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CIRCADIAN RHYTHM OF RAT SPLEEN CYTOPLASMIC THYMIDINE KINASE

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Abstract—The activity of thymidine kinase (TK, EC 2.7.1.21), the first enzyme of the thymidine phosphorylation pathway, was measured at various times over a 24-hr period in the spleens of Sprague–Dawley rats that had been housed under standardized conditions of light and dark for at least 4 weeks before the study. Spleen cytoplasmic TK activity was assayed with [2-^{14}C]thymidine as substrate. Under "normal" light conditions (lights on 6:00 a.m.–6:00 p.m. and lights off 6:00 p.m.–6:00 a.m.), a carcadian variation of TK activity was observed ($P < 0.0001$, Cosinor analysis) with peak activity (1.98 nmol product/hr/mg protein) at 1:00 a.m. (19 hr after light onset, HALO) and trough activity (0.40 nmol product/hr/mg protein) at 1:00 p.m. (7 HALO). Maximum enzyme activity exceeded minimum activity by approximately 5-fold. Reversing the light–dark cycle resulted in a corresponding shift in TK activity. Under these "reverse" conditions (lights on 6:00 p.m.–6:00 a.m. and lights off 6:00 a.m.–6:00 p.m.), a circadian variation in TK activity was also observed ($P < 0.0001$, Cosinor analysis) with peak activity (1.14 nmol product/hr/mg protein) at 12:00 noon (18 HALO) and trough activity (0.32 nmol/hr/mg protein) at 12:00 a.m. (6 HALO). Maximum enzyme activity exceeded minimum activity by approximately 4-fold. In summary, this study demonstrated for the first time that TK activity varies over a 24-hr period in association with the light–dark cycle.

It is recognized that many enzymes involved in metabolism follow a circadian pattern [1–3]. Recently, there has been interest in whether enzymes in the pyrimidine pathway follow a circadian pattern, in particular because of the demonstrated therapeutic advantage of time-modified administration of fluoropyrimidine drugs [4–6]. We have shown previously that dihydropyrimidine dehydrogenase (DPD§, EC 1.3.1.2), the initial and rate-limiting enzyme in pyrimidine catabolism, follows a circadian pattern in both Sprague–Dawley rats [7, 8] and humans [9] and can affect the plasma concentration of the pyrimidine drug 5-fluorouracil [9]. In subsequent studies, we examined thymidine phosphorylase (EC 2.4.2.4) activity in the same species of rat [10] and humans [11] and observed no variation over 24 hr. In another investigation a circadian pattern of uridine phosphorylase (EC 2.4.2.3) activity in mice [12] was demonstrated.

In the present study, we examined thymidine kinase (TK, EC 2.7.1.21), the initial enzyme in the thymidine phosphorylation pathway. This enzyme is of interest not only because of its importance in DNA synthesis [13, 14] but also because of its role in the activation of two important pyrimidine drugs: (1) 5-fluoro-2′-deoxyuridine (FdUrd) used in the treatment of metastatic cancer to the liver [15] and renal cell carcinoma [6, 16]; and (2) 3′-azido-3′-deoxythymidine (AZT) [17], the most commonly used drug in the treatment of AIDS [18].

Our preliminary experience in determination of TK activity in several rat tissues including liver, kidney, and brain suggested that TK activity in those tissues is very low. Therefore, in the present study, we have used rat spleen, since TK activity in the spleen is easily detectable [19], as a source of enzyme to determine whether this enzyme follows a circadian pattern.

MATERIALS AND METHODS

Chemicals and radiochemicals. Unlabeled thymidine, ATP, $MgCl_2$, NaF, dithiothreitol, phosphocreatine, creatine kinase and bovine serum albumin were purchased from the Sigma Chemical Co. (St. Louis, MO). [2-^{14}C]Thymidine (56 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA). The purity of both unlabeled and labeled thymidine was determined by HPLC [20, 21] to be greater than 99%. Solvents were HPLC grade and all other chemicals used in the present study were of the highest grade available.

Animals. In February 1992, male Sprague–Dawley rats weighing 170–190 g (Harlan Laboratories, Indianapolis, IN) were obtained and housed three to a cage with free access to food and water in a light–dark-controlled room with environmental temperature maintained at 25 ± 1° and relative humidity at approximately 50%. For experimental purposes, the animals were randomly divided into

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§ Abbreviations: DPD, dihydropyrimidine dehydrogenase; TK, thymidine kinase; HALO, hours after light onset; FdUrd, 5-fluoro-2′-deoxyuridine; and AZT, 3′-azido-3′-deoxythymidine.

two groups: one group was housed under "normal" light–dark conditions (lights on from 6:00 a.m. to 6:00 p.m. and off from 6:00 p.m. to 6:00 a.m. local time); the other group was housed under "reverse" light–dark conditions (lights off from 6:00 a.m. to 6:00 p.m. and on from 6:00 p.m. to 6:00 a.m. local time). All animals were given at least 4 weeks to adjust to their light–dark cycle.

Preparation of rat spleen homogenates. To determine the variation of TK activity over 24 hr, the animals were killed at various times of the day (i.e. 4:00 a.m., 8:00 a.m., 12:00 noon, 4:00 p.m., 8:00 p.m., and 12:00 midnight) by cervical dislocation in accordance with an institutional approval protocol. The spleen was excised and washed with ice-cold physiologic saline (0.9% NaCl). The spleen cytosol containing TK activity was prepared by a modification of a method previously reported by Cheng [22]. In brief, the removed spleen was weighed, minced and homogenized in 6 vol. of 0.05 M Tris–HCl buffer (pH 7.5) containing 2 mM dithiothreitol, 50 μM thymidine, and 10% glycerol. The resulting homogenate was centrifuged at 100,000 *g* for 60 min at 4°. The supernatant was removed and used for subsequent assays.

Enzyme assay. Spleen cytoplasmic TK activity was assayed using the method described by Cheng [22] and modified by introducing reversed-phase HPLC to determine product (TMP) formation [20, 21]. In brief, the reaction mixture (2 mL, final volume) contained 1.5 mL of the stock assay solution (20 μM [2-^{14}C]thymidine, 2.5 mM ATP, 2.5 mM $MgCl_2$, 12.5 mM NaF, 2 mM dithiothreitol, 4.5 mM phosphocreatine, 6 U/mL creatine kinase, and 1% bovine serum albumin in 0.2 M Tris–HCl, pH 7.5) and 0.5 mL of enzyme solution. This mixture was incubated at 37°. The enzyme reactions were stopped at the designated times by spotting 50 μL of the reaction mixture on a Whatman DE-81 paper disc; the disc was then dropped immediately into 95% alcohol (10 mL/disc) and washed twice with 95% alcohol for 5 min. The disc was then dried and placed in a 7.5-mL vial containing 6.5 mL of scintillation liquid (Econo-Safe, RPI, Mt. Prospect, IL). The radioactivity in each vial was quantitated by liquid scintillation spectrometry (LS 5801, Beckman, Irvine, CA). The blank value was obtained by conducting the assay with denatured enzyme solution which had been heated at 100° for 3 min.

To confirm product formation in the above assay, a reversed-phase HPLC method was employed. Reaction was stopped by adding an aliquot (300 μL) of the reaction mixture to a microcentrifuge tube which was then immersed in a boiling water bath for 3 min. After centrifugation (12,000 *g*, 5 min), the supernatant was filtered through a 0.2 μm Acro Filter (Gelman, Ann Arbor, MI) and then analyzed by reversed-phase HPLC [20, 21], using a Hewlett–Packard 1050 HPLC system equipped with a filter spectrometric detector and chromatographic terminal (HP 3396 Series II integrator). Two hypersil 5-μm columns (Jones Chromatography, Littleton, CO) were used in tandem as the stationary phase. The mobile phase contained 1.5 mM potassium phosphate (pH 8.5) and 5 mM tetrabutylammonium hydrogen sulfate. The flow rate was 1.0 mL/min. Fractions (1 mL) were collected into 7-mL scintillation vials, using a Redifrac fraction collector (Pharmacia LKB Biotechnology, Piscataway, NJ) and were mixed with 5.5 mL of scintillation solvent. The radioactivity in each fraction was quantitated by liquid scintillation spectrometry as described above. Under these conditions, typical retention times for authentic standards of TMP and thymidine were 7 and 35 min, respectively.

There was good agreement between the two methods used in determination of TK activity. HPLC analysis also indicated that no products other than TMP were detectable. As reported by Cheng [22], this reaction was linear for at least 4 hr, providing at least 10% thymidine was still present in the reaction mixture. The TK activity in the spleen was expressed as the amount of product (TMP) per hour per milligram of protein. Protein concentration was determined by the method of Lowry *et al.* [23].

Statistical analysis. Three animals were utilized for each time point. Data from each animal was the mean of five determinations. The time series data were analyzed by the "Cosinor" method [24]. A cosine wave was fitted to the data by the method of

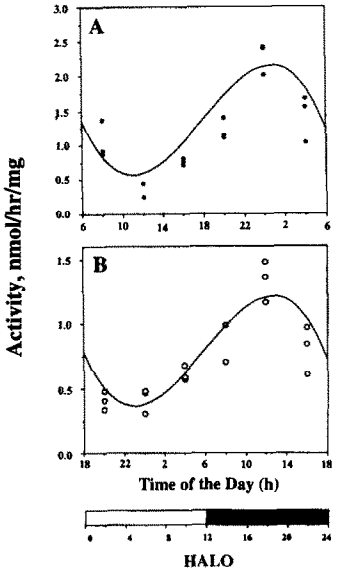

Fig. 1. Circadian variation of rat spleen cytoplasmic TK activity. (A) Data from studies in rats under "normal" light conditions—lights on from 6:00 a.m. to 6:00 p.m. (18:00) and lights off from 6:00 p.m. (18:00) to 6:00 a.m. (B) Data from studies in rats under "reverse" light conditions—lights on from 6:00 p.m. (18:00) to 6:00 a.m. and lights off from 6:00 a.m. to 6:00 p.m. (18:00). Each data point represents the TK activity measured in spleen homogenate of one rat and is the mean of five determinations. Three animals were used for each time point. The curves in both panels A and B were computer-generated by Cosinor analysis [24]. To emphasize the relationship between the circadian pattern of TK activity and the light–dark cycle, the data were plotted by setting the abscissa from 0–24 HALO in both panels A and B. The light bar indicates the period when lights were on (0–12 HALO); the dark bar indicates the period when lights were off (12–24 HALO).

Table 1. Rhythmometric summary of single Cosinor analysis of TK activity in rat spleen under normal and "reverse" light–dark conditions

Group	Mesor* ± SE	Amplitude ± SE	R^2	P
Normal cycle†	1.189 ± 0.105	0.787 ± 0.099	0.807	< 0.0001
Reverse cycle‡	0.729 ± 0.049	0.406 ± 0.060	0.752	< 0.0001

* Rhythm-adjusted mean.

† Normal cycle: lights on 6:00 a.m.–6:00 p.m. (18:00) and lights off 6:00 p.m. (18:00)–6:00 a.m.; N = 18.

‡ Reverse cycle: lights on 6:00 p.m. (18:00)–6:00 a.m. and lights off 6:00 a.m.–6:00 p.m. (18:00); N = 18.

Table 2. Values and times of maximum and minimum TK activity in rat spleen under normal and "reverse" light–dark conditions

Group	Maximum* ± SE	Minimum† ± SE	Maximum/minimum	T_{max}‡ ± SE	T_{min}‡ ± SE
Normal cycle	1.976 ± 0.144	0.401 ± 0.144	4.92	0.61 ± 0.14	12.69 ± 0.14
Reverse cycle	1.135 ± 0.077	0.323 ± 0.077	3.52	11.46 ± 0.05	23.54 ± 0.05

TK activities were determined in 18 animals in each group and expressed as nmol TMP/hr/mg protein.

* Maximum activity was calculated by addition of the amplitude to the mesor.

† Minimum activity was calculated by subtracting the amplitude from the mesor.

‡ Time of maximum activity (T_{max}) and time of minimum activity (T_{min}) were calculated from the acrophase of the Cosinor analysis [24].

least squares. Four parameters were calculated in the analysis, including the mesor (the rhythm adjusted mean), amplitude (maximum or minimum value from the adjusted mean), acrophase (time of maximum or minimum value) and period (the length of one complete cycle).

RESULTS

Normal cycle. In male, Sprague–Dawley rats maintained in standardized conditions of 12 hr light [6:00 a.m. to 6:00 p.m. (18:00)] alternating with 12 hr dark [6:00 p.m. (18:00) to 6:00 a.m.], there was a circadian pattern of TK activity observed in the cytoplasmic fraction of spleen homogenates obtained at specified times over 24 hr (Fig. 1A). Cosinor analysis demonstrated that the pattern of TK activity over 24 hr could easily be fitted to a cosine wave and was highly significant ($P < 0.0001$). These statistical measures in addition to the mesor and amplitude are shown in Table 1. From the fitted data, the peak (maximum) of TK activity (1.98 nmol product/hr/mg protein) was shown to be at approximately 1:00 a.m. (19 HALO, hours after light onset) and the trough (minimum) of TK activity (0.40 nmol product/hr/mg protein) at approximately 1:00 p.m. (13:00; 7 HALO). Maximum enzyme activity exceeded minimum activity by approximately 5-fold. The data on maximum and minimum activities and times are shown in Table 2.

Reverse cycle. In another group of rats maintained under "reverse" light–dark conditions of 12 hr dark [6:00 a.m. to 6:00 p.m. (18:00)] alternating with 12 hr of light [6:00 p.m. (18:00) to 6:00 a.m.], there was also a circadian pattern of TK activity observed (Fig. 1B). Cosinor analysis confirmed that the pattern of TK activity could be easily fitted to a cosine wave and was highly significant ($P < 0.0001$, Table 1). From the fitted data, as illustrated in Table 2, the peak (maximum) of TK activity (1.14 nmol product/hr/mg protein) was shown to be at approximately 12 noon (18 HALO) and the trough (minimum) of TK activity (0.32 nmol product/hr/mg protein) at approximately 12:00 a.m. (24:00; 6 HALO). Maximum enzyme activity exceeded minimum activity by approximately 4-fold.

DISCUSSION

In the present study, we have demonstrated for the first time that TK activity in rat spleen varies over 24 hr. Under a "normal" light–dark cycle, a circadian rhythm in TK activity was shown by Cosinor analysis (Fig. 1A) to be highly significant (Table 1), $P < 0.0001$) with maximum activity at 1:00 a.m. and minimum activity at 1:00 p.m. (13:00, Table 2). Maximum activity exceeded minimum activity by approximately 5-fold. Reversing the light–dark cycle resulted in a corresponding shift (clock hr) in TK activity. Under the "reverse" light–dark cycle, a circadian rhythm in TK activity was also shown by Cosinor analysis (Fig. 1B) to be highly significant (Table 1, $P < 0.0001$) with maximum activity at 12:00 noon and minimum activity at 12:00 a.m. (24:00, Table 2). Maximum activity exceeded minimum activity by approximately 4-fold.

Closer examination of the data demonstrates that TK activity was closely related to the light–dark

cycle. This is shown in Fig. 1 in which the time data on the abscissa of both Fig. 1A and Fig. 1B has been depicted as time in HALO (hours after light onset). As can be seen, the curves are very close with a 1-hr lag in the time of maximum (and minimum). The maximum and minimum values were lower for the "reverse" cycle than for the "normal" cycle with the magnitude of difference between maximum and minimum being less for the "reverse" cycle. These small differences between the two cycles were not statistically significant and may be due to incomplete adaptation of the rats during the 4 weeks on the "reverse" cycle.

The physiologic or biochemical mechanism(s) responsible for the circadian variation of TK and how it is related to the light–dark cycle were not determined in the present study. It is interesting to note that previous studies have shown that ACTH via corticosteroid can result in increased TK synthesis [25]. Since corticosteroids have been shown to have a role in many different circadian rhythms [2, 25], future studies will be needed to determine if this is the mechanism responsible for the circadian variation of TK.

Other enzymes in the pyrimidine pathway are known to follow a circadian pattern [1, 7–12]. Previous studies from our laboratory utilizing Sprague–Dawley rats housed under similar experimental conditions have demonstrated that DPD, known to be the critical rate-limiting enzymatic step in pyrimidine catabolism responsible for the catabolism of thymine, uracil and fluoropyrimidine drugs [26], also follows a circadian pattern [7, 8, 10]. Of particular interest is the inverse rhythmic pattern of DPD compared to TK. At the time TK is at its maximum activity (either "normal" or "reverse"), DPD is approaching its minimum activity. Similarly, at the time TK is at its minimum activity (either "normal" or "reverse"), DPD is approaching its maximum activity. Other studies from our laboratory utilizing Sprague–Dawley rats housed under similar experimental conditions have demonstrated that the amphibolic enzyme, thymidine phosphorylase, responsible for interconversion of thymine and thymidine, does not vary over 24 hr [10]. While there is a need for a more comprehensive analysis of other enzymes in the pyrimidine pathway, there is already a suggestion based on these observed inverse circadian patterns of DPD and TK that regulation of the circadian variation of critical catabolic and anabolic enzymes may be important in maintaining metabolic homeostasis. Thus, it is probably advantageous to decrease catabolic activities in order to increase anabolic activities most efficiently. Previous investigators have emphasized the importance of the balance between anabolism and catabolism in the nucleic acid pathways [27], but to our knowledge the role of circadian variation of critical enzymes in anabolism and catabolism in orchestrating overall pyrimidine metabolism has not been reported previously.

The importance of TK in pyrimidine nucleotide biosynthesis and DNA replication has been demonstrated in several studies [13, 14]. It has been shown previously that DNA synthesis in various tissues follows a circadian pattern [28–31]. In the present study, a circadian pattern of TK activity was also observed, suggesting that there may be a correlation between the circadian pattern of TK activity and DNA synthesis.

A circadian pattern of TK activity in spleen suggests that a similar rhythmic pattern of cytoplasmic TK may also exist in other tissues such as bone marrow and gastrointestinal mucosa. A circadian variation of TK in these tissues may affect the anabolism of pyrimidine nucleoside drugs and may explain the circadian variation of toxicities observed with time-modified FdUrd chemotherapy in humans [4–6, 16] and rats [32]. Future studies are needed to evaluate whether a circadian variation of TK exists in these tissues as well as in tumor tissue in order to further evaluate a possible rationale for time-modified chemotherapy.

In summary, to our knowledge, the present study is the first in which a circadian variation of TK activity was demonstrated and shown to be related to the light–dark cycle. Since TK is important in pyrimidine metabolism, DNA synthesis, and anabolism of pyrimidine analogues used in cancer and AIDS chemotherapy, its rhythmic variation may have therapeutic significance.

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