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CIRCADIAN RHYTHM OF RAT LIVER DIHYDROPYRIMIDINE DEHYDROGENASE

POSSIBLE RELEVANCE TO FLUOROPYRIMIDINE CHEMOTHERAPY*

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Abstract—The activity of dihydropyrimidine dehydrogenase (DPD), the initial, rate-limiting enzyme in pyrimidine catabolism, was measured at various times over a 24-hr period in the livers of rats housed under standardized conditions of light and dark. Under "normal" conditions, i.e. lights on from 6:00 a.m. to 6:00 p.m. and off from 6:00 p.m. to 6:00 a.m., a circadian rhythm of DPD activity was observed ($P < 0.0001$, Cosinor analysis) with the peak of activity at 4:00 p.m. (2.96 nmol catabolites/min/mg) and the trough at 4:00 a.m. (0.40 nmol catabolites/min/mg). Maximum enzyme activity exceeded minimum activity by more than 7-fold. Reversing the light–dark cycle (i.e. lights on from 6:00 p.m. to 6:00 a.m. and off from 6:00 a.m. to 6:00 p.m.) resulted in a corresponding shift in enzyme activity. Under these "reverse" conditions, a circadian rhythm was observed ($P < 0.0001$, Cosinor analysis) with the peak of activity at 6:00 a.m. (2.87 nmol catabolites/min/mg) and the trough at 6:00 p.m. (0.92 nmol catabolites/min/mg). These studies demonstrated that DPD activity in rat liver varies over a 24-hr period in association with the light–dark cycle.

Since introduced 30 years ago by Heidelberger and colleagues [1], fluoropyrimidines (FPs‖) have become important in the management of several common malignancies including carcinoma of the breast, ovary, and colon [2]. 5-Fluorouracil (FUra) and 5-fluorodeoxyuridine (FdUrd) are the most common FPs used clinically. Over the past three decades there have been numerous studies of FP metabolism, and it is now clear that the cytotoxic activity of FPs appears to be mediated by anabolism within the cell [3].

Until recently, very little attention has been given to the examination of FP catabolism (in contrast to anabolism). Dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2) is the major, rate-limiting enzyme of pyrimidine catabolism. Since greater than 85% of administered FUra is catabolized by this route [3], pyrimidine catabolism has a major role in determining the bioavailability of FPs. The need for examination of FP catabolism is further emphasized by studies with "modulators" of pyrimidine catabolism such as thymine and uracil [4, 5]. These pyrimidines can compete with FUra for catabolic enzymes and thus influence the bioavailability of FUra. The severe toxicity observed with concurrent use of thymidine with FUra [6] has been suggested to be due to competition between thymine and FUra for catabolic enzymes [7]. The toxicity resulting from an alteration in DPD activity is further emphasized by a recent report from our laboratory [8] which noted severe FUra-induced toxicity in a patient deficient in DPD activity.

Roemeling and Hrushesky [9] recently studied a "time-modified" continuous infusion regimen in which the infusion of FdUrd was varied over a 24-hr period (i.e. two-thirds of a daily dose was administered between 3:00 p.m. and 9:00 p.m.). This resulted in a therapeutic advantage with decreased host toxicity compared to FdUrd given as a "flat" continuous infusion (dose not altered during infusion), permitting an increased dose of FdUrd to be administered. The biochemical basis for the reduced host toxicity of this "time-modified" regimen was not described in this study.

Given the apparent importance of pyrimidine catabolism in determining FP bioavailability and the increased effectiveness of the "time-modified" continuous infusion regimen, a variation in the enzymes of pyrimidine catabolism over a 24-hr period may have a role in the therapeutic advantage observed with the "time-modified" regimen. The purpose of this study was to investigate the activity of rat liver DPD over 24 hr to determine if a circadian pattern may exist. A variation in rat liver DPD, the rate-limiting enzyme of pyrimidine catabolism, may provide a possible biochemical explanation for the increased effectiveness of "time-modified" continuous infusion FP regimens.

MATERIALS AND METHODS

Chemicals. FUra was obtained from the Sigma Chemical Co. (St. Louis, MO). [6-^{3}H]FUra (sp. act. = 26 Ci/mmol) was obtained from Moravek

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‖ Abbreviations: FP, fluoropyrimidine; FUra, 5-fluorouracil; FdUrd, 5-fluorodeoxyuridine; and DPD, dihydropyrimidine dehydrogenase.

Biochemicals (Brea, CA). Chemical and radiolabeled purity of these compounds was quantitated by HPLC and found to be greater than 99%. All solvents were HPLC grade, and all other chemicals were of the highest grade available.

Preparation of rat liver homogenates. In the fall of 1987, male Sprague–Dawley rats weighing 170–200 g (~7 weeks old) were obtained and housed four to a cage with free access to food and water. For experimental purposes, the animals were divided into two groups of eighteen—one group was housed under "normal" light conditions, i.e. lights on from 6:00 a.m. to 6:00 p.m. and lights off from 6:00 p.m. to 6:00 a.m., and the other group was housed under "reverse" light conditions, i.e. lights off from 6:00 a.m. to 6:00 p.m. and lights on from 6:00 p.m. to 6:00 a.m. Each group was given a minimum of 3 weeks to adapt to their environment (light–dark cycle). The animals were killed at various times of the day by cervical dislocation in accordance with an institutional approved protocol. Prior to removal of the liver, the portal vein was cannulated and the liver perfused with ice-cold 0.15 M KCl. Upon removal, the liver was weighed, minced, and homogenized in 5 vol. of 0.25 M sucrose buffer (35 mM potassium phosphate, 2.5 mM $MgCl_2$, and 5 mM β-mercaptoethanol) using a motor-driven Potter homogenizer. This homogenate was centrifuged at 100,000 *g* for 60 min at 4° in a Beckman model L8-80 ultracentrifuge. The supernatant fraction was carefully removed and filtered (Whatman No. 1 filter paper) under gentle suction. The resulting clear, lipid-free filtrate was used for subsequent enzyme assays.

Enzyme assay. Enzyme activity was determined by HPLC using a method we have described previously [8]. Aliquots (25 μl, 50 μl, and 100 μl) of the above filtrate were incubated at 37° for 30 min in the presence of 250 μM NADPH and 20 μM [6-^{3}H]FUra (3.5 μCi/μmol). Total incubation volume was 2 ml. The reaction was stopped by removing an aliquot (300 μl) at the designated time and adding it to an equal volume of ice-cold 100% ethanol. The sample was centrifuged (12,000 *g*) for 1 min to pellet precipitated protein, filtered (0.2 μm pore size), and analyzed for the presence of FUra and FUra catabolites by HPLC as described previously [10]. Enzyme activity was calculated from the slope of five time points over the initial 30 min during which the reaction was linear. DPD activity was expressed as nanomoles of total catabolites formed per minute per milligram of protein. Protein concentration was calculated by the method of Lowry *et al.* [11].

Statistical analysis of data. The time series data in the present study were analyzed by the "Cosinor" method [12]. The data were fitted to a cosine wave by the method of least squares. Four parameters were quantitated in this analysis. These include the mesor (i.e. the rhythm-adjusted mean), the amplitude (i.e. maximum or minimum value from the mean), the acrophase (i.e. time of maximum or minimum value) and the period (i.e. length of one complete cycle).

RESULTS

In male, Sprague–Dawley rats maintained in standardized conditions of 12 hr light (6:00 a.m. to 6:00 p.m.) alternating with 12 hr dark (6:00 p.m. to 6:00 a.m.), there was a circadian pattern of DPD activity observed in liver homogenates obtained at specified times over 24 hr (Fig. 1A). The values of the Cosinor analysis are shown in Table 1. When samples were taken every 4 hr, the peak of DPD activity was at approximately 4:00 p.m. and the trough at approximately 4:00 a.m., resulting in a 7-fold difference in enzyme activity between the two time points (Table 2). Reversing the light–dark cycle so that the lights came on at 6:00 p.m. and went off at 6:00 a.m. resulted in a corresponding shift in enzyme activity (Fig. 1B). Under the reversed conditions of 12 hr dark (6:00 a.m. to 6:00 p.m.) alternating with 12 hr light (6:00 p.m. to 6:00 a.m.), the peak of DPD activity was at approximately 6:00 a.m. and the trough at approximately 6:00 p.m., resulting in a 3-fold difference in enzyme activity (Table 2). In addition to the reversal of the pattern of DPD activity, there was a 2-hr lag in the time of maximum and the time of minimum for the "reverse" light–dark cycle.

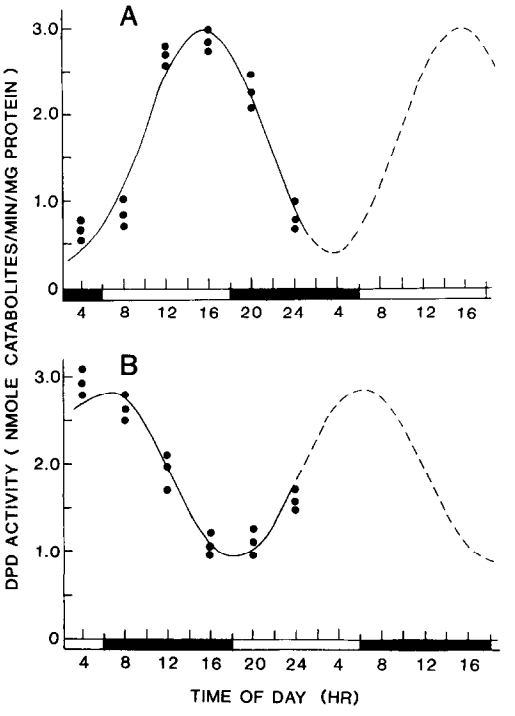

Fig. 1. Pattern of circadian variation of rat liver DPD under "normal" conditions, i.e. lights on from 6:00 a.m. to 6:00 p.m. and lights off from 6:00 p.m. to 6:00 a.m. (A) and under "reverse" conditions, i.e. lights on from 6:00 p.m. to 6:00 a.m. and lights off from 6:00 a.m. to 6:00 p.m. (B). For each time, three data points are shown. Each data point represents the DPD activity measured in the liver homogenate of one rat and is calculated from the mean of two or three determinations at different enzyme concentrations. The curve is a computer-generated cosine curve which best fits the data as determined by the Cosinor method.

While maximum enzyme activity was similar under both light–dark cycles, the minimum enzyme activity

Table 1. Rhythmometric summary of single Cosinor analysis of DPD activity in rat liver under normal and reversed conditions of light and darkness

Cycle	P	r^2	Mesor* ± SE	Amplitude ± SE
Normal†	< 0.0001	0.953	1.682 ± 0.115	1.282 ± 0.163
Reverse‡	< 0.0001	0.955	1.894 ± 0.087	0.975 ± 0.123

* Rhythm-adjusted mean.

† Normal cycle: light, 6:00 a.m. to 6:00 p.m.; dark, 6:00 p.m. to 6:00 a.m. Values were obtained from a group of eighteen animals.

† Reverse cycle: light, 6:00 p.m. to 6:00 a.m.; dark, 6:00 a.m. to 6:00 p.m. Values were obtained from a group of eighteen animals.

Table 2. Maximum and minimum DPD activity under normal and reversed conditions of light and darkness

Cycle	Maximum ± SE (nmol catabolites/ min/mg protein)	Time of maximum ± SE	Minimum ± SE (nmol catabolites/ min/mg protein)	Time of minimum ± SE	Maximum/Minimum
Normal	2.964* ± 0.199	15.636† ± 0.485	0.400‡ ± 0.199	3.636† ± 0.485	7.41
Reverse	2.867* ± 0.151	6.259† ± 0.481	0.919‡ ± 0.151	18.259† ± 0.481	3.12

Activities were determined in eighteen animals in each group under normal and reversed conditions of light and darkness.

* Calculated by addition of the amplitude to the mesor (rhythm-adjusted mean).

† Calculated from the acrophase parameter of the Cosinor analysis.

‡ Calculated by subtracting the amplitude from the mesor (rhythm-adjusted mean).

under the "normal" cycle was lower than the minimum activity under the "reverse" cycle leading to a discrepancy in the overall difference in enzyme activity (Table 2). This may be due to incomplete adaptation of the animals under the reverse light cycle.

DISCUSSION

It has been recognized that the time of drug administration may markedly influence drug effect and toxicity [13]. Studies with several different anticancer drugs including FPs [14–16] have emphasized the importance of the time of drug administration to host toxicity. The biochemical basis for this varied drug response over 24 hr is unknown. One possible explanation could be a circadian variation in the enzymes responsible for drug activation or deactivation.

Because of the importance of pyrimidine catabolism in determining the bioavailability of FPs, we examined the activity of DPD over 24 hr to determine if this enzyme had a circadian pattern. DPD is the major, rate-limiting enzyme of pyrimidine catabolism and, therefore, has a primary role in the balance between pyrimidine catabolism and anabolism [17]. The cytotoxicity of FPs, such as FUra or FdUrd, in host and tumor cells occurs following anabolism to nucleotides in actively proliferating cells. The availability of FP for anabolism, however, is determined by catabolism. Because the primary site of systemic FP catabolism is the liver, DPD activity in the liver has an important role in determining the systemic bioavailability of FPs.

In the present study we demonstrated that DPD activity in rat liver varies over 24 hr. A circadian rhythm of DPD activity was observed (Fig. 1A, $P < 0.0001$) with maximum activity at 4:00 p.m. and minimum activity at 4:00 a.m. (Table 2). Maximum activity exceeded minimum activity by more than 7-fold (Table 2). Reversing the light–dark cycle resulted in a corresponding shift in DPD activity (Fig. 1B) with maximum activity at 6:00 a.m. and minimum activity at 6:00 p.m. Maximum activity exceeded minimum activity by 3-fold (Table 2). Under the "reversed" conditions of the light–dark cycle, there was a 2-hr lag in the time of maximum (and minimum), and minimum activity was higher than observed in the "normal" cycle. Therefore, it is clear that the pattern of DPD activity in rat liver is determined by the light–dark cycle. The physiological mechanisms responsible for this regulation is unclear but may include patterns of cell proliferation, sleep/wake (activity), and feeding/fasting.

Extrapolation of circadian rhythms from rodents to humans must always consider differences in the life cycles of the two species. Rats are nocturnal animals and, therefore, are more active in the dark and less active (resting) in the light. However, humans are just the opposite, i.e. they are more active in the light and less active during the dark. In the above data, the peak of DPD activity in rat livers occurred at the end of the resting span and the trough at the end of the activity span for both the "normal" and "reversed" light–dark cycles. This agrees with the pattern of DPD in human peripheral mononuclear cells found in a study from our laboratory [18]. In this study and in subsequent experiments with a total of twenty healthy subjects, DPD activity was found to vary insignificantly during the day with

a significant decrease in activity (trough) at the end of the activity and into the early part of the resting span (time of minimum = 12.00 a.m.). A recent study by Tuchman *et al.* [19] reported a circadian rhythm of DPD activity in human blood mononuclear cells from seven healthy subjects. In this study a significant circadian variation was demonstrated throughout the 24-hr period and the timing of the peak values (time of maximum = 1:00 a.m.) was different from that in rat liver and from our study of human peripheral mononuclear cells. In the study by Tuchman *et al.*, the peak occurred at the end of the activity span and into the early part of the resting span. The reason for this discrepancy is not clear but emphasizes the variability that may exist between patients. It is interesting to note that a recent report by Petit *et al.* [20] determined the plasma concentration of FUra in seven patients receiving FUra by continuous infusion for 5 consecutive days. When samples were taken every 3 hr on days 1, 3, and 5, FUra plasma concentration exhibited a circadian pattern with a peak at 1:00 a.m. (early part of resting span).

Knowledge of circadian patterns that alter host toxicity is important to long-term continuous infusion regimens. Utilization of such information in a clinical setting is much more feasible due to the recent development of a variety of programmable drug-administration devices. These systems can deliver drugs via intraarterial or intravenous infusion in time-modified regimens with excellent precision [9]. These devices allow the patient to receive increased drug when they are most resistant to toxic side effects and decreased drug when they are more susceptible to drug-induced toxicity. Tumors may also exhibit a temporal pattern of sensitivity (and resistance) to FPs which may also be important in optimizing FP chemotherapy. Therefore, careful attention to circadian patterns of enzymes will optimize tumor kill by maintaining optimal drug concentration throughout the infusion decreasing the occurrence of unwanted side effects.

In summary, this study demonstrated that the level of DPD activity in rat liver varies over a 24-hr period. Since liver catabolism has a major role in determining the systemic level of drug following FP administration, the circadian pattern of DPD activity should be considered in planning FP continuous infusion schedules, particularly by programmable pumps.

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