Circadian Variation of 5-Fluorouracil Catabolism in Isolated Perfused Rat Liver

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ABSTRACT

The catabolism of 5-fluorouracil (FURA) was measured in isolated perfused rat liver (IPRL) at various times of the day. IPRLs were prepared from rats sacrificed at 3-h intervals and the elimination rate of FURA and FURA catabolites (i.e., rate leaving the IPRL in the effluent perfusate) following infusion of [1-14C]FURA was analyzed for circadian periodicity. Animals were housed under standardized conditions of light and dark and divided into two groups of 24 animals each. The first group was housed under "normal" light conditions (light on from 6:00 a.m. to 6:00 p.m.; off from 6:00 p.m. to 6:00 a.m.), while the second group was housed under "reverse" light conditions (light on from 10:00 p.m. to 10:00 a.m.; off from 10:00 a.m. to 10:00 p.m.). A circadian rhythm was observed in the elimination rate of FURA and FURA catabolites by both groups (P < 0.0001, Cosinor analysis). Under "normal" light conditions, peak and trough elimination rate of FURA was at 19 h after light onset (HALO: 183.8 ± 3.4 nmol/min/g liver) and 7 HALO (123.8 ± 3.4 nmol/min/g liver), respectively. There was a reciprocal relationship between the elimination rates of FURA and FURA catabolites with peak and trough values for FURA catabolites at 7 HALO (70.5 ± 3.6 nmol/min/g liver) and 19 HALO (17.5 ± 3.6 nmol/min/g liver), respectively. Animals housed under the "reverse" conditions of light and dark also exhibited a circadian pattern. Under the "reverse" conditions, the peak and trough elimination rate of FURA was at 18.5 HALO (170.0 ± 1.7 nmol/min/g liver) and 6.5 HALO (130.0 ± 1.7 nmol/min/g liver), respectively. The peak and trough elimination rate of FURA catabolites under these conditions occurred at 6.5 HALO (64.3 ± 2.2 nmol/min/g liver) and 18.5 HALO (29.7 ± 2.2 nmol/min/g liver), respectively. These results demonstrate that the elimination rate of FURA and FURA catabolites by IPRL varies over a 24-h period with a circadian rhythm in association with the light/dark cycle. Such a variation in the hepatic elimination rate of FURA in humans could result in a variation in the systemic level of drug during chemotherapy thus affecting the therapeutic efficacy of FURA. This study suggests that a circadian pattern in the hepatic catabolism of FURA needs to be considered when planning chemotherapeutic regimens with FURA.

INTRODUCTION

FURA is a cancer chemotherapeutic agent used in the management of several malignancies, primarily cancers of the breast and gastrointestinal tract (1). Over the past three decades, investigations into the cellular metabolism of FURA have demonstrated that the anabolism of FURA to nucleotide analogues is responsible for the observed cytotoxic effects (2). In contrast to anabolism, very little attention has been given to the importance of catabolism of FURA despite the fact that it has been estimated that greater than 80% of administered FURA is rapidly catabolized (3). Catabolism of FURA is thought to be carried out primarily by the same three enzymes which degrade uracil and thymine (4).

The importance of catabolism in FURA chemotherapy has been demonstrated in a number of recent studies. Clinical studies with "modulators" of pyrimidine catabolism such as thymidine and uridine demonstrated that coadministration with FURA resulted in increased cytotoxicity due to increased availability of FURA (5). In addition, a recent study from our laboratory (6) identified a patient with severe neurological toxicity who was completely deficient in DPD activity and exhibited elevated and prolonged plasma levels of FURA following an i.v. bolus of FURA.

Although pyrimidine catabolism occurs in several tissues (7), the primary site of FURA catabolism appears to be the liver (8). Given the apparent importance of pyrimidine catabolism to FURA chemotherapy, we recently measured DPD activity in rat liver homogenates and reported a variation in enzyme activity over a 24-h period (9). DPD activity varied with a circadian pattern in association with the light/dark cycle with a peak of activity at the end of the animals resting phase (lights on) and a trough at the end of their activity phase (lights off). However, an alteration in liver DPD activity may or may not result in a relevant change in overall hepatic catabolism of pyrimidines. Many factors may influence the hepatic catabolism of FURA such as transport, oxidation state of the cell, metabolite pools, etc. Therefore, the IPRL was chosen to examine FURA catabolism in the liver.

The purpose of this study was to determine if the hepatic elimination rate of FURA and total FURA catabolites (i.e., rate leaving the IPRL in the effluent perfusate) by IPRL exhibited a similar circadian pattern as that observed for DPD activity in rat liver homogenates.

MATERIALS AND METHODS

Chemicals. FURA was obtained from Sigma Chemical Co. (St. Louis, MO). [6-14C]FURA (26 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Chemical and radiolabeled purity was greater than 99% as determined by HPLC. All solvents were HPLC grade and all other chemicals were of the highest grade available.

Animals. Male Sprague-Dawley rats weighing 170–200 g (~7 weeks old) were obtained from Harlan Laboratories, Inc. (Indianapolis, IN). The animals were divided into two groups of 24 each and housed four to a cage with free access to food and water. The first group was housed under "normal" light conditions, i.e., lights on from 6 a.m. to 6 p.m. and lights off from 6 p.m. to 6 a.m.; while the second group was housed under "reverse" light conditions, i.e., lights on from 10 p.m. to 10 a.m. and lights off from 10 a.m. to 10 p.m. The light/dark cycle for each set of animals was automatically controlled by a mechanical timer. Animals were housed under these conditions for a minimum of 3 weeks prior to being used for liver perfusion studies so that they could adapt to their respective environments. At the time the perfusion studies were begun, the rats ranged in weight from 250 to 300 g.

IPRL. Prior to isolation of the liver, the rats were anesthetized with pentobarbital (35 mg/kg i.p.). Animals were anesthetized at 3-h intervals corresponding to the following clock times: 1:00 a.m., 4:00 a.m., 7:00 a.m., 10:00 a.m., 1:00 p.m., 4:00 p.m., 7:00 p.m., and 10:00 p.m. The surgical procedure required less than 30 min and liver perfusion was carried out in a nonrecirculating system as described previously (10) with a flow rate of 40 ml/min to maintain sufficient oxygenation of the liver. The perfusion buffer was Krebs-Henseleit bicarbonate buffer (pH 7.4) heated to 37°C and saturated with a gas mixture of oxygen (95%) and carbon dioxide (5%). Sampling ports were positioned so...
that the perfusate could be sampled just prior to entering the liver and immediately after exiting the liver. The liver preparation was perfused for a period of 20 min prior to the infusion of [6-3H]FUra.

Infusion of [6-3H]FUra. Initially, the concentration of FUra in the perfusing buffer was evaluated over the range of 1 to 200 µM. Fifty µM was chosen as the concentration to be used in the studies described below since the elimination rate of FUra and total FUra catabolites reached a maximum level (see “Results”). For each IPRL, [6-3H]FUra was infused into the perfusing buffer at a rate of 1 µCi/min. Samples of the perfusing buffer were taken every 10 min to determine the rate (nmol/min/g liver) of [6-3H]FUra entering the liver. The specific activity of [6-3H]FUra during the infusion was approximately 0.51 µCi/nmol.

Determination of FUra Metabolites in the IPRL Perfusion. Effluent perfusate samples were collected every 5 min during the infusion of [6-3H]FUra. An aliquot (200 µl) of each sample was analyzed for FUra and FUra catabolites using an HPLC method previously described (11). FUra and FUra catabolites were separated using two 5-µm C18 reversed-phase columns (25 x 0.45 cm) in tandem. Elution was carried out isocratically at 1 ml/min with a mobile phase of 5 mM tetraethylammonium hydrogen sulfate and 1.5 mM potassium dihydrogen phosphate (pH 8.0). FUra catabolites (FUH2, FUPA, FBAL) were quantitated separately but the values were pooled and expressed as total FUra catabolites in this study. With this HPLC method, FUra and FUra catabolites had the following retention times: FBAL = 6.7 min; FUH2 = 7.8 min; FUPA = 13.2 min; and FUra = 20.2 min. Elimination rate of FUra and total FUra catabolites (i.e., rate leaving the IPRL in the effluent perfusate) was expressed as nmol/min/g liver.

Determination of Hepatic Extraction Ratio of FUra. Hepatic clearance is the product of blood flow and the extraction ratio. Therefore, at a constant flow (40 ml/min), hepatic clearance can be determined experimentally in IPRL from the extraction ratio of a drug, that is, the difference between the concentration of drug entering the liver versus the concentration of drug leaving the liver divided by the concentration of drug entering the liver.

Statistics. The values obtained for the hepatic elimination rate of FUra and total FUra catabolites by IPRL every 3 h over a 24-h period were analyzed for circadian periodicity. The data was fitted to a “Cosinor” model by the method of least squares (12, 13). Three parameters were quantitated in this analysis: mesor (i.e., the rhythm-adjusted mean), amplitude (i.e., maximum or minimum value from the mesor), and acrophase (i.e., time of maximum or minimum value from a given phase of reference). In the present study, the acrophase data is expressed as HALO.

RESULTS

Determination of Infusion Concentration of FUra in IPRL. In order to observe an alteration in catabolism due to a periodicity of enzyme activity, substrate concentration cannot be rate-limiting. Therefore, various concentrations of FUra (1-200 µM) were infused into IPRLs to determine the concentration of infused FUra that would yield maximum catabolism. For each of these determinations the animals were anesthetized at the same time of the day (i.e., 9:00 a.m.). When samples were taken from the effluent perfusate every 5 min, a constant, maximum elimination rate of FUra catabolites was reached after 30 min of perfusion and maintained throughout the 60-min perfusion period. In this study, the elimination rate of FUra and total FUra catabolites was determined from this plateau achieved in the final 30 min of perfusion. As the concentration of FUra infused was increased, there was a proportional increase in the elimination rate of FUra catabolites in the effluent perfusate up to an infusion concentration of 25 µM (data not shown). As FUra concentration was increased from 25 to 200 µM, the elimination of FUra catabolites in the effluent perfusate approached a maximum level at approximately 50 µM indicating saturation (data not shown). In addition, to monitor the viability of the IPRL preparation at these various concentrations of FUra (1-200 µM), an oxygen probe was placed in the effluent perfusate and oxygen uptake by the liver was measured. The rate of oxygen uptake by the liver also increased with increasing concentration of FUra up to a concentration of 50 µM after which a maximum level of oxygen uptake was achieved (data not shown). Based on these observations, all subsequent studies were carried out with 50 µM FUra.

Infusion Rate of FUra and Elimination Rate of FUra and Total FUra Catabolites in IPRL. Infusion of FUra at a final concentration of 50 µM in the perfusing buffer yielded an infusion rate (i.e., rate of FUra entering the IPRL) of 203.2 ± 9.3 nmol FUra/min/g liver for the 48 animals in this study. The elimination rate of FUra and total FUra catabolites was determined as described in the previous section. Elimination rate of FUra and total FUra catabolites was quantitated from the average of seven determinations taken from the effluent perfusate 30 to 60 min (every 5 min) following infusion of FUra into each of the IPRL preparations. The elimination rate of FUra and FUra catabolites was determined in IPRLs over 60 min at all time points (see “Materials and Methods”) over 24 h in animals housed under “normal” and “reverse” conditions of the light/dark cycle. The elimination rate of FUra ranged from 121.4 to 193.8 nmol/min/g liver and the elimination rate of total FUra catabolites ranged from 10.6 to 78.2 nmol/min/g liver. Fig. 1 shows the pattern observed in the elimination rate of both FUra and total FUra catabolites obtained at peak and trough times. The time of peak (1:00 p.m., 7 HALO) and trough (1:00 a.m., 19 HALO) elimination of FUra was determined from the mean ± SD of three animals housed under “normal” light conditions and are given in Fig. 1A; peak (4:00 a.m., 6 HALO) and trough (4:00 p.m., 18 HALO) values from three animals (mean ± SD) housed under “reverse” condition are given in Fig. 1B. There was a reciprocal relationship between the elimination rate of FUra and FUra catabolites with peak and trough values for FUra catabolites at 7 HALO and 19 HALO, respectively, for the “normal” cycle (Fig. 1A) and 18 HALO and 6 HALO, respectively, for the “reverse” cycle (Fig. 1B).

Variation in the Elimination Rate of FUra and Total FUra Catabolites by IPRL. The elimination rate of FUra and total FUra catabolites at a given time of the day was quantitated from the mean ± SD of three IPRL preparations. In the present study, the elimination rate of FUra and total FUra catabolites under both “normal” and “reverse” conditions of the light/dark cycle exhibited a circadian periodicity (P < 0.0001, Cosinor analysis; Fig. 2). In all of the studies, the total amount of infused radioactivity was accounted for by the elimination of FUra and total FUra catabolites into the effluent perfusate. The parameters determined by Cosinor analysis are listed in Table 1.

Variation in the Hepatic Extraction Ratio of FUra by IPRL. A variation was observed in the hepatic extraction ratio of FUra by IPRL ([FUraout] - [FUrain])/[FUrain] determined at each of the time points for both light/dark cycles (Fig. 3). Animals housed under “normal” light conditions exhibited a circadian pattern in the hepatic extraction ratio of FUra (P < 0.0001) such that a peak was observed at 1:00 p.m. (0.36 ± 0.03) corresponding to 7 HALO and a trough at 1:00 a.m. (0.05 ± 0.02) corresponding to 19 HALO (Fig. 3). The parameters obtained by Cosinor analysis are given in Table 1. Animals housed under “reverse” light conditions also exhibited a circadian pattern in the hepatic extraction ratio of FUra with peak values at 4:30 a.m. (0.34 ± 0.04; 6.5 HALO) and trough values

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DISCUSSION

Several recent studies have demonstrated that time modification of fluoropyrimidine infusion (14, 15) results in a decrease in fluoropyrimidine toxicity thereby permitting an increase in the "dose intensity." The biochemical basis for this potential therapeutic advantage in fluoropyrimidine chemotherapy is still not known. Since fluoropyrimidine catabolism has a major role in determining the availability of fluoropyrimidines, it is conceivable that a circadian variation in catabolism may be responsible. It has been demonstrated that circadian rhythms regulate the activity of numerous liver enzymes in drug metabolism (16). This has been shown with the initial enzyme of the major pyrimidine catabolic pathway, DPD, in rat liver homogenates (9). However, many factors influence overall pyrimidine catabolism within intact cells and an alteration in DPD activity in liver homogenates may or may not correspond to an alteration in hepatic pyrimidine catabolism.

The present study demonstrates a circadian variation in the elimination rate of FUra and total FUra catabolites by IPRL. Studies with IPRL were carried out with FUra infused at 50 μM. Following infusion of [6-3H]FUra, the elimination rate of FUra and total FUra catabolites was quantitated in IPRLs from animals sacrificed every 3 h over a 24-h period. For all animals in this study, the elimination rate of FUra and total FUra catabolites by the IPRL accounted for the total amount of infused radioactivity suggesting that catabolism was responsible for the hepatic clearance of FUra with no detectable anabolism of FUra. In addition, a reciprocal relationship was noted in the elimination rate of FUra and total FUra catabolites such that as the elimination rate of one increased, the elimination rate of the other decreased. This relationship suggests that FUra catabolism may be a major factor in determining the elimination rate of FUra in IPRL.

With continuous infusion of [6-3H]FUra into an IPRL at 50 μM, the hepatic extraction ratio of FUra varied in IPRL with the light/dark cycle with a peak in the middle of the light period and a trough in the middle of the dark period. This pattern was observed when rats were housed under “normal” light conditions and “reverse” light conditions. The corresponding shift in the circadian pattern of the hepatic extraction ratio with the shift in the light cycle suggests that the entrainment of this rhythm is associated with the light/dark cycle or some aspect that is related to the light/dark cycle (e.g., activity cycle, feeding habits, etc.). In the present study we utilized the IPRL to examine FUra catabolism over 24 h. To insure that an observed variation in catabolism was not due to a variation in substrate concentration, saturated conditions (>50 μM) were determined and used in this study. While pharmacological conditions (<50 μM) similar to those obtained in the clinical administration of FUra cannot be directly addressed by this study, it provides a possible explanation for the observed variation in systemic levels of FUra following constant-rate continuous infusion (17).

Interpretation of biochemical data from rodents for application in humans must always consider differences in the light/dark cycles of the two species. Rats are nocturnal animals and are more active in the dark and less active in the light. Humans, on the other hand, are typically just the opposite, that is, more active in the light and less active in the dark. However, many factors influence overall pyrimidine catabolism within intact cells and an alteration in DPD activity in liver homogenates may or may not correspond to an alteration in hepatic pyrimidine catabolism.

at 4:30 p.m. (0.12 ± 0.03; 18.5 HALO) suggesting that the circadian pattern is associated with the light/dark cycle.
active during hours of light and less active during hours of darkness. The present study demonstrates that the hepatic extraction of FUra in rodents has a circadian pattern with a peak at approximately 7 HALO and trough at approximately 19 HALO for both light/dark cycles studied. Therefore, peak hepatic extraction of FUra occurred during the middle of the resting portion of the animals light/dark cycle, whereas, trough extraction occurred during the middle of the active portion. This agrees with our previous data on DPD activity in rat liver homogenates (9) with peak and trough enzyme activity occurring in approximately the same portions of the light/dark cycle.

In summary, this study demonstrates that the hepatic elimination rate of both FUra and total FUra catabolites in IPRL varies over a 24-h period. Since liver catabolism may have a major role in determining the availability of FUra, a circadian pattern of FUra catabolism could affect the efficacy of fluoropyrimidine chemotherapy. With the recent development of programmable pumps, utilization of information on circadian patterns may be useful in planning continuous infusion schedules of fluoropyrimidines.

The basis for this periodicity in survival was not determined. However, from this information a “time-modified” infusion of 5-fluorodeoxyuridine was tested in humans with a resulting decrease in toxicity and an increase in the dose intensity, that is, the amount of drug that could be administered to the patient per day (14, 15). Therefore, in this case, time-modification of fluoropyrimidine infusion resulted in an increase in the therapeutic efficacy of the drug.

Knowledge of circadian rhythms that alter host toxicity to chemotherapeutic agents may be important in both bolus and continuous infusion schedules of drug delivery. While adjustment of the time of administration for a bolus dose may appear to be simple, utilization of circadian rhythms in continuous infusions schedules is obviously more complex and may be limited by patient compliance and/or the pump used to deliver the drug. The recent development of a variety of programmable pumps which can deliver drugs via intraarterial or i.v. infusion in a time-modified schedule has made utilization of such information more feasible. These devices allow the patient to receive more drug during the time of the day when they are more resistant to drug-induced side-effects and less drug when they are more susceptible to toxic side-effects.

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