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Short communication

Feeble awake effects of plasminogen activator inhibitor type-1 in mice

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ABSTRACT

Plasminogen activator inhibitor-type 1 (PAI-1) is involved in the fibrinolytic system and shows its increased levels in diseases, e.g., obesity and sleep apnea syndrome. The aim of the study is to investigate whether PAI-1 affects sleep–wake patterns in mice. When recombinant mouse PAI-1 was administered intraperitoneally, only rapid but short increases in time spent awake were observed after 20 or 100 μg/kg, although its plasma concentration was kept high for an hour. The results suggest that PAI-1 may serve its role rather as a marker than an initiator of disturbed sleep.

Plasminogen activator inhibitor-type 1 (PAI-1) is a primary physiological inhibitor of tissue-type plasminogen activator (t-PA) that degrades fibrin *via* activation of plasminogen to plasmin, and consequently reduces fibrinolytic actions [1]. Therefore, the elevation of plasma PAI-1 concentration is associated with a variety of thrombotic conditions, such as myocardial infarctions and deep venous thrombosis [2]. Serious adverse cardiovascular events cause sudden cardiac death, and its incidence rate reaches a peak during the morning hours; the frequency of such cardiac failures is 1.5–3 times higher in the morning hours than that during the other time period [3,4]. One of the possible reasons why cardiac death often happens during the morning hours could be an involvement of elevated PAI-1, because plasma PAI-1 concentration shows typical diurnal variations with a peak during the early morning in humans (at the beginning of the dark period in nocturnally active rodents) [3,5–7].

In the last decade, further physiological roles of the t-PA and PAI-1 pathway in the regulation of fibrinolysis have been speculated. The gene expression of PAI-1 is increased in aging, particularly in association with obesity, diabetes, immune activation, sleep apnea syndrome, and even emotional or psychosocial stress [8–11]. In the case of stress-induced PAI-1, its elevated expression was also demonstrated in several animal studies [12–14], proposing that PAI-1 might serve as a potential stress marker. Indeed, the incident ratio of myocardial infarction and thrombosis is higher in the elderly [15], therefore elevated PAI-1 by mental stressors possibly increases a risk for those diseases. Furthermore, it is well documented that stress often disturbs sleep. In patients with stress-related disorders, e.g., depression, impaired sleep is a hallmark symptom [16]. However, the possibility whether a stress marker, PAI-1, could affect sleep has been fully unclear so far.

In this study, we investigated effects of PAI-1 administration on sleep–wake patterns in mice. First, we clarified time-dependent changes in plasma PAI-1 concentration after intraperitoneal administration of recombinant mouse PAI-1. In the following, we prepared mice for electroencephalogram (EEG) and electromyogram (EMG) recordings and differences in their sleep–wake patterns were compared before and after PAI-1 treatment.

Male C57BL/6J mice (6 weeks old, Japan SLC, Shizuoka) were housed in groups of five per cage with free access to tap water and certified diet for mice (MF) from Oriental Yeast Co., Ltd. (Tokyo, Japan). Each cage was kept in the individual animal breeding system for mice (LP-30CCFL-8ARS, Nippon Medical & Chemical Instruments, Osaka, Japan). The environment of the individual chambers was regulated at 23 ± 1 °C and a 12 h light/12 h dark cycle (light on at 11:00 h; Zeitgeber time (ZT) 0). A cold cathode fluorescent lamp (4.2 W) was used as an illumination source in the chamber with

Abbreviations: NREM, non-rapid eye movement; PAI-1, plasminogen activator inhibitor-type 1; REM, rapid eye movement; ZT, Zeitgeber time; t-PA, tissue-type plasminogen activator.

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an intensity of 250 lx. After acclimatization for 4 weeks, recombinant mouse PAI-1 (American Diagnostic Inc., Stanford, CT, USA) was intraperitoneally injected at a dose of 20 μg/kg body weight in the mice at ZT 6. Blood samples were individually collected from the abdominal vein under ether anesthesia shortly before (0 min), and 10, 30, 60 or 120 min after PAI-1 injection, and were immediately treated with 0.1 M citric acid (100 μL/mL of collected blood). The same volume of physiological saline (10 mL/kg body weight) was injected as vehicle solvent. Plasma was separated by centrifugation at 1200 × *g* for 10 min, and maintained at a temperature under −80 °C until PAI-1 analysis. All experimental procedures were in accordance with the guideline of the University of Shizuoka, Japan, for the Care and Use of the Laboratory Animals, based on those of the American Association for Laboratory Animal Science.

Plasma PAI-1 concentrations were determined in duplicate using a Murine PAI-1 Total Antigen Assay kit (Molecular Innovations, Novi, MI, USA) according to the manufacturer's protocol.

Male C57BL/6J mice (Harlan Winkelmann GmbH, Borchen, Germany) were group housed before surgery with free access to tap water and certified diet. Animals, weighing 23-27 g (10–12 weeks old) were used for sleep-recording experiments. After surgery, animals were individually housed in a sound-attenuated recording chamber maintained at a regulated ambient temperature of 23 ± 1 °C on a 12 h light/12 h dark cycle (light on at 5:00 h; ZT 0). All studies regarding sleep recordings were conducted according to the guidance of the European Community Council Directive, and experimental protocols were approved by the local commission for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Details of surgical procedure and vigilance classification were described in our previous reports [17,18]. Briefly, the mice were anesthetized with an intraperitoneal injection of a ketamine–xylazine mixture (115 mg and 11.5 mg/kg, respectively) and chronically implanted with EEG and EMG electrodes for polysomnographic recordings. The implant consisted of six gold-wire electrodes soldered to an 8-pin connector; 4 electrodes were placed on the dura for EEG recordings and 2 electrodes were inserted into neck muscles for EMG recordings. After 2 weeks of recovery in the environment mentioned above, the lead wires of the EEG and EMG electrodes were connected to an electric swivel through a flexible tether. The weights of the swivel and tether were outbalanced through a mechanical device, therefore the mice could move unrestrictedly. EEG and EMG signals were amplified (10,000×), filtered (EEG 0.5–29 Hz, 48 dB per Oct.; EMG underwent root mean square rectification), and digitized by a high-speed analog-to-digital converter at a sampling rate of 64 Hz. Then, the signals were processed by a PC equipped with a LabVIEW program, especially designed for sleep EEG analysis (National Instruments, SEA, Köln, Germany). All stored polygraphic data were processed after the recording with the LabVIEW-based acquisition program. Using the aid of a Fast Fourier Transform algorithm, vigilance states were classified semi-automatically as wake, rapid eye movement (REM) sleep and non-REM (NREM) sleep in 4-s epochs. Defined vigilance states were further confirmed visually and corrected if necessary.

After recovery from surgery baseline recordings were performed for 24 h from the beginning of the light period. Then, individual amounts of recombinant mouse PAI-1 (2, 20 or 100 μg/kg body weight, respectively) were intraperitoneally injected into the mice at ZT 6 while EEG and EMG were continuously monitored. For vehicle control, the same volume of physiological saline solution (10 mL/kg) was intraperitoneally injected. On the following day, PAI-1 was tested first at 20 μg/kg (*n* = 8), and then the same animals were used for a further treatment at either 2 or 100 μg/kg (*n* = 4 each) with an interval of 10 days after vehicle injection. The recordings continued until a withdrawal day after each PAI-1 treatment.

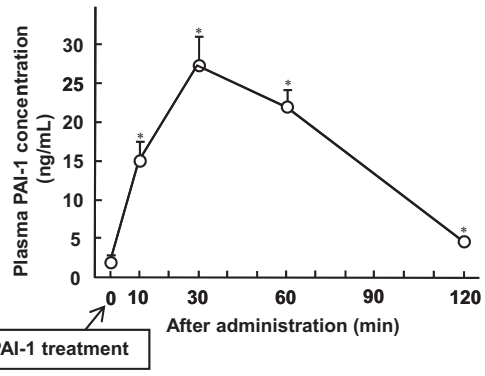

Fig. 1. Time-dependent changes of plasma PAI-1 concentrations. Recombinant mouse PAI-1 was administered intraperitoneally at a single dose of 20 μg per kg body weight. Plasma samples were collected at the given time points from different animals (*n* = 3), and PAI-1 concentrations were determined by ELISA. Values are indicated as means ± SEM. **P* < 0.05 *vs* the group before administration (0 min).

Time spent in WAKE, NREMS and REMS was calculated in consecutive 10-min intervals, and effects of different doses and time intervals on these vigilance states were statistically evaluated for significance in each time period and each mouse group by both one-way and two-factorial analyses of variances (ANOVAs) with a repeated measures design using the software program Stat View for Windows (Version 5.0, SAS Institute, Cary, NC, USA). The 2 influential factors were treatment and time; within-subjects factors were 5 levels (baseline, saline, 2, 20, and 100 μg PAI-1) and 13 levels (10-min intervals after PAI-1 administration for 2 h), respectively. When ANOVA showed statistical significance in main or interaction effects, a post hoc test for simple effects (Dunnett's test) was applied in order to locate significant differences among the levels of the within- or between-subjects factors, respectively. For the analysis of PAI-1 concentration in blood, Student's *t*-test was employed. The results were considered significant if the possibility of error was less than 5%.

PAI-1 concentration in blood is known to demonstrate a typical diurnal variation, which in mice peaks at the beginning of the dark period [3,5,6]. In this study, we tested PAI-1 in mice at ZT 6, the time point in the middle of their inactive phase, when plasma PAI-1 concentration is supposed to be at nadir [3,6]. The level of endogenous plasma PAI-1, obtained shortly before administration (ZT 6), was 1.4 ± 0.26 ng/mL (Fig. 1), indicating an almost similar level to those reported by other researchers earlier [3,6]. After injecting PAI-1 intraperitoneally (20 μg/kg body weight) into mice, plasma concentrations immediately increased to 14.8 ± 2.6 ng/mL within 10 min, and reached a maximum of 27.2 ± 3.8 ng/mL after 30 min of administration. These elevated levels were, to some extent, comparable to those found in several disease models such as obesity in mice ranging between 4 and 14 ng/mL [19–21]. Later, plasma PAI-1 concentration sharply decreased and returned to almost basal levels by 2-h postinjection time. This result demonstrated that PAI-1 injected intraperitoneally circulates in the blood stream, but disappears within 2-h postinjection time.

Fig. 2 indicates the corresponding hypnograms of four individual mice (1–4) under basal condition (A), on the day when vehicle (saline) was intraperitoneally injected at ZT 6 (B), and on another day when 20 μg/kg of PAI-1 was injected at ZT 6 (C), respectively. Compared with baseline and saline control days, wakefulness was clearly induced after PAI-1 administration, indicating that PAI-1 might promote waking. However, this effect did not last long, although mice number 2 and 4 tended to be awake again towards the end of the light period. Thereupon, we focused on analyzing short-term effects of PAI-1 during only 2 h after administration. Sleep–wake patterns for intervals of 10 min were plotted in Fig. 3