MOLYBDENUM HYDROXYLASE SUPER FAMILY SHOWS CIRCADIAN ACTIVITY FLUCTUATION IN MICE LIVER: EMPHASIS ON ALDEHYDE HYDROXYLASE AND XANTHINE OXIDASE

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ABSTRACT

Non-CYP oxidase enzymes are important system in biotransformation of drugs and environmental pollutants. Molybdenum containing oxidase enzymes such as aldehyde oxidase and xanthine oxidase are constitutive tissue enzymes that metabolize several drug moieties. Herein, we evaluated the circadian rhythm of these two enzymes in mice liver using different substrate/oxygen donor couples. Aldehyde oxidase showed typical rhythmic fluctuation with peak activity at night cycle and minimum activity at light cycle using pthalazine/ferricyanide and 3-methylisoquinoline/ferricyanide substrates. On the other hand, xanthine oxidase showed interrupted diurnal rhythm, however peak and minimum enzyme activities were similar to aldehyde oxidase circadian rhythm. In conclusion, diurnal rhythm of both molybdenum hydroxylase enzymes was confirmed and validated in mice liver tissue that might provide further insights in the experimental evaluation of phase-I pharmacokinetics for new drugs.

Keywords: Molybdenum hydroxylase, aldehyde oxidase, xanthine oxidase, diurnal rhythm.

INTRODUCTION

CYP450-mediated metabolic biotransformation is well known oxidative pathway for phase-I activation/ inactivation of xenobiotics (Strolin Benedetti *et al*., 2006). However, non-CYP-mediated oxidative pathways have been discovered and their exact mode of action has been recently elucidated (Hille, 2005; Hille *et al*., 1998). Molybdenum hydroxylase super family is important non-CYP oxidative enzyme family that was considered mainly for degradation of purine derivatives into uric acid catabolites (Beedham, 1985; Pritsos, 2000). Aldehyde oxidase and xanthine oxidase are major members of molybdenum hydroxylase super family with molybdenum co-factor that utilize water oxygen for oxidation rather than molecular oxygen species (Hille, 2005). Molybdenum containing oxidases are constitutive enzymes in a wide range of tissues that show less vulnerability to drug induction/depression compared to classical CYP oxidative enzyme family (Beedham *et al*., 1987a; Critchley *et al*., 1992). Yet, molybdenum hydroxylase enzymes are considered main constitutive arch in biotransformation (activation/inactivation) of several anticancer drugs and environmental pollutants (Rashidi *et al*., 2007; Strolin Benedetti *et al*., 2006; Ueda *et al*., 2005).

Despite inter-organ variability of molybdenum oxidases enzymes activity, interspecies variability and poor relevance to human data has been reported as well (Beedham *et al*., 1987b; Beedham *et al*., 1995). Guinea pig and rabbit are the most widely used animal species for studying the in-vivo activity of aldehyde oxidase and xanthine oxidase on newly synthesized drug moieties, such as, anticancer drugs (Beedham *et al*., 1995; Beedham *et al*., 1989) Mice, despite its facile experimental use in terms of animal care and breeding, are less frequently used for studying molybdenum containing enzymes. It is worth mentioning that, electron acceptor agent and enzyme substrate used in the oxido-reductive assays of molybdenum hydroxylase is significantly influenced by the enzyme substrate binding affinity and specificity (Beedham *et al*., 1987a; Beedham *et al*., 1995).

Biorhythmic diurnal cycle for wide range of hormone levels and enzyme activities has been deeply investigated in several experimental animal species (Brusco *et al*., 1998; Reppert *et al*., 1979; Rivest *et al*., 1989) The influence of circadian rhythm on metabolic pathways might improve studying the pharmacokinetic profile of newly discovered moieties. The aim of the current work is to study the circadian fluctuation in the activity of molybdenum hydroxylase enzymes, aldehyde oxidase and xanthine oxidase, in mice liver using different substrates and oxygen donors.

MATERIALS AND METHODS

Chemicals

Phenanthridine (PHE), pthalazine (PHT), and xanthine were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). 3-methylisoquinoline (MIQ) was gifted from ICN pharmaceuticals Inc. (Irvine, CA, USA). All other chemicals were of the highest available commercial grade.

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Animals

Adult male MFI mice weighing 25-35 g were obtained from King Fahad Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. Animals were acclimatized in groups (n=6) in our animal house facility for at least two weeks prior to experimentation. Animals were kept at controlled environment during the whole experiment (temperature: 17±1°C; relative humidity: 65±10%; and regular day/night cycles: 06:00-18:00 light; 18:00-06:00 dark). Standard food pellets and water were supplied *ad labium*.

Enzyme preparation

Hepatic molybdenum hydroxylase enzymes were partially purified as previously reported with minor modifications (Johnson *et al*., 1987). Briefly, at the specified circadian time points, animals were euthanized by cervical dislocation; liver tissues were immediately isolated, weighed and snap frozen at -80°C until extraction. Frozen tissues were let to thaw at room temperature and finely sliced in homogenization solution (potassium chloride solution (1:15 w/v) containing 10^{-4} M ETDA). Sliced liver tissues were adjusted to 25% tissue homogenate concentration and homogenized for 15 min in homogenization solution. Tissue homogenates were incubated at 50-55°C for 10 min and immediately cooled to 10°C by dipping in ice bath. Liver tissue homogenates were centrifuged at 15,000 *g* for 45 min at 4°C. The supernatant was filtered and saturated with ammonium sulfate powder (35.4% w/v final concentration) by continuous stirring in ice bath (4°C). Precipitated protein suspensions were collected by further centrifugation at 6,000g for 20 min at 4°C. Protein pellets were collected and rinsed twice with distilled water and finally dissolved in the minimum volume of 10^{-4} M EDTA solution. Partially purified enzymes were stored at -80°C to be assayed.

Enzyme assay

Enzyme activity of aldehyde oxidase was measured spectrophotometrically using three different substrate/oxygen donor couples as previously reported (Johnson *et al*., 1984). Briefly, partially purified enzymes were incubated with 1 mM pthalazine/potassium ferricyanide, 1mM 3-methylisoquinoline/potassium ferricyanide, or phenanthridine/O_2 for at least 2 min at 37°C. The color adducts of potassium ferricyanide and phenanthridine were measured at 420 and 322 nm, respectively.

The activity of xanthine oxidase was measured spectrophotometrically as well by incubation with 0.1 mM xanthine/O_2 for at least 2 min at 37°C. The color product was measured at 295 nm (Beedham *et al*., 1989).

The color stability of all color adducts was confirmed over the whole duration of measurement. Enzyme activities were normalized by protein concentration in each sample measured by Biuret method (Simonian and Smith, 2006).

Statistical analysis

Data are presented as means ± SD. Student's *t*-test was used as a test of significance using SPSS® for windows, version 13.0.0. $P \leq 0.05$ was taken as a cut off value for significance.

RESULTS AND DISCUSSION

Molybdenum hydroxylase enzymes are considered recently main arch in non-CYP oxidative machineries for biotransformation of drugs and environmental pollutants. Yet, molybdenum hydroxylase enzymes, aldehyde oxidase and xanthine oxidase have been studied in several experimental animals, however poorly studied in mice (Beedham *et al*., 1987a; Beedham *et al*., 1995). Herein, we studied the circadian rhythm of two major molybdenum containing oxidase enzymes, in mice liver using different substrate/oxygen donor couples.

Aldehyde oxidase assayed in liver partially purified samples using PHT/ferricyanide couple showed rhythmic fluctuating activity between 18.4-100% of the full enzyme activity as a function of time. Each peak enzyme activity was significantly different from the corresponding trough with 3 h fluctuation rhythm. Peak aldehyde oxidase enzyme activity was detected at time 03:00 h (night cycle) while minimum enzyme activity was observed at time 12:00 (day cycle) (fig. 1). The circadian rhythm of aldehyde oxidase enzyme in mice liver was confirmed with another substrate adduct (MIQ)/ferricyanide oxygen donor. Aldehyde oxidase showed rhythmic fluctuating activity between 26.9-100% of the full enzyme activity as a function of time. Enzyme activity maxima were significantly different from corresponding minima with 3 h fluctuation rhythm. Peak aldehyde oxidase enzyme activity was detected at time 03:00 h (night cycle) while minimum enzyme activity was observed at time 12:00 (day cycle) (fig. 2).

On the other hand, aldehyde oxidase activity showed different rhythmic profile when assayed using PHE/O_2 (substrate/donor) couple. Diurnal rhythm was also noticed, however peak enzyme activity was noticed at time 21:00 (night cycle) and minimum activity was noticed at time 24:00 (night cycle). Enzyme activity ranged from 12.8-100% of the full enzyme activity (fig. 3).

The difference in rhythmic profile of the same enzyme assayed by different methods might be attributed to the different isozymes of aldehyde oxidase enzyme with different substrate specificity. Two different isozymes of the molybdenum containing hydroxylase and aldehyde

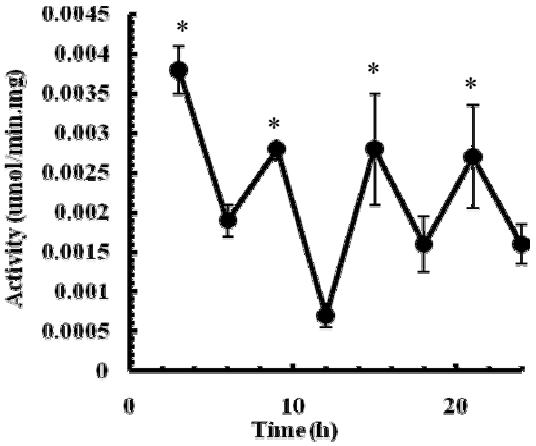

Fig. 1: Circadian rhythmic activity of aldehyde oxidase enzyme was assessed at physiological temperature (37°C) and expressed as µmolar PHT oxidized/min.mg protein. Potassium ferricyanide (1 mM) was used as an electron acceptor. Data are expressed as mean ± SD (n=6). Peaks were significantly different from corresponding troughs at $p<0.05$.

*: significantly different from corresponding trough.

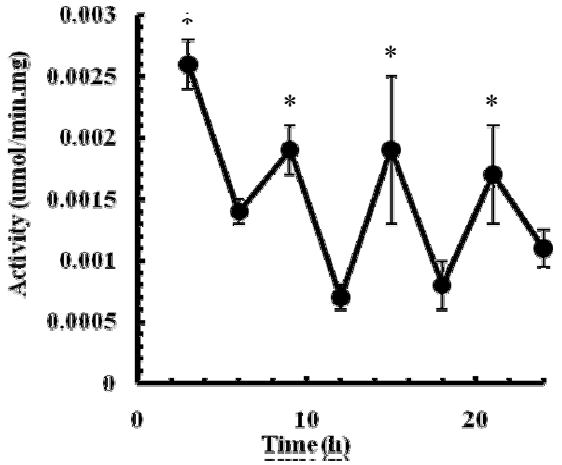

Fig. 2: Circadian rhythmic activity of aldehyde oxidase enzyme was assessed at physiological temperature (37°C) and expressed as µmolar MIQ oxidized/min.mg protein. Potassium ferricyanide (1 mM) was used as an electron acceptor. Data are expressed as mean ± SD (n=6). Peaks were significantly different from corresponding troughs at $p<0.05$.

*: significantly different from corresponding trough.

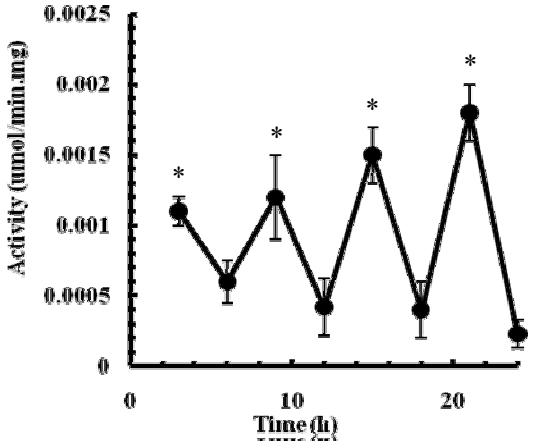

Fig. 3: Circadian rhythmic activity of aldehyde oxidase enzyme was assessed at physiological temperature (37°C) and expressed as µmolar PHE oxidized/min.mg protein. Oxygen was used as an electron acceptor. Data are expressed as mean ± SD (n=6). Peaks were significantly different from corresponding troughs at $p<0.05$.

*: significantly different from corresponding trough.

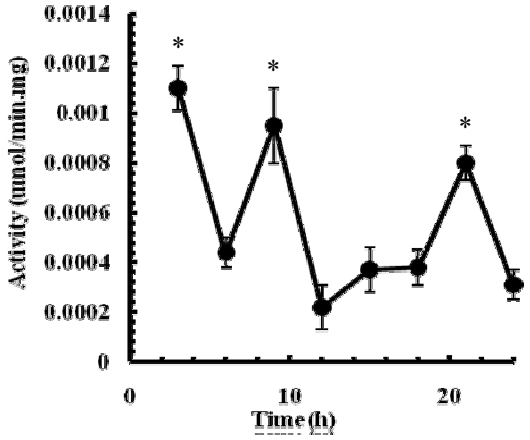

Fig. 4: Circadian rhythmic activity of xanthine oxidase enzyme was assessed at physiological temperature (37°C) and expressed as µmolar XAN oxidized/min.mg protein. Oxygen was used as an electron acceptor. Data are expressed as mean ± SD (n=6). Peaks were significantly different from corresponding troughs at $p<0.05$.

*: significantly different from corresponding trough.

oxidase have been identified electrphoretically in the mouse liver tissue (Holmes, 1978; Lush, 1978). Also, enzyme substrate specificity of molybdenum hydroxylase super family in general and aldehyde oxidase in particular has been an important issue in designing assay methods for these unique oxidase systems (Beedham *et al*., 1995). It is worth mentioning that in real biosystem, aldehyde oxidase enzyme does not utilize molecular O_2 for oxidation which might partially explain the ambiguity in assaying aldehyde oxidase using O_2 as oxygen donor in contrast to ferricyanide (Hille, 2005).

In contrast to aldehyde oxidase, the circadian rhythm of xanthine oxidase activity in mouse liver was interrupted from time 12:00 h to 18:00 h (day cycle) and systematic during night cycle (18:00 h-09:00 h). Xanthine oxidase showed fluctuating activity between 20-100% of the full enzyme activity as a function of time. Peak enzyme

activity was detected at time 03:00 h (night cycle) while minimum enzyme activity was observed at time 12:00 (day cycle) (fig. 4).

In the current work, two important non-CYP molybdenum containing hydroxylase enzymes have been investigated in a widely used laboratory animal (mouse), in contrast to previous studies in tissues of guinea pig, rat, baboon, dog, cows and other less experimentally impact animal, however similar circadian profile has been detected (Beedham *et al*., 1989; Johnson *et al*., 1984) Circadian biorhythm is important phenomenon that affects many hormone and enzyme systems such as cortisone, melatonin and pineal secretions (Attanasio *et al*., 1986; Rivest *et al*., 1989). Herein, we are evaluating the influence of circadian day/night rhythmic cycles on metabolizing enzyme system that might have potential importance in studying drug pharmacokinetic and activation/inactivation of environmental pollutants.

In conclusion, diurnal rhythm of molybdenum hydroxylase enzymes has been identified in mice liver tissue using mutual validation of different couples of substrate/oxygen donors in partially purified enzyme preparation. This work might carry further insights into detailed investigation of Phase-I pharmacokinetics and activation/inactivation of drugs and environmental pollutants.

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