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Changes in Human Gene Expression After Sleep Deprivation

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Objectives Sleep is fundamental to maintaining homeostatic control and has behavioral and psychological effects on humans. To better understand the function and pathophysiology of sleep, specific gene expressions in reference to sleep deprivation have been studied. In this study, we investigated the gene expression of peripheral blood mononuclear cells after sleep deprivation to better understand the functional consequence of sleep.

Methods In eight healthy men, 24 h sleep deprivation was induced. Blood was sampled at 14:00, before and after sleep deprivation. mRNA was isolated and analyzed via microarrays. cDNAs before and after sleep deprivation were coupled to Cy3 or Cy5, respectively, and normalized cDNAs were selected with a ratio greater than two as a significant gene. Results are expressed as mean.

Results Among 41174 transcripts, 38852 genes were selected as reliable, and only a small minority (< 1%) of the genes were up-or down-regulated. Total six and eleven genes were selected as significant upregulated and downregulated genes, respectively. *Protein tyrosine phosphatase receptor type O* was most upregulated (6.9-fold), and *low-density lipoprotein receptor-related protein 5-like protein* showed the most substantial inhibition (0.06-fold).

Conclusions This study showed significant associations between sleep deprivation and the immune system. Acute sleep deprivation affects pathways in proinflammatory cytokines as well as metabolic pathways of glutamate and purine, neurotransmitters related to sleep and wake cycle.

Keywords Sleep deprivation; Gene expression; Immune system.

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Introduction

Sleep is a complex and intricate physiological and behavioral process. It can be defined by a distinct change in the electroencephalogram (EEG) from the waking state accompanied by behavioral change. Sleep is organized in two different states, rapid eye movement and non-rapid eye movement, which have different biological manifestations. Although unambiguous in its meaning, the function and exact pathophysiology of sleep are currently unknown.

There are several hypotheses to explain the crucial role of sleep. For instance, sleep may be necessary for brain protein synthesis[1] or to promote memory consolidation.[2] Another possibility is that sleep promotes physical restoration. The Energy Allocation Model of Sleep[3] suggests that limited energy resources are optimally distributed during sleep to essential biologic processes and decrease the total energy expenditure. After sleep deprivation in healthy control, 24 h energy expenditure increased by 7% compared to baseline.[4] Recent studies support sleep enhances the early stage of the immune response. After 24 h sleep deprivation, monocyte production of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) increased,[5] suggesting an association between sleep loss and inflammatory cytokine activity.

Along with an effort to understand the function of sleep, many studies explored sleep's physiological and biochemical mechanisms. Neurons in the suprachiasmatic nucleus express a set of clock transcription factors that comprise the circadian feedback loop to create a daily oscillating cycle.[6] In addition to the circadian system, multiple chemical mechanisms run the homeostatic

systems. Adenosine accumulates during wake time to activate the inhibitory A1 receptor to enhance wake neurotransmitter.[7)8)] Among multiple neurotransmitters involved in the homeostatic sleep-pressure system, the importance of glutamatergic and GABAergic networks has been emphasized in recent years. GABAergic system targets the supramammillary nucleus, lateral hypothalamus, and the basal forebrain to promote wake.[9)]

Identifying the expression changes of the genes between sleep and wakefulness may help clarify the core function of sleep at the cellular level. Previous studies have completed microarrays of RNA transcripts during sleep deprivation to identify functional categories of genes. Several gene expressions, including somatostatin,[10)] IL-1β,[11)] and those related to glycogen metabolism,[12)] peptidoglycan recognition protein,[13)] were identified as sleep state-dependent genes. After sleep deprivation, circadian genes *per1* and *per2* mRNA levels in the cerebral cortex increased while *dbp* expression decreased.[14)] Transcriptomics studies have concluded that gene expression in the brain changes considerably after sleep deprivation, and about 1% to 10% of gene expression showed changes in the array studies.[15)16)] However, most studies are done on rats and flies, and expression profiling is expected to be different in humans.

To address the changes in gene expression after sleep deprivation in humans, we report a comprehensive microarray analysis of gene expression peripheral blood mononuclear cells (PBMC) to identify sleep state-dependent genes and highlight the functional consequence of sleep.

Methods

Subjects

After giving a written informed consent, eight healthy, normal male subjects aged 25–35 were included in the study. Before the study, an interview with a psychiatrist ensured that all participants had a regular sleep-wake rhythm. A battery of clinical tests, including vital signs, blood tests, and urine analysis were performed to ensure that subjects were physically and physiologically healthy. Exclusion criteria are the following: 1) abnormal values in blood test including basic chemistry, metabolic panel, and urine test, 2) comorbid mental health problems assessed by a psychiatrist, 3) use of drugs including alcohol, caffeine, psychotropic drug, and Z-drugs within ten days that could affect the results. The study was performed in October 2008. This study was carried out as recommended in the Helsinki Declaration. All participants signed an informed consent form, and the study received approval by the Ethical Review Committee (IRB No.: KMC GS-0808-03).

Protocol

Each subject participated in a sleep deprivation experiment that lasted for 24 h. A visual analog scale to assess appetite and mood and blood samples were done twice upon admission to Kyung Hee Medical Science Research Institute at 14:00 and after 24 h of sleep deprivation. The investigators were continuously present during the experiment to ensure that the subjects remained alert and would not fall asleep for 24 h of sleep loss. Kyung Hee Medical Science Research Institute is a hospital bed setting where subjects were allowed to lie down or engage in activities such as reading and playing video games. The subject was exposed to typical fluorescent lighting during the experiment.

Culture of human PBMC

Obtained human peripheral venous blood was centrifuged (400 g) onto Histopaque (Sigma-Aldrich, St Louis, MO, USA). Cells were washed in phosphate-buffered saline, resuspended in RPMI 1640 medium, and cultured (5×10^6 cells/mL) in 10-cm flat-bottomed culture plates, in medium supplemented with 25 mM HEPES, 10% fetal bovine serum, 2 mM l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (WelGENE Inc., Seoul, Korea). After incubation at 37°C, 5% CO_2 air for 24 h, cells were collected in an Eppendorf tube and stored at -70°C.

cDNA synthesis

Total RNAs of eight samples were extracted from PBM using the ZYMO research RNA isolation kit (Zymo Research, Irvine, CA, USA). Total RNA concentration and quality were accessed with the Agilent 2100 Bioanalyzer System (Agilent Technologies, Palo Alto, CA, USA). We used Agilent's Low RNA Input Linear Amplification kit PLUS to synthesize target cDNA probes and hybridization. T7 promoter primer mix and total RNA (1 μg) were incubated at 65°C for 10 min. cDNA master mix solution (5X first strand buffer, 0.1 M DTT, 10 mM dNTP mix, RNaseOut, and MMLV-RT) was mixed with the primer mixture and RNA. Reverse-transcription and synthesis of double-stranded cDNA (dsDNA) were finished by incubating the mixtures at 40°C for 2 h. After incubating the mixture at 65°C for 15 min, all the reaction was terminated. The transcription master mixture (4X transcription buffer, 0.1 M DTT, NTP mix, 50% PEG, RNaseOut, Inorganic pyrophosphatase, T7-RNA polymerase, and Cyanine 3/5-CTP) was added to the dsDNA reaction mixture. For dsDNA transcription, the mixture was incubated at 40°C for 2 h. Two cRNA samples before and after sleep deprivation were labeled with Cy3-CTP and Cy5-CTP, respectively. For fragmentation of cRNA, the mixture was incubated at 60°C for 30 min with 10X blocking agents and 25X fragmentation buffer. A 44 K Mouse Oligo Microarray (Agilent Technologies) kit containing

41174 transcripts and variants was used for microarray analysis. Scanned images in all groups were analyzed using Feature Extraction software (Agilent Technologies).

Microarray data analysis and statistical analysis

Transcriptional analysis of 41174 transcripts and variants of PBMCs was done before and after sleep deprivation. The ratio (CY5/CY3) of signal intensities generated in each microarray was normalized using Loewss models (Agilent's GeneSpring Software). Flag values were assigned to eliminate probes with a value lower than background signal intensity and achieve reliable gene expression profiles, and 38852 genes were identified as reliable genes. The signal intensity ratio (CY5/CY3) was computed and further log-transformed on the base of 2 into $M = \log_2$ (CY5/CY3) in order to treat the up-or down-regulation of identical magnitude as numerically equal but with opposite direction. Probes with a normalized average ratio greater than two in all eight participants were selected as significant genes. Statistical analysis was carried out using the program SPSS, release 12.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as mean. Paired t-test was used to calculate statistical significance. Significance was determined if the p value was less than 0.5.

Results

We tested for changes in gene expression with 24 h of sleep deprivation in healthy subjects. Among 41174 transcripts, 38852 genes were selected as reliable. We selected upregulated or downregulated genes with more than twofold changes after sleep deprivation because gene expression changes in peripheral blood are generally minor.[17] Table 1 summarizes six gene expressions that were upregulated after sleep deprivation. Among the upregulated genes, *protein tyrosine phosphatase receptor type O (PTPRO)* was most upregulated (6.9-fold, $p = 2.16 \times 10^{-5}$). Sleep deprivation increased the expression of the *Fc fragment of IgG receptor IIIa, and IIIB (FCGR3A and FCGR3B)* more than three-fold in PBMCs, which are related to natural killer (NK) cell-mediated cytotoxicity. Genes that were downregulated more than two-fold after sleep deprivation are a total of eleven (Table 2). Of the downregulated genes, *low-density lipoprotein receptor-related protein 5-like protein (LRP5L)* showed the most substantial inhibition (0.06-fold, $p = 4.47 \times 10^{-4}$). Nuclear receptor subfamily 4, group A, members 1 and 3 (NR4A1, NR4A3) were downregulated by 0.21 and 0.22-fold ($p = 1.30 \times 10^{-5}$, 7.48×10^{-5}) respectively.

Discussion

Sleep deprivation affects man's biochemical, physiological, and psychological aspects, and due to high prevalence in the general public, it has become a significant concern. Although numerous studies examine the effects of sleep deprivation in each aspect, including cognition, mood, immune, and more, the over-

Table 1. List of genes upregulated after 24 h of sleep deprivation in peripheral blood mononuclear cells

Gene name	GenBank number	Fold	Description
PTPRO	NM_000569	6.88539	Protein tyrosine phosphatase, receptor type, O
IL8RBP	NM_000570	4.87087	Interleukin 8 receptor, beta pseudogene
FCGR3A	NM_006163	4.158534	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)
ADAMTS15	BC033255	4.04061	ADAM metallopeptidase with thrombospondin type 1 motif, 15
NFE2	NM_030667	3.99195	Nuclear factor (erythroid-derived 2), 45kDa
FCGR3B	NM_139055	3.68866	Fc fragment of IgG, low affinity IIIb, receptor (CD16b)

Table 2. List of genes downregulated after 24 h of sleep deprivation in peripheral blood mononuclear cells

Gene name	GenBank number	Fold	Description
SERPINB9	NM_004155	0.498507	Serpin proteinase inhibitor, clade B (ovalbumin), member 9
THBS1	NM_003246	0.496154	Thrombospondin 1
EGR3	NM_015900	0.462562	Early growth response 3
PLA1A	NM_006203	0.381567	Phospholipase A1 member A
NR4A3	NM_173198	0.223925	Nuclear receptor subfamily 4, group A, member 3
NR4A1	NM_002135	0.210709	Nuclear receptor subfamily 4, group A, member 1
ATF3	NM_004024	0.199089	Activating transcription factor 3
PDE4D	NM_006203	0.187013	cAMP-specific phosphodiesterase 4D
SERPINB2	NM_002575	0.140867	Serpin peptidase inhibitor, clade B (ovalbumin), member 2
GFPT2	NM_005110	0.128161	Glutamine-fructose-6-phosphate transaminase 2
LRP5L	NM_182492	0.060075	Low density lipoprotein receptor-related protein 5-like

all understanding of gene expressions and general effects on human biology are obscure. This study investigates the gene expression pattern after 24 h of sleep deprivation in PBMC of healthy human subjects. Due to rare accessibility to brain tissue, we identified gene expression in peripheral blood nuclear cells that share more than 80% of the transcriptome with brain tissues.[18] Of the 41174 genes assessed, only a small minority (< 1%) of the genes were up-or down-regulated (changes ranging up to two-fold) before and after 24 h of sleep deprivation.

Sleep deprivation resulted in an elevation in the expression of the following inflammatory genes: *PTPRO*, *IL8RBP*, *FCGR3A*, and *FCGR3B*. PTPRO is one of the receptor types of phosphor tyrosine phosphatases and plays critical roles in acute inflammation via nuclear factor kappa B (NF-kB) signaling pathway in T lymphocytes[19] and macrophages.[20] *PTPRO* expression changes involve various types of cancers, including lung cancer,[21] breast cancer,[22] hepatocellular carcinoma,[23] esophageal squamous cell carcinoma[24] as well as inflammatory response, particularly toll-like receptor 4 mediated inflammation.[25][26]

Sleep deprivation increases the secretion of pro-inflammatory cytokines as well as increased white blood cells, most notably neutrophils.[27] Previous studies reported notable elevation in IL-6 concentration[28-30] after sleep deprivation and its role in quantity and depth of sleep. In our study, *IL-6* increased by 1.8 fold, while *IL8RBP* gene expression showed a 4.87-fold change. IL-8 and cytokines such as IL-2, IL-6, IL-15, and IL-18 are reported to increase non-rapid eye movement sleep.[31] Increased blood IL-8 concentration is observed in sleep disorders including narcolepsy[32] and obstructive sleep apnea.[33] However, gene expression and protein levels lack correlation,[34] and further studies are required to understand the pathophysiology between sleep deprivation and *IL-8* gene expression.

CD16 (FCγRIII) is expressed on macrophages, NK cells, neutrophils and is involved in diverse immune responses, including antibody-dependent cell-mediated cytotoxicity.[35] Wilder-Smith et al.[36] reported a significant decrease of CD 16 after 10 h-partial sleep deprivation and then increase above the baseline after prolonged sleep deprivation. The lag in NK activity in response to sleep deprivation is also consistent with other studies. A similar result is expected if we extend the observation time.[37]

Down-regulated genes can be predominantly grouped into two functional pathways, metabolic and inflammatory pathways. Our study indicates possible effects on translation in the downregulation of glutamate and purine metabolism, including *phospholipase A1 Member A (PLA1A)*, *phosphodiesterase 4D (PDE4D)*, *glutamine-fructose-6-phosphate transaminase 2 (GFPT2)*. *GFPT2* codes enzymes that catalyze the conversion of glutamine to glutamate.[38][39] Glutamate is an essential primary excitatory neurotransmitter that affects the sleep-wake regulatory system. Glutamate activates pre-synaptic glutamate receptors, thus increasing GABAergic inhibitory tone.[40] Also, glutamate secretion affects astrocytes' production of ATP, critical gliotransmitters in regulating sleep demand.[41]

The orphan nuclear receptor 4A subfamily, including Nur77 (NR4A1), Nurr1 (NR4A2), and NOR1 (NR4A3), is emerging as a key regulator of inflammatory response. NR4A1 in monocytes and macrophages inhibits NF-κB activation by TNFα and IL-1β.[42] It is also involved in T cell regulation and CD81 T cells development.[43][44] In cancer, NR4A1 exhibits pro-oncogenic activities via TCR-induced apoptosis in T cell hybridoma.[45] NR4A receptors all engage in inhibition of the inflammatory responses through various regulatory mechanisms. After sleep deprivation, two NR4A genes, *NR4A1* and *NR4A3*, were downregulated, suggesting disengagement of inflammation inhibition response.

PDE4D is a cAMP-specific phosphodiesterase widely expressed in the human brain.[46] Studies have shown that PDE45 interacts with several signaling proteins, most notably RACK1, β-arrestin2, MAPK signaling proteins, and oxidative-stress kinase. A possible functional role for PDE4D involves β2-adrenergic signaling,[47] immunity/inflammation,[48] and depressive disorder and cognition.[49] Also, there seems to be a significant association between single-nucleotide polymorphism within *PDE4D* gene expression and sleep duration.[50] Downregulation of *PDE4D* was observed in patients with atrial fibrillation.[51]

In addition to the change in immune-related gene expression, 19 clock genes were upregulated. Sleep deprivation is associated with changes in the expression of circadian clock-related genes in the cerebral cortex and PBMCs.[52][53] None of the clock genes had a normalized average ratio greater than two in all participants in this study. Interindividual gene expression variability can be explained by polymorphic variants of clock genes.[54] Mechanisms for gene expression changes induced by sleep and sleep deprivation in PBMCs and other tissues remain unclear, and further studies are required. PBMCs best represent the current status of the immune system and the inflammatory response, while the correlation of expression in blood with expression in the brain is imperfect. Given this, it may be possible that the immune-related gene expression change is more noticeable than the clock-related gene expression change.

Our findings suggest that of the 41174 genes assessed, some were specifically related to immune functions via up-regulation and down-regulation of immune and inflammatory responses in the absence of 24-h sleep deprivation. The study limitations are as follows: 1) Investigators were continuously present to monitor the participants to keep them alert during the study periods, but we were unable to record continuous EEG recording to en-

sure that the participants did not sleep. 2) The genome-wide expression analysis results need to be confirmed with a more extensive study number. Further studies to group the studied genes in biologically meaningful pathways to better understand the pathophysiological mechanism of sleep deprivation. 3) Small study samples and poor statistical analysis limits generalizability and interpretation of the data, and further studies to replicate the data are required. 4) Confounding variables such as food intake and activity level are not fully controlled. 5) In an ideal study, because acute sleep deprivation can alter the circadian expression of genes, blood samples should be drawn multiple times to understand the course of gene expression changes fully.

The strengths of this study lie in that the subject is human, and PBMC can represent genetic changes in different organs after sleep deprivation, while previous studies are mostly done with animals such as rats. Also, the large number of genes allowed us to explore different possible pathways involved in sleep deprivation.

In conclusion, the RNA-based approach provides a comprehensive gene expression profiling in PMBCs of sleep deprivation. There is a clear interaction between sleep deprivation and immune cascades via modification of inflammatory meditators level. These data should motivate further investigations to define mechanisms that explain the associations between sleep disturbance and inflammation.

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Conflicts of interest

The authors have no financial conflicts of interest.

Author Contributions

Conceptualization: Jong Woo Kim, Sung-Vin Yim. Data curation: Jong Woo Kim, Sung-Vin Yim. Formal analysis: Jong Woo Kim, Sung-Vin Yim. Investigation: Jong Woo Kim, Sung-Vin Yim. Methodology: Jong Woo Kim, Sung-Vin Yim. Project administration: Jong Woo Kim, Sung-Vin Yim. Resources: Sung-Vin Yim. Supervision: Jong Woo Kim. Visualization: Je Young Sun, Miae Oh, Won Sub Kang. Writing—original draft: Je Young Sun, Won Sub Kang. Writing—review & editing: all authors.

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