Chronic sleep deprivation markedly reduces coagulation factor VII expression

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ABSTRACT

Chronic sleep loss, a common feature of human life in industrialized countries, is associated to cardiovascular disorders. Variations in functional parameters of coagulation might contribute to explain this relationship. By exploiting the mouse model and a specifically designed protocol, we demonstrated that seven days of partial sleep deprivation significantly decreases (-30.5%) the thrombin generation potential in plasma evaluated upon extrinsic (TF/FVIIa pathway) but not intrinsic activation of coagulation. This variation was consistent with a decrease (-49.8%) in the plasma activity levels of factor VII (FVII), the crucial physiologicalal trigger of coagulation, which was even more pronounced at the liver mRNA level (-85.7%). The recovery in normal sleep conditions for three days completely restored thrombin generation and FVII activity in plasma.

For the first time, we demonstrate that chronic sleep deprivation on its own reduces, in a reversible manner, the FVII expression levels, thus influencing the TF/FVIIa activation pathway efficiency.

Key words: Hodgkin, transplantation, refractory.

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Introduction

Chronic sleep debt affects millions of people in more developed countries and it is emerging as a co-factor in the development of metabolic and endocrine dysfunctions, as well as of cardiovascular and cerebrovascular pathologies.[1-5] One of the well-characterized causes of chronic partial sleep deprivation (PSD), sleep apnea, has been clearly associated with cardiovascular disease.[6-8]

Among many factors, variations in the plasma levels of key proteins able to shift the hemostatic balance might contribute to the association between sleep deprivation and cardiovascular disorders. A number of studies have reported the association between levels of procoagulant (soluble Tissue Factor, fibrinogen, von Willebrand factor) and anti-fibrinolytic (Plasminogen activator inhibitor-1) molecules and sleep apnea.[9-14] On the other hand, little information is available on the coagulant impact of sleep deprivation on its own.[15]

In this paper we exploited the mouse model and a well established protocol to induce partial sleep deprivation[16] to demonstrate that it strongly affects, in a reversible manner, the expression levels of factor VII (FVII), the serine-protease triggering the coagulation process.[17]

Design and Methods

Animal colony, maintenance and housing conditions

Experiments were performed with C57BL/6J mice (n=44; Jackson Laboratory, Bar Harbor, ME, USA) kept in a 12h light : 12h dark cycle (LD 12:12; lights on at 07:00). It is conventional to divide the 24-hour LD cycle into 24 one-hour Zeitgeber time (ZT) units and indicate the time of lights on as ZT0 and the time of lights off as ZT12. Mice had free access to food and water. The housing and sleep recording environments were sound attenuated and temperature controlled (21°C). The sleep deprivation wheels are 9.0 inch diameter stainless steel rotating wheels (Nalgene, Pittsburgh, PA, USA). Each one is fixed between a solid steel plate on one side and a clear piece of plastic on the other. The wheel cages are designed to allow simultaneous wheel rotation and free access to food and water. Wheel speed was maintained at 1.0 or 1.7 revolutions per minute (r.p.m.).

Experimental design

Mice (n=18) underwent 20h of sleep deprivation using a slowly rotating wheel (1.0 r.p.m. x 20h/day = 1,200 total wheel revolutions/day) after which they were transferred to their home cage for a 4-hour sleep opportunity in the first part of the light phase from ZT0 to ZT4. Mice were subdivided in 3 groups (n=6 each group) and

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subjected to three days of sleep deprivation, seven days of sleep deprivation or seven days of sleep deprivation followed by a recovery in normal conditions for three days. Non-sleep deprived mice (n=18; 6 for each sleep deprived group) were placed in a non-rotating wheel for 20h/day and in a home cage for 4h/day for experimental control over the recording environments.

A second group of mice (n=8) was exploited to investigate the effect of forced activity on coagulation. A group (n=4) was subjected for seven days to forced activity (1.7 r.p.m.) for 12h/day from ZT12 to ZT24. This daily amount of activity was equivalent to the amount of activity performed by mice subjected to partial sleep deprivation (1,224 wheel revolutions/day). Mice were subsequently transferred to their home cage for a 12-hour sleep opportunity during the light phase (from ZT0 to ZT12). The control mice (n=4) were housed for seven days in a non-rotating wheel for 12h/day and in a home cage for 12h/day.

The last day of treatment, at ZT0, all experimental and control mice were anesthetized with isoflurine, subjected to retro-orbital bleeding to isolate plasma, and then sacrificed to isolate livers, as previously described.[18]

Mice subjected to sleep deprivation showed a modest reduction in weight (about 2-3% after three days and about 5-6% after seven days of PSD). The mice regained most of the weight loss after three days of recovery. All treatments were conducted under the guidelines established by the Institutional Animal Care and Use Committee of the Morehouse School of Medicine.

Functional assays in plasma

Thrombin generation assays, optimized to evaluate the efficiency of the extrinsic or the intrinsic activation coagulation pathways, as well as the FVII activity assays were conducted in mouse plasma as previously described.[18-21] Student's t-test was used to determine significant differences ($P<0.05$).

q-PCR

DNase-treated total RNA was isolated from mouse liver using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and used to perform cDNA synthesis (iScriptTM cDNA synthesis kit, Biorad, Milan, Italy). cDNA was PCR-amplified with a Chromo4 real-time PCR Detection System using iQTM SYBR Green Supermix (Biorad, Milan, Italy). Primers for mouse FVII mRNA quantifications were 5'-GACTTTGACGGTCGGAACTGTG-3' and 5'-GCGGCTGCTGGAGTTTCTTT-3'. Mouse GAPDH (5'-AACTTTGGCATTGTGGAAGG-3' and 5'-ACA-CATTGGGGGTAGGAACA-3') was used as housekeeping gene. The comparable amplification efficiencies of FVII and GAPDH transcripts prompted us to exploit the comparative threshold cycle method (CT), as previously described.[18] Each CT value used for these calculations is the mean of three replicates of the same reaction. One-way ANOVA was used to determine significant differences ($P<0.05$) and the Dunnett's *post hoc* test was applied to compare experimental groups with the control group.

Results and Discussion

We have previously shown that the circadian clock plays an important role in the modulation of the coagula-

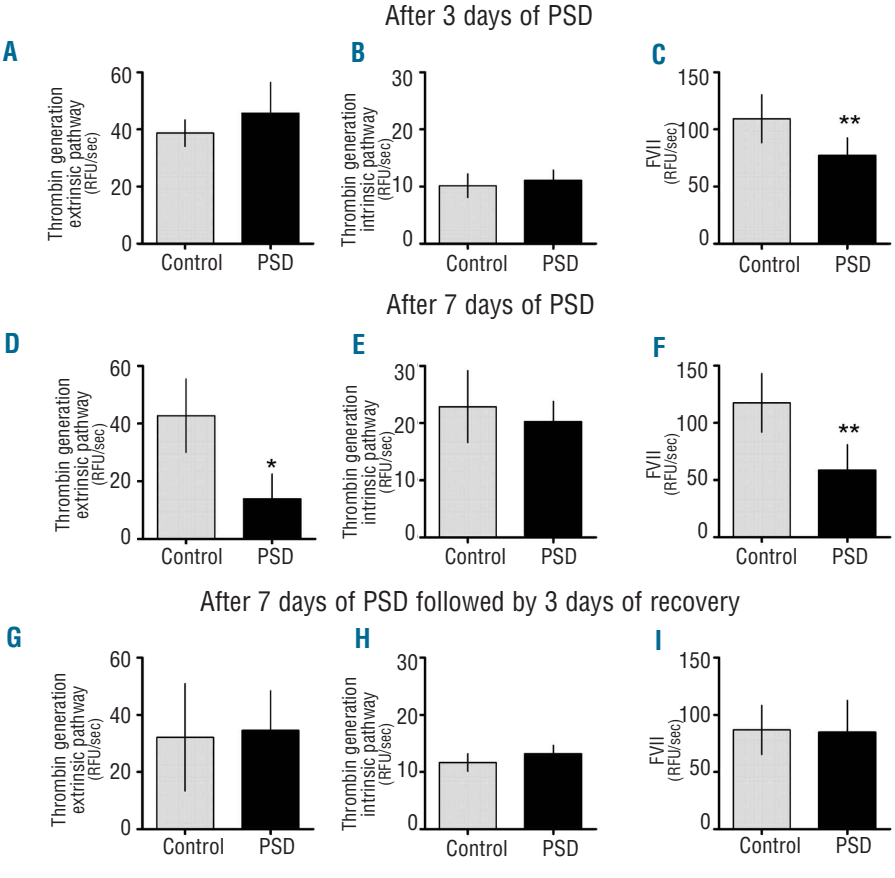

Figure 1. Effects of PSD on thrombin generation activity levels upon extrinsic (A,D,G) or intrinsic (B,E,H) activation, and on FVII activity (C,F,I) levels. For thrombin generation assays, the coagulation in diluted mouse plasma (1:40) was triggered via the extrinsic or the intrinsic pathway by adding an excess of Innovin™ (Dade Behring, Deerfield, IL) as a source of tissue factor, calcium, and phospholipids, or aPTT reagent (Actin-FS, Dade Behring, Germany) as a source of cephalin and negatively charged surfaces, respectively. The fluorogenic substrate (200 μM) for thrombin (Benzoil-Phe-Val-Arg-AMC; ICN Biomedicals, Costa Mesa, CA, USA) was then added and relative fluorescence units (RFU) monitored over time. FVII activity was evaluated by measuring the generation of activated factor X (FXa) in mouse plasma diluted 1:40 in human FVII depleted plasma (Dade Behring). Upon triggering of coagulation with an excess of Innovin, the fluorogenic substrate (200 μM) for FXa (MeSO2-D-CHA-Gly-Arg-AMCAcOH; American Diagnostica, USA) was added and relative fluorescence units monitored over time. In each assay the activity was evaluated as the initial rate expressed as RFU per second. Values represent the mean ± SEM of 6 samples per group (*$P<0.01$; **$P<0.001$).

tion cascade efficiency, mainly by modulating the TF/FVIIa pathway.[18-21] Since sleep and wake cycles and the circadian system closely interact with each other, we investigated whether partial sleep deprivation has any effect on the thrombin generation activity in plasma, a parameter defining the coagulation cascade efficiency.[22]

To this purpose, we exploited the mouse model and a recently developed PSD protocol.[16] This is a very effective method to induce partial sleep deprivation in rodents and although micro-sleep events may occur, animals lose about 60% of their normal daily sleep during the PSD protocol (KN Paul, unpublished data, 2009).

In mice subjected to three days of partial sleep deprivation, we did not observe a statistically significant variation of thrombin generation activity levels in plasma, neither upon extrinsic nor intrinsic activation of coagulation (Figure 1A and B; $P>0.1$ and $P>0.4$, respectively). When the PSD period was prolonged to seven days, we detected a significant reduction (-30.5 %; $P<0.01$) of thrombin generation activity levels upon extrinsic activation (Figure 1D). Conversely, upon intrinsic activation, these levels were comparable to those of control mice (Figure 1E).

To corroborate these findings we monitored the thrombin generation activity upon extrinsic trigger in the PSD mice after three days of undisturbed sleep and found that it returned to levels that were undistinguishable from those of controls (Figure 1G-I; $P>0.1$).

These data indicate that partial sleep deprivation significantly affects, in a reversible manner, the efficiency of the extrinsic activation pathway, characterized by the activity of the TF/FVIIa complex. Since, in our functional assay, TF is added in excess to mouse plasma, the variation of FVII activity levels likely explains the observed phenomenon. Other features of FVII support our mechanistic hypothesis: 1) FVII has a very short half-life (2-4h) which allows quick level fluctuations over time; and 2) FVII gene expression is directly controlled by the circadian clock machinery[18] which is strongly interlaced with the sleep/wake mechanisms.

In accordance with this hypothesis, the activity levels of FVII in plasma from mice subjected to three and seven days of partial sleep deprivation were reduced by 29.2% (Figure 1C; $P<0.001$) and 49.8% (Figure 1F; $P<0.001$) when compared to controls, respectively. Furthermore, three days of undisturbed sleep was able to completely restore FVII activity levels (Figure 1I; $P>0.1$) in line with the short half-life of this factor.

To corroborate this finding and to provide insights into the regulation at the transcriptional level, we investigated FVII gene expression in liver mRNA previously isolated from PSD mice (Figure 2). We observed a clear relationship between PSD conditions and a reduction in FVII mRNA levels in the liver, particularly after seven days (-85.7%;

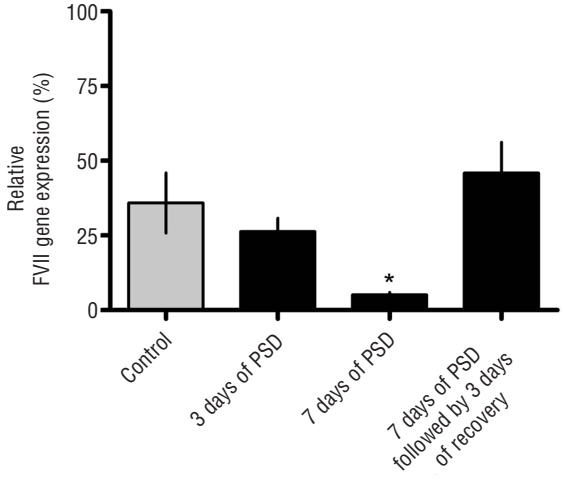

Figure 2. Liver FVII mRNA expression in PSD mice. Values were normalized with respect to the maximum value (100%) measured for each condition. The mean ± S.E.M. is shown. Dunnett's *post hoc* test was applied. No statistical differences in FVII mRNA expression were found among control groups ($P>0.50$), indeed the data for the 3 control groups of mice (N=18) were pooled. (*$P<0.01$).

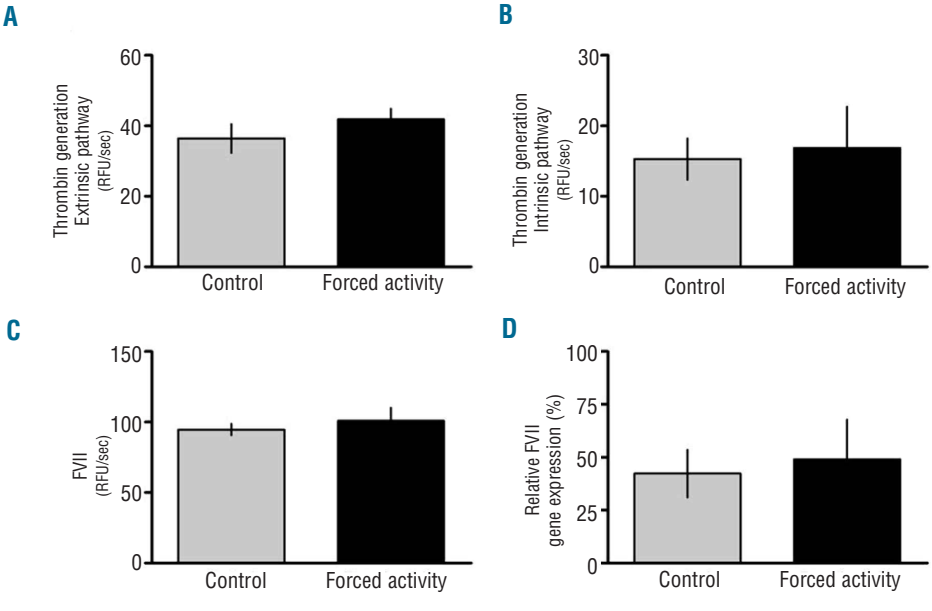

Figure 3. Effects of forced activity on thrombin generation activity levels upon extrinsic (A) or intrinsic (B) activation, on FVII activity levels (C) and on liver FVII mRNA expressions (D). Values represent the mean ± SEM of 4 samples per group. The coagulation parameters in the control group were not statistically different from those measured in the control mice in the PSD study ($P>0.1$ in all cases).

$P<0.01$). As observed for FVII activity, the normal FVII mRNA expression was restored by three days of undisturbed sleep.

One might argue that the variations reported here are due to increased levels of physical activity rather than lack of sleep. We, therefore, investigated the effect of forced activity on thrombin generation and FVII activity levels. The forced activity (12h/day from ZT12 to ZT24 for seven days) did not result in a significant change in any of the parameters under investigation (Figure 3 A-D; $P>0.1$ in all cases) thus supporting our main findings on the effects of partial sleep deprivation.

Very recently, Liu *et al.*[15] suggested that a one-day sleep deprivation results in a slight shortening of coagulation times (PT, APTT) in 10 healthy humans. Other studies, mainly focused on sleep apnea patients, reported a positive association between daily sleep disturbance/reduction and levels of specific prothrombotic factors (i.e. soluble Tissue Factor, von Willebrand factor, fibrinogen)[9-10,12-14,23] thus supporting a relationship between sleep deprivation and cardiovascular risk. Our data do not fit this hypothesis and would instead support a counteracting mechanism. We could speculate that the reduction of FVII levels may combine with the hemostatic balance through compensatory mechanisms operating in the coagulation/hemostasis pathways. A similar mechanism has been suggested by the parallel temporal oscillations in levels of FVII and of its direct inhibitor tissue factor pathway inhibitor.[19] Further studies aimed at evaluating the impact of sleep deprivation on plasma levels of a wide panel of coagulation and fibrinolytic factors are needed to address the physiopathology of this complex pathway.

For the first time, through the use of a well controlled animal model and a specifically designed experimental protocol, we demonstrated that chronic sleep deprivation on its own strongly affects and reduces, in a reversible fashion, the expression levels of FVII, thus influencing the TF/FVIIa activation pathway efficiency. These data further highlight the complexity of the modulation of the clotting cascade.

Authorship and Disclosures

MP, CB, FB, KNP and GT designed research; KB, SC, EF, NC, AB and JCE performed research; MP, CB, KNP, KB, SC, EF, NC, AB and GT analyzed and interpreted data; MP, CB, KNP, JCE, FB and GT wrote the paper.

The authors declare no conflict of interest.

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