appropriate change in the UV absorption profile with the appearance of uracil and the disappearance of the uridine from the HPLC chromatogram. Uridine concentrations were independently determined by ion-exchange HPLC methods and the results concurred. Finally, the uridine peak in the HPLC chromatograms of tissue samples was completely eliminated by treatment with Br2-water (18). Analysis of tissue extracts for other pyrimidine nucleosides by HPLC methods revealed that dThd and cytosine concentrations were very low (<5 nmol/g wet weight). The ATP content of these tissue extracts ranged from 0.55 μmol/g wet weight (tumor) to 2.90 μmol/g wet weight (liver) with kidney, gut, and spleen averaging 1.80 μmol/g wet weight, indicating that the method of tissue preparation did not result in anoxic conditions that might result in the degradation of uracil nucleotides to uridine (21, 22).

Tissue uridine pools were quickly and efficiently labeled following a single i.v. injection of a tracer dose of [3H]uridine (Fig. 1). Since we and others had reported that [3H]uridine rapidly disappears from plasma with a half-life of approximately 2 min (11, 17, 23), these findings suggested that, in addition to catabolism by the liver, distribution into tissue pools played a major role in the clearance of uridine from plasma. Furthermore, tissue [3H]uridine was cleared from these pools with half-lives ranging from 13 to 18 h (Fig. 1). Thus, it would

Table I Uridine concentration in various tissues obtained from colon tumor 38-bearing female C57 mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Uridine content (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>3.2 ± 1.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>38.0 ± 4.7</td>
</tr>
<tr>
<td>Kidney</td>
<td>31.2 ± 4.0</td>
</tr>
<tr>
<td>Liver</td>
<td>27.1 ± 4.8</td>
</tr>
<tr>
<td>Intestine</td>
<td>12.6 ± 2.3</td>
</tr>
<tr>
<td>Colon tumor 38</td>
<td>7.3 ± 1.1</td>
</tr>
</tbody>
</table>

Tissues were removed from anesthetized mice, immediately frozen in liquid nitrogen, and homogenized in ice cold 15% TCA. Uridine values were determined by reverse-phase HPLC analysis of the acid-soluble material and represent the mean ± SE of five samples.

3491
be predicted that a tracer dose of \([^{3}H]\)uridine should rapidly equilibrate with the relatively stable tissue pools and consequently sustain \([^{3}H]\)uridine in plasma for many hours. Indeed, when plasma clearance of a high specific activity tracer dose of \([^{3}H]\)uridine was examined, biphasic clearance was observed (Fig. 1) with the \(\alpha\) phase half-life probably representing the composite of tissue distribution, renal clearance, and catabolism. The half-life of the \(\beta\) phase, an aspect of uridine kinetics that has not been considered previously, is approximately 22 h and probably reflects the composite of efflux from tissues, its reuptake by tissues, and catabolism.

Since large doses of exogenous uridine (24, 25) or administration of BAU (11) increase the plasma concentration of uridine, their effect on the tissue pools of uridine were determined. A single 240-mg injection of BAU/kg increased plasma concentrations of uridine 15-fold, to 45 \(\mu\)M 4 h after injection and slowly returned to normal over the next 4 h. This dose of BAU increased the uridine pool in all tissues surveyed from 2- to 5-fold (Fig. 2A) 4 h after BAU injection. During this period, the tissue:plasma ratio of uridine dropped to approximately one-half that seen in untreated animals, after which both the tissue uridine pool size and the tissue:plasma uridine ratio slowly returned to normal over the next 4 h. Injection of a nonphysiological dose of uridine (250 mg/kg) achieved an initial plasma concentration of over 1 mM which decreased rapidly so that after 1 h the uridine concentration was below 10 \(\mu\)M. Uridine pools in all tissues expanded in response to this increase in the plasma concentration of uridine. Five min after injection, a 4- to 20-fold increase occurred in all tissues except the kidney, where the uridine concentration increased 70-fold (Fig. 2B). Unlike the effect of BAU, tissue uridine pools returned to normal within 4 h although somewhat less rapidly than plasma, and always sustaining a tissue:plasma ratio greater than or equal to that seen under physiological conditions. These results suggest that intracellular uridine metabolism as well as uridine access from plasma can both play significant roles in the generation and maintenance of tissue uridine pools.

Since the proximal enzymes that affect the concentration of uridine are uridine phosphorylase or uridine kinase, these enzymatic activities in different tissues were compared with the steady state tissue pools of uridine. Uridine kinase activity, as determined in this study, was relatively similar in all the tissues surveyed with the highest activity detected in tumor (Table 2). On the other hand, a wide variation in the uridine phosphoryl-
Cells in the log phase of growth (1.0 to 5.0 x 10^5 cells/ml) were centrifuged through oil into 15% TCA. The uridine content of the TCA extracts was determined by reverse-phase HPLC. Splenocytes were obtained from female C57 mice by dispersing intact spleens into Fischer's medium, adjusted to a cell density of 2 x 10^6 cells/ml, and incubated for 45 min at 37°C. The uridine content was then determined as described above. Each value represents the mean ± SE of three to four determinations except in spent media determinations for HCT-8, P388, L5178Y, and Sarcoma 180 cell cultures, where only one determination was made.

The finding of 13-fold greater tissue concentrations of uridine in tissues as well as the rapidity with which they return to normal after being artificially expanded. The uridine kinase activity, important though it may be for salvage of uridine, does not correlate with the size of the tissue pools of uridine. The present findings do suggest, however, that uridine access from plasma plays a significant role in the generation and maintenance of tissue uridine pools.

The high uridine concentrations in tissues have several important implications with respect to the action of a variety of therapeutic agents. For example, these large tissue reservoirs of uridine may explain the poor clinical response to agents, such as N-(phosphoracetyl)-L-aspartate (PALA) (29, 30) and pyrazofurin (31), that are potent inhibitors of de novo uridine synthesis, and may be the basis for the pronounced tissue selectivity that BAU "rescue" regimens impart to FUra therapy (11). Further, tissue uridine pools may determine the susceptibility of these tissues to FUra toxicity, particularly where activation of FUra occurs through condensation with ribose 1-phosphate and consequent formation of fluorouridylic acid. In this regard, tissues with high pools of uridine, such as liver, kidney, and spleen, are less susceptible to FUra-induced toxicity than tissues with low uridine pools such as gut and tumor. Thus the development of means to control these tissue uridine pools may have important physiological and therapeutic applications.

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REFERENCES


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