The effect of experimental diabetes on the circadian pattern of adenosine deaminase and myeloperoxidase activities in rat liver

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Abstract. This study investigated time-dependent variations in the activities of adenosine deaminase (ADA), an adenosine-metabolizing enzyme, and myeloperoxidase (MPO), an oxidation reaction-catalyzing enzyme, in control and streptozotocin (STZ)-induced diabetic rat liver. The animals were sacrificed at six different times of day (1, 5, 9, 13, 17 and 21 hours after lights on – HALO). The hepatic activity of ADA did not change depending on the STZ treatment whereas MPO activity was significantly higher in the diabetics than in the controls. Hepatic ADA activity was dependent on the time of sacrifice with the lowest activity at 21 HALO and the highest activity at 5 HALO. Both enzyme activities failed to show any significant interaction between STZ treatment and time of sacrifice, which means that diabetes does not influence the 24 h pattern of these activities. Since MPO, a heme protein localized in the leukocytes, is involved in the killing of microorganisms, increased MPO activity in diabetic rat liver may reflect leukocyte infiltration secondary to diabetes. A reduction in ADA activity during the dark (activity/feeding) period will presumably lead to high concentrations of adenosine in the liver, possibly contributing to changes in some metabolic processes, such as glycogen turnover and oxygen supply.

Key words: Adenosine deaminase — Myeloperoxidase — Circadian — Rat — Liver

Introduction

Adenosine, a purine molecule, plays an important role in the regulation of many metabolic processes and acts as a signal for certain physiological events. Adenosine may act as a “local hormone” in mammalian tissues, showing effects on modulation of blood flow (Bouma et al. 1997), metabolism of adipocyte tissue (Pelleg and Porter 1990), inflammation (Sands and Palmer 2005; Akkari et al. 2006), neurotransmission and pain (Sawynok and Liu 2003), ischemia (Bouma et al. 1997), sleep-wake cycle (Chagoya de Sanchez et al. 1993) and glycogen turnover in the liver (Pelleg and Porter 1990). Moreover, adenosine can modulate hormone release, including insulin (Feldman and Jackson 1974), glucagon (Weir et al. 1975), and corticosterone (Formento et al. 1975), and the synthesis of steroids (Wolf and Cook 1977). Adenosine has been reported to be a potent antagonist of insulin action in various tissues (McLane et al. 1990). It is well known that the sensitivity of several tissues to adenosine, including the kidney (Pflueger et al. 1995), heart (Li 1992), and brain (Morrison et al. 1992), increases as a result of insulin-dependent diabetes.

Adenosine deaminase (ADA), a polymorphic enzyme, irreversibly deaminates adenosine to inosine, contributing to the regulation of intracellular and extracellular concentrations of adenosine. Diabetes mellitus may affect the metabolism of adenosine by changing the activities of the principal enzymes involved in nucleoside production and degradation (Rutkiewicz and Gorski 1990; Pawelczyk et al. 2000; Karasu et al. 2006).

It has been postulated that the physiological meaning of the circadian variations in adenosine and its metabolism is related to maintenance of the energetic homoeostasis of the tissues, regulation of feeding and fasting metabolic pattern, modulation of membrane structure and function, sleep-wake
cycle, and modulation of the hormone action (Chagoya de
Sanchez 1995). For this reason, these authors have suggested
that adenosine could be a molecular oscillator involved in
the circadian pattern of biological activity in the rat. They
have also shown that the concentrations of adenosine and
adenosine-metabolizing enzymes such as ADA in rat blood
and liver are modified at certain times of day (Chagoya de
Sanchez et al. 1983; Chagoya de Sanchez et al. 1991).

Myeloperoxidase (MPO), an iron-containing heme protein
that accounts for 5% of the total neutrophil protein localized
in the azurophilic granules of neutrophil granulocytes and in
the lysosomes of monocytes, is involved in the killing of several
microorganisms and foreign cells. The enzyme is released by
degranulation of activated leukocytes. Phagocyte activation
and MPO secretion are accompanied by an oxidative burst
where superoxide (O_{2}^{•–}) and its dismutation product, hyd-
rogen peroxide (H_{2}O_{2}), are formed by the NADPH oxidase
complex. The presence of increased reactive oxygen species
such as H_{2}O_{2} in diabetic vasculature is well documented, and
the activation, adhesion, and infiltration of leukocytes into
the vessel wall is a critical component in the development of
diabetic complications (Zhang et al. 2004). Circadian varia-
tions in MPO activity and free radical concentrations might
accompany some other time-dependent events, such as lipid
peroxidation which plays a role in the occurrence of diabetic
complications. In addition, possible circadian changes in MPO
activity, an index of tissue neutrophil infiltration, may reflect
variations in the susceptibility to infection in both control and
diabetic rats during the daily rhythm.

Streptozotocin (STZ) is an antibiotic extracted from the
bacterium *Streptomyces achromogenes*. It is widely used to
induce experimental diabetes in rodents (e.g. Karasu et al.
1995). Rakieten et al. (1963) were the first to report that STZ,
when given intravenously, caused diabetes mellitus in rats
and dogs. STZ treatment has been shown to result in changes
in various hepatic enzyme systems and in hepatic function
(Watkins and Sanders 1995). Increased oxidative stress has
been suggested to be a contributory factor in development and
complications of STZ-induced diabetes (Ceriello 2003).
Although hepatic function has been found to be altered in di-
betes, no information exists on the circadian activity pattern
of ADA and MPO enzymes in diabetic conditions. Therefore, it
is of interest to investigate the role of rhythmicity in a disease
model associated with oxidative stress-mediated pathologies
such as STZ diabetes mellitus. Circadian rhythms in normal
physiological functions and in the occurrence of several un-
favorable cardiovascular events are well established (Touti-
tou and Haus 1994). Furthermore, endogenous circadian and
exogenously driven daily rhythms of antioxidative enzyme
activities have been described in various organisms (Harde-
land et al. 2003). Previously, we revealed that the activities of
NADPH-generating enzymes (6-phosphogluconate dehy-
drogenase and glucose-6-phosphate dehydrogenase) exhibit
24 h variation in the rat liver and the STZ diabetes model
used does not modify the temporal pattern of these enzymes
(Ulus et al. 2005). Additionally, knowledge of chronobiologic
variations in ADA and MPO activities in diabetic conditions
can add new information regarding oxidative systems and the
replenishment of the adenosine pool in the liver through the
24 h cycle and can lead to a better understanding of diabetes
mellitus. In the present study, time-dependent variations in
the activities of ADA and MPO in isolated rat liver obtained
from control and diabetic animals were investigated. Our
experiments focused on the question of whether circadian
regulation of ADA and MPO enzyme activities in rat liver is
influenced by experimental diabetes.

Materials and Methods

Experimental protocol

The experiments were performed on male Wistar rats, weigh-
ing 250–300 g. They were fed a standard rat chow diet, had
access to water *ad libitum*, and were housed in controlled
environmental conditions (light, temperature, feeding time,
etc.). The animals were synchronized to a light-dark cycle
(lights on from 08:00 h to 20:00 h) beginning at least two
weeks before the commencement of the experiments. The rats
were randomly divided into two groups: control and diabetic
animals. Diabetes was induced by a single intraperitoneal
injection of STZ (55 mg/kg) to animals fasted overnight.
Diabetes was verified 48 h later by measuring the tail vein
blood glucose concentration. The rats with blood glucose
concentration of 13.8 mmol/l or higher were considered dia-
betic. Glycemia was measured again in the days just before the
animals were sacrificed. Blood glucose concentrations were
measured by an Accutrend® GCT meter (Roche Diagnosis,
Mannheim, Germany). For a span of 8 weeks after the STZ
injection, subgroups of animals were anesthetized with sodium
pentobarbital (30 mg/kg) and sacrificed at one of six different
times during the 24 h: 1, 5, 9, 13, 17, or 21 hours after lights
on (HALO) and their livers were removed. All experiments
were performed during January to March to avoid the pos-
sibility of seasonal rhythms affecting the findings. The study
was performed in compliance with the ethical standards and
principles of chronobiological research (Touitou et al. 2006)

Biochemical analyses

Determination of liver ADA activity

Liver tissues were prepared as described by Adams and Hark-
ness (1976). Shortly, 10% tissue homogenates in a 0.1 mol/l
phosphate buffer (pH 7.0) were prepared. Homogenates were
centrifuged at 20,000 × g for 30 min at 2–3°C and supernatants
were separated. Protein concentrations in the supernatants were determined using a Lowry assay (Lowry et al. 1951). Concentrations were calculated from a standard curve constructed using bovine serum albumin and expressed as mg/ml.

ADA activity in the supernatant was determined spectrophotometrically as described by Goldberg (1965). The optical density of a solution containing 20 mg of adenosine per ml of 0.1 mol/l phosphate buffer (pH 7.0) was read at 265 nm in a 1 cm light path immediately after addition of supernatant (0.05 ml / 3 ml buffer – substrate mixture) and again after incubation at 37°C for 1 h, the spectrophotometer being set at zero, using 0.05 ml of supernatant in 3 ml of 0.1 mol/l phosphate buffer (pH 7.0) as a blank which was incubated in parallel with the test solution. The units given herein represent the fall in optical density under the above conditions, and liver ADA activity was calculated as nmol/min/mg protein.

Determination of liver MPO activity

Liver MPO activity was determined as described by Schierwagen et al. (1990). Liver tissue was homogenized in 20 mmol/l potassium phosphate buffer (pH 7.4) and centrifuged for 5 min at 10,000 × g at 4°C. The supernatant was discarded, and the pellet was resuspended in 50 mmol/l potassium phosphate buffer (pH 6.0), containing 0.5% hexadecyltrimethylammonium bromide. The suspension was frozen and, it was then sonicated once only for 10 s, incubated for 2 h in a water bath at 60°C, and then centrifuged at 10,000 × g for 5 min. The supernatants were used for the MPO assay.

MPO activity was assessed by measuring H$_2$O$_2$-dependent oxidation of O-dianisidine. One unit of enzyme activity was defined as the amount of MPO in tissue that caused a change in absorbance of 1/min at 410 nm and 37°C (Glowickj and Kaplan 1955).

Chemicals

All the chemicals were analytical grade and obtained from Sigma (USA).

Statistical analyses

Data are presented as means ± SEM. Differences due to biological time-dependencies in the control and diabetic groups were assessed by one-way analyses of variance (ANOVA) followed by a post-hoc Dunn test. Treatment, circadian time, and interaction between treatment and circadian time were tested by two-way ANOVA followed by post-hoc Tukey tests. p < 0.05 was considered statistically significant.

Results

Hepatic ADA and MPO enzyme activities failed to show any significant interaction between STZ treatment and the time of sacrifice (Table 1, p > 0.05). The lack of interaction between time and treatment observed in both ADA and MPO enzyme activities by two-way ANOVA means that diabetes does not influence the 24 h pattern of enzyme activity, as shown in Figures 1 and 2.
When we evaluated the overall data of the two experimental groups (control and diabetes), two-way ANOVA revealed that hepatic ADA activity was dependent on the time of sacrifice (the lowest activity at 21 HALO and the highest activity at 5 HALO, $p < 0.05$) but not STZ treatment (Table 1). Hepatic ADA activity was $6.74 \pm 0.2$ and $6.34 \pm 0.2$ nmol/min/mg protein which are the means of six time points (1, 5, 9, 13, 17 and 21 HALO) in control and diabetic animals, respectively (data not shown). The difference between these two values is not significant.

No significant circadian rhythm in the activity of ADA was seen in either control or diabetic livers (Table 1).

**ADA activity**

When we evaluated the overall data of the two experimental groups (control and diabetes), two-way ANOVA revealed that hepatic ADA activity was dependent on the time of sacrifice (the lowest activity at 21 HALO and the highest activity at 5 HALO, $p < 0.05$) but not STZ treatment (Table 1). Hepatic ADA activity was $6.74 \pm 0.2$ and $6.34 \pm 0.2$ nmol/min/mg protein which are the means of six time points (1, 5, 9, 13, 17 and 21 HALO) in control and diabetic animals, respectively (data not shown). The difference between these two values is not significant.

No significant circadian rhythm in the activity of ADA was seen in either control or diabetic livers (Table 1).

**MPO activity**

Hepatic MPO activity was dependent on the treatment but not on the time of sacrifice (Table 1, two-way ANOVA). Activity of MPO was significantly higher in the diabetics than in the controls ($772 \pm 29$ and $382 \pm 27$ µmol/min/g tissue, respectively, $p < 0.001$, Figure 2).

Similar to the ADA results, no significant dependence on the circadian rhythm was observed in MPO activity in control or diabetic livers (Table 1).

**Discussion**

The effect of diabetes experimentally induced by STZ treatment on the 24 h pattern in hepatic ADA and MPO activities has to the best of our knowledge not been previously assessed. The present results give the first evidence that experimental diabetes does not change the 24 h pattern of these enzymes.

**ADA activity**

In our study, the hepatic activity of ADA did not vary depending on the STZ treatment, whereas the activity of MPO was significantly higher in the diabetics than in the controls. In parallel with our results, Pawelczyk and colleagues (2000) showed that the cytosolic activity of ADA was unchanged in the liver of diabetic rats as compared to normal rats. Singh and Sharma (1998) also reported that alloxan-induced diabetes increases ADA activity in the liver in 15-day-old mice, while it has no significant effect in 60-day-old animals. A similar result was observed in intestinal ADA activity. In contrast, ADA activity was moderately increased in the spleen of animals of both ages, however, no significant influence of alloxan was observed on ADA activity in the stomach independent of the age of the mice. The authors concluded that alloxan-induced diabetes increased ADA activity in an age- and tissue-specific manner. We observed that ADA activity did not significantly vary in the liver of adult (2.5–3 months) diabetic male rats and thus our findings are in accordance with those of previous studies. In contrast, Rutkiewicz and Gorski (1990) postulated that acute STZ diabetes showed significant elevations in liver ADA activity, but their study is not comparable with ours due to the different STZ treatment method of the rats, which were sacrificed 3 h after intravenous administration of STZ.

In the present study, we found that hepatic ADA activity was dependent on the time of sacrifice. Many early previous

**Table 1.** Diurnal variations in adenosine deaminase (ADA) and myeloperoxidase (MPO) activity in the rat liver obtained from control and diabetic animals at six different times of day

<table>
<thead>
<tr>
<th>HALO</th>
<th>ADA (nmol/min/mg protein)</th>
<th>MPO (µmol/min/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetes</td>
</tr>
<tr>
<td>1</td>
<td>6.2 ± 0.2</td>
<td>6.7 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>7.98 ± 0.7</td>
<td>6.6 ± 0.3</td>
</tr>
<tr>
<td>9</td>
<td>6.6 ± 0.6</td>
<td>6.7 ± 0.4</td>
</tr>
<tr>
<td>13</td>
<td>7.2 ± 0.9</td>
<td>7.1 ± 0.6</td>
</tr>
<tr>
<td>17</td>
<td>6.6 ± 0.3</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>21</td>
<td>5.9 ± 0.2</td>
<td>5.5 ± 0.2</td>
</tr>
</tbody>
</table>

One-way ANOVA | n.s. | n.s. | n.s. | n.s.

Two-way ANOVA

<table>
<thead>
<tr>
<th>time</th>
<th>treatment</th>
<th>interaction</th>
<th>ADA</th>
<th>MPO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$* p &lt; 0.05$</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM ($n = 4–5$). HALO, hours after lights on; ANOVA, analysis of variance; n.s., not significant; $* p < 0.05$ (two-way ANOVA, control versus diabetes in ADA activity); $** p < 0.001$ (two-way ANOVA, control versus diabetes in MPO activity).
Circadian pattern of hepatic ADA and MPO activities in experimental diabetes

studies showed that the concentration of adenosine and its catabolites (inosine, hypoxanthine, and uric acid), adenosine synthesis (S-adenosylhomocysteine hydrolase and S'-nucleotidase), degrading ADA and nucleotide-forming (adenosine kinase) enzymes as well as adenine nucleotides (AMP, ADP, and ATP) undergo statistically significant fluctuations over 24 h in human blood (Chagoya de Sanchez et al. 1996) and in rat blood and liver (Chagoya de Sanchez et al. 1983, 1991). Chagoya de Sanchez and colleagues (1983) found that the concentration of adenosine in the liver was high during the night. Although we did not measure adenosine levels in the liver, these results agree with ours because as soon as there is a decrease in purine catabolism we can expect that adenosine is accumulated. Thus, we found that hepatic ADA activity was low during the night.

The significance of the circadian variations in ADA activity in the rat liver is not well understood. Since adenosine has many biological effects (Peleg and Porter 1990), such as suppression of the heart pacemaker activity, modulation of renal and liver blood flow, coronary vasodilation, bronchoconstriction, stimulation of glycogenolysis in the liver, inhibition of lipolysis in adipocyte, and tissue protection in hypoxic and/or inflamed environments (Linden 2005), we suggest that the circadian variations in ADA activity in rat liver could participate in some physiological and/or pathologic processes in which adenosine is involved. The lowest activity of ADA in the liver during activity/feeding period in rats (21 HALO) may be related to: i) the replenishment of the adenosine pool that contributes to glycogen turnover, ii) tissue protection and repair through increasing oxygen supply and activation of cellular antioxidant enzymes. On the other hand, adenosine acts directly to stimulate insulin activity via several processes including glucose transport, lipid synthesis, pyruvate dehydrogenase activity and cyclic nucleotide phosphodiesterase activity (McLane et al. 1990; Rutkiewicz and Gorski 1990). For example, fat cells release adenosine spontaneously and adenosine is thought to sensitize the cell to insulin action (Joost and Steinfeld 1982). Glucose transport stimulation is also modulated by adenosine in the adipocytes (Joost and Steinfeld 1982). For these reasons, it is thought that adenosine and ADA play an important role in the modulation of insulin action on glucose metabolism in various tissues.

In the present study, since we demonstrated that there is a basic 24 h pattern in the hepatic ADA activity in rats that still exists in diabetes, we can postulate that there might be similar circadian patterns in some metabolic processes in which adenosine is involved in both control and diabetic rats.

MPO activity

MPO activity is often elevated in patients during inflammatory conditions in plasma (Biasucci et al. 1996) or in the vitreous humor of patients suffering from diabetic retinopathy (Augustin et al. 1993). Similarly, hepatic MPO activity is also found to be higher in experimental inflammation models (Sener et al. 2006), in ischemia/reperfusion-induced oxidative stress models (Kacmaz et al. 2005), and in STZ-induced diabetic rat tissues (Kamalakkannan and Stanely Mainzen Prince 2006). In the present study, the activity of MPO in the rat liver was significantly higher in the diabetics than in the controls. This is in accordance with the results reported by Bruce et al. (2003), who demonstrated that plasma MPO content was higher in diabetic patients compared with control subjects. On the other hand, Uchimura et al. (1999) found that MPO activity was significantly reduced in leukocytes from patients with diabetes mellitus and they proposed that changes in MPO activity may affect the susceptibility to infection and immunocompetence of patients with diabetes.

Recent advances in chronopharmacology demonstrate that the toxicity of many chemical and non-chemical agents varies considerably according to the biological time of their administration (Lemmer 1989). Moreover, many reports reveal that all major antioxidative enzymes are 24 h rhythmic in several animals and tissues, such as superoxide dismutase in the mouse liver (Hodoglugil et al. 1995), hemoperoxidase/catalase in the rat liver (Kampschmidt and Upchuch 1970), glutathione peroxidase in the rat brain (Barlow-Walden et al. 1995) and the mouse liver (Davies et al. 1983), and glutathione reductase in the mouse liver (North et al. 1981). Investigations carried out in the last 30 years have indicated clearly that the activities of hepatic enzymes involved in the metabolism of drugs and other xenobiotics are not constant over a 24 h period (Tunon et al. 1992).

Rhythmic changes in oxidative damage of protein and lipid molecules have also been reported (Belanger et al. 1991). Chronobiological variations in hepatic defense systems against oxidative processes have important implications for agents, whose hepatodamaging effects are thought to be dependent on the production of reactive intermediates during their metabolism. Thus the susceptibility to toxic effects of oxidative processes can vary at different times of day and night. Thus, circadian variations in MPO activity can modulate susceptibility to diseases and xenobiotics. Kitoh et al. (2005) demonstrated that both the number of polymorphonuclear neutrophils (PMN) in the liver and hepatic MPO concentration, which reflects the number of PMN in the liver, were significantly greater after lipopolysaccharide (LPS) dosing at 14 HALO than at 2 HALO. They suggested that the LPS-induced accumulation of PMN in liver tissues is dependent on the circadian time of treatment, which partially contributes to the dosing time-dependent differences in the severity of liver injury. In our study, activity of MPO in the liver was significantly higher in the diabetic rats compared to the controls, however, we did not observe a time-dependent variation in MPO activity over 24 h in
control or STZ-treated diabetic rat livers. On the other hand, consistent with our ADA results, since the interaction between treatment and time of sacrifice was not significant for MPO activity, we can speculate that the temporal pattern of neutrophil infiltration to the rat liver was not influenced by STZ-induced diabetes.

In summary, hepatic ADA activity in experimental diabetic rats did not differ from that in control rats, whereas MPO activity was significantly higher in the diabetic animals. Since MPO, a heme protein localized in the leukocytes, is involved in the killing of several microorganisms, increased MPO activity in diabetic rat liver may reflect leukocyte infiltration secondary to diabetes. While hepatic MPO activity was not dependent on the time of sacrifice, ADA activity was lowest during the dark period and highest during the light period. A reduction in ADA activity during the dark period (activity/feeding period in rats) will presumably lead to high concentrations of adenosine in the liver, which may contribute to increases in liver blood flow, oxygen supply and glycogen turnover. Furthermore, STZ-induced diabetes does not play any role in the 24 h pattern of hepatic ADA or MPO activities.

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