Research letter

Association between glucose metabolism, the circadian cycle and hypoxia: Evaluation of the NPAS2 and Rev-Erb-α protein serum levels in obstructive sleep apnea patients – a pilot study

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Dental and Medical Problems, ISSN 1644-387X (print), ISSN 2300-9020 (online) *Dent Med Probl*. 2024;61(3):465–469

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Funding sources
Student scientist minigrant organized as part of the project "Operacja Integracja!" – Integrated Program of the Medical University of Lodz, Poland, co-financed by the European Union within the European Social Fund.

Conflict of interest
None declared

Acknowledgements
None declared

Received on December 31, 2023
Reviewed on February 27, 2024
Accepted on March 6, 2024

Published online on May 28, 2024

Cite as
Karuga FF, Jaromirska J, Sochal M, Białasiewicz P, Gabryelska A. Association between glucose metabolism, the circadian cycle and hypoxia: Evaluation of the NPAS2 and Rev-Erb-α protein serum levels in obstructive sleep apnea patients – a pilot study. *Dent Med Probl.* 2024;61(3):465–469. doi:10.17219/dmp/185718

DOI
10.17219/dmp/185718

Abstract

Background. Obstructive sleep apnea (OSA) is one of the risk factors for diabetes mellitus type 2 (DM2). As OSA is associated with the disruption of the circadian rhythm, it affects circadian clock proteins, including neuronal PAS domain protein 2 (NPAS2) and nuclear receptor subfamily 1 group D member 1 (Rev-Erb-α). These proteins have been shown to be related to metabolic abnormalities, i.a., insulin resistance.

Objectives. The present pilot study aimed to investigate the NPAS2 and Rev-Erb-α protein serum levels in the groups of patients with severe OSA and severe OSA+DM2 in comparison with healthy controls, taking into account correlations with polysomnography (PSG) parameters (e.g., oxygen saturation (SpO_2) variables).

Material and methods. A total of 40 participants were included in the study. They were split into 3 groups as follows: the OSA group ($n = 17$; apnea–hypopnea index (AHI) >30, no DM2); the OSA+DM2 group ($n = 7$; AHI > 30 and DM2); and the control group ($n = 16$; AHI < 5, no DM2). All participants underwent a nocturnal PSG examination and had their blood collected the following morning. The serum levels of NPAS2 and Rev-Erb-α proteins were assessed using the enzyme-linked immunosorbent assay (ELISA).

Results. The mean NPAS2 protein level was significantly lower in the OSA group as compared to healthy individuals ($p = 0.017$). Additionally, the OSA group presented with lower NPAS2 protein levels as compared to the OSA+DM2 group, but only a tendency was observed ($p = 0.094$). No differences in the Rev-Erb-α protein concentration were noticed. Furthermore, a negative correlation between AHI during rapid eye movement (REM) sleep and the NPAS2 protein serum level was observed ($r = -0.478$; $p = 0.002$).

Conclusions. Serum NPAS2 protein might be involved in metabolic dysregulation present among OSA patients, while the mechanism itself may be associated with REM sleep.

Keywords: hypoxia, circadian cycle, glucose metabolism, NPAS2, Rev-Erb-α

Introduction

Obstructive sleep apnea (OSA) is characterized by recurrent pauses in breathing during sleep, leading to intermittent hypoxia, arousal and sleep fragmentation.[1] The gold standard in the OSA diagnosis is a polysomnography (PSG) examination, which enables the assessment of OSA severity through the apnea–hypopnea index (AHI).[2] The available literature recognizes OSA as an independent risk factor for various comorbidities, including glucose metabolism impairment, insulin resistance and diabetes mellitus type 2 (DM2).[3,4] These metabolic complications can be related to electrolyte and vitamin dysregulation,[5] serotoninergic system dysregulation[6–8] or the alteration of circulating adipokines.[9]

Circadian clocks are endogenous coordinators of the 24-hour rhythm of behavioral and molecular processes.[10] Each circadian clock comprises a set of genes that function as activators, namely circadian locomotor output cycles kaput (*CLOCK*) and basic helix–loop–helix ARNT-like (*BMAL1*).[11] Through binding to regulatory elements containing E-boxes, they activate the transcription of repressor proteins period (encoded by *Per*) and cryptochrome (encoded by *Cry*).[12] Neuronal PAS domain protein 2 (NPAS2), similar to circadian clock activators and hypoxia-inducible factors, belongs to the helix–loop–helix Per/Armt/Sim (PAS) transcription factor family. Due to this structural similarity, it can substitute the function of CLOCK.[13] In mice, which serve as a mammalian model of the circadian clock,[14] the activation of CLOCK–BMAL1 occurs in the daytime, which subsequently causes the transcription of both *Per* and *Cry* in the afternoon, and the accumulation of PER and CRY proteins in the evening. The proteins translocate to the nucleus at nighttime and interact with the CLOCK–BMAL1 complex, which results in the repression of their transcription. Following a decrease in the protein concentration at night, the CLOCK–BMAL1 complex starts a new cycle in the morning.[15] The regulatory complex of the circadian clock also includes a supporting loop that involves nuclear receptor subfamily 1 group D member 1 (Rev-Erb-α), which interacts with the CLOCK–BMAL1 complex, taking part in the interlocked feedback loops of activators and repressors, thereby creating a canonical positive- and negative-feedback gene network.[16] Studies have shown that the dysregulation of the circadian clock can result in metabolic complications, including glucose metabolism impairment.[17]

Therefore, this pilot study aimed to investigate the NPAS2 and Rev-Erb-α protein serum levels in patients with severe OSA, severe OSA and DM2, and in healthy individuals, and to assess their relationship with PSG parameters.

Material and methods

This cross-sectional study was conducted at the Department of Sleep Medicine and Metabolic Disorders of the Medical University of Lodz, Poland, between January and June 2021 – recruitment and data collection. Patients were recruited according to the inclusion criteria (age above 18 and below 70 years, body mass index (BMI) of 20–40 kg/m^2, and providing informed written consent to participate in the study). The exclusion criteria comprised withdrawing of the consent, chronic respiratory diseases, chronic inflammatory diseases, infection within 1 month of blood collection, active cancer or a history of cancer, a lifetime history of diagnosed sleep disorders other than OSA, employment in the changing shift system, caffeine intake >900 mg per day, and the use of hypnotic medications or any medications known to affect sleep during 2 weeks before the sleep laboratory examination. Patients referred with suspected OSA syndrome underwent a standard medical examination and a nocturnal PSG examination. The DM2 diagnosis was based on the patient's medical history. After the examination, venous blood samples were collected into serum separator blood collection tubes. Forty participants were qualified for the study. Based on the PSG and clinical data, the participants were assigned to one of 3 study groups: OSA (severe OSA – AHI > 30, no DM2); OSA+DM2 (severe OSA – AHI > 30 and DM2); and control (no OSA – AHI < 5, no DM2). The study was approved by the Bioethical Committee of the Medical University of Lodz (RNN/432/18/KE).

Polysomnography

The patients were admitted to the sleep laboratory at about 9 p.m. (±0.5 h), where standard physical and subjective examinations were conducted, and body mass, height, blood pressure, and pulse were measured. Standard nocturnal PSG was performed by recording the following channels: electroencephalography (EEG) (C4\A1, C3\A2); electromyography (EMG) of the chin muscles and tibialis anterior; electrooculography (EOG); measurements of the oronasal airflow (a thermistor gauge); snoring; body position; respiratory movements of the chest and the abdomen (piezoelectric gauges); unipolar electrocardiogram (ECG); and hemoglobin oxygen saturation (SpO_2) (Alice 6; Phillips Respironics, Murrysville, USA). Polysomnographic events were scored by the same experienced physician. Sleep stages were scored according to the criteria based on the 30-second epoch standard.[2]

Biochemical analysis

Peripheral blood samples were collected in the morning following PSG (between 6 a.m. and 7 a.m., within 10 min of awakening) into blood collection tubes with a clot activator and ethylenediaminetetraacetic acid (EDTA).

Blood samples with a clot activator were centrifuged immediately after blood draws at 4°C. Serum was collected and stored at –80°C. The serum protein concentration was assessed using an enzyme-linked immunosorbent assay (ELISA) kit (EIAaB, Wuhan, China) and the absorbance was measured at wavelength λ = 450 nm, using an absorbance reader (BioTek 800 TS; Agilent Technologies Inc., Santa Clara, USA).

Statistical analysis

Statistical analysis was conducted with the use of Statistica 13 PL (StatSoft Polska, Krakow, Poland). The assessment of distribution (normal/non-normal) for continuous variables was performed with the Shapiro–Wilk test. Parametric independent variables were assessed with Student's *t* test, and the Mann–Whitney *U* test was used for nonparametric independent variables. Spearman's correlation test was applied for correlations. A *p*-value <0.05 was considered statistically significant.

Results

The baseline characteristics of the pilot study participants are shown in Table 1, and the results, including the serum levels of NPAS2 and Rev-Erb-α proteins, are presented in Table 2. No statistically significant differences were noticed in the Rev-Erb-α protein concentration among all study groups ($p = 0.624$). The mean NPAS2 protein level was significantly lower in the OSA group as compared to healthy individuals ($p = 0.017$). Additionally, the OSA group presented with lower NPAS2 protein levels as compared to the OSA+DM2 group, but only a trend was observed, as the difference did not reach statistical significance ($p = 0.094$). Of the evaluated correlations, only a weak negative correlation between AHI during rapid eye movement (REM) sleep and the NPAS2 protein serum level was observed ($r = –0.478$; $p = 0.002$).

Table 2. Concentrations of circadian clock proteins

Parameter	OSA group (*n* = 17)	OSA+DM2 group (*n* = 7)	Control group (*n* = 16)	*p*-value
NPAS2 level [ng/mL]	117.07 ±55.29	198.28 ±259.83	186.22 ±166.31	0.037* 0.017*[a] 0.446[b] 0.094[c]
Rev-Erb-α level [ng/mL]	240.93 ±73.46	271.31 ±89.66	272.04 ±92.81	0.624 0.368[a] 0.947[b] 0.505[c]

Data presented as mean ± standard deviation (*M* ±*SD*).
NPAS2 – neuronal PAS domain protein 2; Rev-Erb-α – nuclear receptor subfamily 1 group D member 1; * statistically significant ([a] control group vs. OSA group, [b] control group vs. OSA+DM2 group, [c] OSA group vs. OSA+DM2 group).

Table 1. Baseline characteristics of the study population

Parameter		OSA group (*n* = 17)	OSA+DM2 group (*n* = 7)	Control group (*n* = 16)	*p*-value
Age [years] *Me* (*IQR*)		53 (44.50–59.50)	64 (56.00–72.00)	46 (33.75–56.50)	0.003*
Sex *n* (%)	M	14 (82.35)	6 (85.71)	11 (68.75)	0.548
	F	3 (17.65)	1 (14.29)	5 (31.25)	
BMI [kg/m^2] *Me* (*IQR*)		33.95 (30.99–37.54)	35.89 (32.08–42.67)	27.33 (24.27–28.88)	<0.001*
TST [h] *Me* (*IQR*)		6.50 (6.10–7.08)	5.46 (5.20–6.40)	6.20 (5.70–6.47)	0.050
Arousal index [events/h] *Me* (*IQR*)		23.70 (19.60–28.75)	28.10 (20.90–38.60)	12.25 (7.07–17.30)	<0.001*
AHI in REM sleep [events/h] *Me* (*IQR*)		38.97 (24.44–53.01)	47.51 (29.14–73.88)	1.64 (0.00–7.70)	<0.001*
AHI in NREM sleep [events/h] *Me* (*IQR*)		38.58 (32.33–61.15)	45.66 (35.69–62.89)	1.06 (0.35–1.64)	<0.001*
Total AHI [events/h] *Me* (*IQR*)		51.40 (35.9–64.15)	51.70 (45.70–63.40)	1.45 (0.52–3.00)	<0.001*
Desaturation index [events/h] *Me* (*IQR*)		50.0 (34.8–76.1)	60.0 (51.2–63.0)	2.0 (1.0–3.0)	<0.001*
SpO_2 during desaturation events [%] *Me* (*IQR*)		86.9 (80.5–90.1)	87.0 (83.8–88.0)	91.8 (90.5–93.1)	<0.001*

Me – median; *IQR* – interquartile range; OSA – obstructive sleep apnea; DM2 – diabetes mellitus type 2; M – male; F – female; BMI – body mass index; TST – total sleep time; AHI – apnea–hypopnea index; REM – rapid eye movement; NREM – non-rapid eye movement; SpO_2 – oxygen saturation; * statistically significant.

Discussion

The dysregulation of circadian clock genes has been shown to cause not only sleep disorders, such as sleep phase syndrome,[18] but also metabolic abnormalities, including metabolic syndrome[19] or insulin resistance.[20] Zhang et al. revealed that diabetic rats had significantly higher Rev-Erb-α protein levels in adipose tissue as compared to nondiabetic rats.[21] Their work suggests that Rev-Erb-α may be one of the key molecules potentially causing metabolic syndromes through circadian clock disruption.[21] Additionally, in a study by Kooner et al., which included 5,561 patients with DM2, *NPAS2* genes were also associated with the development of DM2.[22] Literature concerning circadian clock disruption among patients suffering from OSA is limited, both in terms of number of publications and only several circadian clock genes being investigated.[23] Canales et al., in a cross-sectional study on 49 patients, compared groups with OSA or nocturnal hypoxemia (defined as ≥10% of total sleep time (TST) spent at SpO_2 < 90%) to those without such conditions, and found that the mRNA expression of *Rev-Erb-α* and *NPAS2* was reduced in patients with nocturnal hypoxemia.[24] Nevertheless, the study was a sub-analysis of a cohort suffering from severe kidney dysfunction. Since it has been established that kidney function greatly influences the expression of circadian clock genes,[25] it is impossible to state whether OSA severity, hypoxia or kidney dysfunction had a key impact on the obtained results.

Our results from a pilot study show the same tendency as reported in research by Xie et al., where *NPAS2* gene expression was decreased in untreated OSA patients with AHI > 15.[26] However, their results did not present statistical significance, contrary to our study, where the difference in the NPAS2 protein levels between the OSA group and the control group was significant ($p = 0.017$). Extended research that would include both NPAS2 gene expression and protein level, together with CLOCK–BMAL1 investigations, might help define exact relationships between circadian clock activators in response to hypoxia occurring in the REM phase.

Limitations

Our study has its limitations, including a small sample size (only 40 participants). We aimed to conduct a small-scale study to test the research design and feasibility to obtain significant results, which was achieved. Therefore, we stand prepared to expand the investigation. As the research was conducted as a pilot study and the group of participants was small, it was hard to choose subjects that would perfectly match all parameters; thus, the age of the subjects varied. Another limitation that should be mentioned are the conditions in which the study was conducted, namely sleeping in the hospital PSG laboratory; TST might have been influenced more by the attitude and the psychological aspects of adjusting to a new sleep environment than by typical circadian-related difficulties in falling asleep.

Conclusions

Serum NPAS2 protein might be involved in metabolic dysregulation present among OSA patients, while the mechanism itself may be associated with REM sleep. The findings of our pilot study may help better understand the molecular mechanism responsible for circadian cycles, the reaction to hypoxia, and their influence on disturbances of glucose metabolism, as well as interactions between them. However, to confirm the role of NPAS2 and Rev-Erb-α in the development of DM2, the continuation of the study is necessary.

Ethics approval and consent to participate

The study was approved by the Bioethical Committee of the Medical University of Lodz, Poland (RNN/432/18/K). All subjects provided informed written consent to participate in the study.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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