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Sleep and circadian rhythm regulate circulating complement factors and immunoregulatory properties of C5a

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ABSTRACT

The sleep-wake cycle is characterized by complex interactions among the central nervous, the endocrine and the immune systems. Continuous 24-h wakefulness prevents sleep-associated hormone regulation resulting in impaired pro-inflammatory cytokine production. Importantly, cytokines and hormones also modulate the complement system, which in turn regulates several adaptive immune responses. However, it is unknown whether sleep affects the activation and the immunoregulatory properties of the complement system. Here, we determined whether the 24-h sleep-wake cycle has an impact on: (i) the levels of circulating complement factors; and (ii) TLR4-mediated IL-12 production from human IFN-γ primed monocytes in the presence or absence of C5a receptor signaling. For this purpose, we analyzed the blood and blood-derived monocytes of 13 healthy donors during a regular sleep-wake cycle in comparison to 24 h of continuous wakefulness. We found decreased plasma levels of C3 and C4 during nighttime hours that were not affected by sleep. In contrast, sleep was associated with increased complement activation as reflected by elevated C3a plasma levels during nighttime sleep. Sleep deprivation prevented such activation. At the cellular level, C5a negatively regulated TLR4-mediated IL-12p40 and p70 production from human monocytes. Importantly, this regulatory effect of C5a on IL-12p70 production was effective only during daytime hours. Thus, similar to hormones, some complement factors and immunoregulatory properties of C5a are influenced by sleep and the circadian rhythm. Our findings that continuous wakefulness has a negative impact on complement activation may provide a rationale for the immunosupportive functions of sleep.

1. Introduction

Sleep is a recurrent state of reduced consciousness and body rest that is intimately associated with the circadian system, characterized by physiological oscillations within a period of approximately 24-h. The combined effect of such cycles has been shown to affect interactions between the central nervous, the endocrine and the immune systems (Hastings et al., 2008). Recent findings show that sleep regulates the function of the neuroendocrine system by modulating the levels of several hormones such as growth hormone (GH), prolactin, cortisol and catecholamines. In turn, the oscillating levels of hormones modulate immune responses by regulating the production of cytokines, the trafficking and activation of leukocytes and the proliferation and differentiation of T cells (Lange et al., 2010). As such, acute sleep deprivation has been described to affect both humoral as well as cellular immunity. Indeed, continuous wakefulness is associated with increased levels of IgE in allergic patients, altered natural killer cell activity, antigen uptake, phagocytosis and secondary antibody responses as well as reduced pro-inflammatory cytokine production from antigen presenting cells (APC) and T cells (Lorton et al., 2006).

It is well appreciated that hormonal (Coulpier et al., 1995; Jacobi et al., 2001; Lemercier et al., 1992) and cytokine (Katz et al., 1995, 2000; Kulics et al., 1990) networks modulate the production of several factors and regulators of the complement system. However, the impact of sleep and circadian rhythm on the production of complement factors and/or the expression of complement receptors on immune cells remains elusive. The complement system, together with Toll-like receptors (TLRs) and other danger sensors, belongs to the group of ancient pattern recognition molecules (PRR) (Köhl, 2006). In contrast to cell associated PRRs, the complement system becomes activated when soluble C1q, Mannan-binding lectin (MBL) or one of the members of the ficolin family sense conserved exogenous (i.e., microorganisms) or

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endogenous (i.e., altered-self) danger-motifs. Depending on the nature of the danger motif, either the classical (via C1q) or the lectin pathway (via MBL or ficolins) becomes activated. A third pathway, the alternative pathway, is activated in response to direct proteolysis of C3 (C3 "tickover"). All complement activation pathways converge at the level of C3 leading to the dissociation of C3 into C3a and C3b fragments, the assembly of C5 convertases and further release of C5a and C5b fragments. C5b is then able to associate with C6, C7, C8 and C9 molecules resulting in the formation of the membrane attack complex (Müller-Eberhard, 1985).

C3a and C5a, the so called anaphylatoxins (ATs), exert a plethora of pro-inflammatory and immunoregulatory functions through their G-protein-induced signaling downstream of the C3a receptor (C3aR) or the C5aR, both of which are broadly expressed on myeloid as well as on tissue cells (Guo and Ward, 2005). In addition to C5aR, C5a and its degradation product C5adesArg bind to C5L2, another seven-transmembrane receptor sharing the broad tissue distribution with C5aR. The role of C5L2 in AT biology is sill enigmatic (Lee et al., 2008).

In addition to their pro-inflammatory properties, ATs regulate APC function and adaptive immune responses (Klos et al., 2009). C3a and C5a have been shown to modulate the expression levels of MHC class II and costimulatory molecules on dendritic cells (DCs) (Strainic et al., 2008; Weaver et al., 2010). Further, ablation of C3aR or C5aR signaling in DCs results in reduced TLR-induced IL-12 production and impaired $CD4^+$ T helper (Th) differentiation towards a type 1 phenotype (Peng et al., 2008, 2009; Strainic et al., 2008; Weaver et al., 2010). Furthermore, combined C5aR/C5L2 blockade in purified human monocytes results in impaired IL-12 production following stimulation with *Staphylococcus aureus* (Karp et al., 2000). In contrast, stimulation of tissue macrophages with C5a down-regulates TLR4-induced production of IL-12 family cytokines via extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) which translates into a decreased Th1 response *in vitro* and *in vivo* (Hawlisch et al., 2005).

Such studies suggest a complex role for ATs in the regulation of TLR-driven cytokine production depending on the cell type and the TLR pathway that is triggered. As sleep and the circadian rhythm regulate the production of cytokines by APCs, we hypothesized that they also control the production and/or activation of complement factors, resulting in modulation of APC function. Here we show for the first time distinct effects of sleep and the circadian system on complement levels and the immunoregulatory properties of C5a.

2. Materials and methods

2.1. Subjects and experimental design

Thirteen physically and mentally healthy men (age: 22.5 ± 0.62 years, body weight: 82.86 ± 1.57 kg, height: 186 ± 2 cm, body mass index: 23.96 ± 0.4), non-smokers and presenting a normal nocturnal sleep pattern participated in this study. All subjects were adjusted to the experimental setting by spending one adaptation night in the sleep laboratory. The study was approved by the Ethics Committee of the University of Lübeck (Lübeck, Germany).

Each subject was evaluated during two 24-h periods each starting at 6 pm, one including a regular sleep-wake cycle (lights off: 11 pm; lights on: 7 am) and the other 24 h of continuous wakefulness. In both conditions subjects were confined to bed from 6 pm to 1 pm. Standardized meals were supplied at appropriate times for breakfast (8 am), lunch (12 pm) and dinner (6 pm). Both conditions were separated by at least 2.5 weeks (range 2.5–15.7 weeks) and the order of conditions was balanced among subjects. To keep subjects awake in the 'wake' condition, they were allowed to watch TV, read and play board games at normal room light (300 lx)."

EEG was recorded continuously using a Neurofax amplifier (Nihon Kohden GmbH; www.nihonkohden.de) and polysomnography was performed according to standard criteria (Rechtschaffen and Kales, 1968). Sleep stages were determined off-line by an experienced scorer blinded to the study hypothesis. Sleep variables confirmed that subjects slept normally under laboratory conditions with slow wave sleep (SWS) predominant during the first night-half and rapid eye movement (REM) sleep dominating the second night-half (data not shown). In addition, EEG recordings confirmed that subjects did not fall asleep in the 'wake' condition.

Blood was sampled in EDTA tubes as described (Stöve et al., 1995) at 6 pm, and every 3 h until 6 pm on the following day with an additional blood draw at 1:30 am. During sleep periods, blood was drawn via an intravenous forearm catheter connected to a long thin tube, which enabled blood collection from an adjacent room without sleep disturbance. Blood samples were always processed immediately after sampling. For analyses of complement factors and hormones, plasma and serum were isolated immediately by centrifugation at $3000 \times g$ 10 min at 4 °C. Samples were stored at −80 °C and thawed just before use.

2.2. Whole blood stimulation

Blood–EDTA samples (200 μl) were primed with 50 ng/ml IFN-γ (R&D Systems, Wiesbaden, Germany) for 30 min and treated with 1 μg/ml LPS (from *Escherichia coli* – Sigma, Munich, Germany) for 6 h at 37 °C 5% CO_2. Where indicated, 5 μM of the C5aR antagonist $A8^{\Delta 71-73}$(Otto et al., 2004), 100 nM C5a (Hycult Biotech, Uden, The Netherlands) and 1.5 mM carboxypeptidase N (CPN) inhibitor DL-2-mercaptomethyl-3-guanidino-ethylthiopropanoic acid (MERGE-TPA – EMD Biosciences, Darmstadt, Germany) were also used.

2.3. Flow cytometry

Surface expression of C3aR, C5aR (CD88), CD14 and HLA-DR was determined using the following mAbs: C3aR-PE (Santa Cruz, Heidelberg, Germany), CD88-Alexa Fluor® 647 (clone P12/1, AbDserotec, Düsseldorf, Germany), CD14-FITC (clone 61D3, eBiosciences, Jena, Germany) and HLA-DR-PE-Cy7 (clone LN3, eBiosciences). Alternatively, cells were stained with the respective isotypes. Red blood cells were lysed in 155 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA and immune cells were fixed in 1% formaldehyde PBS/1% BSA. Samples were then stained for 30 min at 4 °C and analyzed using the LSRII flow cytometer (BD Biosciences) and FCS Express software (3.0 De Novo software). Intracellular cytokine detection in the monocyte population ($CD14^+HLA\text{-}DR^+$) was determined using anti-IL-12p40-PE (clone C8.6, eBiosciences). Fixed cells were permeabilized in PBS 1% BSA, 0.5% saponin for 30 min, followed by staining with the appropriate mAb in PBS 1% BSA, 0.5% saponin for 30 min at 4 °C.

2.4. Determination of IL-12p70

IL-12p70 levels were determined in plasma samples after incubation of whole blood for 6 h using the conditions described above. Cytokine concentrations were measured by ELISA with the BD Opt-EIA Human IL-12p70 ELISA set (BD Biosciences) as per manufacturer's instructions. The assay sensitivity was 7.8 pg/ml and intra-assay and inter-assay coefficients of variation were less than 10%.

2.5. Measurement of complement levels

Plasma levels of C3 and C4 were determined by nephelometry. C3a levels were measured in plasma by ELISA with the Human C3a