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Microarray Studies of Genomic Oxidative Stress and Cell Cycle Responses in Obstructive Sleep Apnea

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ABSTRACT

Obstructive sleep apnea (OSA), the commonest form of sleep-disordered breathing, is characterized by recurrent episodes of intermittent hypoxia and sleep fragmentation. This study evaluated microarray measures of gene transcript levels in OSA subjects compared to age and BMI matched healthy controls. Measurements were obtained before and after: (a) a night of normal sleep in controls; and (b) a night of untreated apnea in OSA patients. All subjects underwent full polysomnography. mRNA from the whole blood samples was analyzed by HG-U133A and B Affymetrix GeneChip™ arrays using Spotfire™ 7.2 data analysis platform. After sleep in OSA patients, changes were noted in several genes involved in modulation of reactive oxygen species (ROS), including heme oxygenase 1, superoxide dismutase 1 and 2, and catalase. Changes were also observed in genes involved in cell growth, proliferation, and the cell cycle such as cell division cycle 25B, signaling lymphocyte activating molecule (SLAM), calgizzarin S100A11, B-cell translocation gene, Src-like adapter protein (SLAP), and eukaryotic translation initiation factor 4E binding protein 2. These overnight changes in OSA patients are suggestive of activation of several mechanisms to modulate, and adapt to, increased ROS developing in response to the frequent episodes of intermittent hypoxia. *Antioxid. Redox Signal*. 9, 661–669.

INTRODUCTION

Obstructive sleep apnea (OSA) is the commonest sleep-related breathing disorder, with an ~25% prevalence in the adult United States population (50). OSA is characterized by recurrent episodes of cessation of respiratory airflow during sleep, leading to sleep fragmentation and periodic and often severe intermittent hypoxia (IH). OSA has been linked to an increased incidence of cardiovascular diseases, including hypertension (23, 28), atrial fibrillation (13), arrhythmias (6), coronary artery disease (19–21), and heart failure (15, 38). Sympathetic activation (39) and systemic inflammation (25, 26, 36) elicited by OSA have been implicated as possible causes of increased cardiovascular risk.

Heightened oxidative stress found in OSA may also be an important contributor to altered cellular signaling pathways (16, 43). Recurrent hypoxia and reoxygenation characteristic of OSA could indeed potentially cause increased reactive oxygen species (ROS) generation. Higher levels of thiobarbituric acid-reactive substance (TBARS) (2), oxidized LDL (16), and urinary excretion of 8-hydroxy-2'-deoxyguanosine (49), and data suggesting higher ROS production in phorbol ester-stimulated monocytes and granulocytes derived from OSA patients (5), are suggestive of increased ROS in OSA. On the other hand, several studies relating to susceptibility of LDL to oxidative stress, glutathione and lipid peroxidation, osmotic fragility of red blood cells, increased number or activity of neutrophils, and concentrations of oxLDL, TBARS,

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Table 1. Baseline Characteristics of Healthy Controls versus OSA Patients

	Healthy (n = 4)	*OSA (n = 4)*	*p*
AGE	37.5 ± 5.06	40.7 ± 9.59	NS
BMI (kg/m^2)	32.6 ± 3.59	35.5 ± 1.29	NS
Basal O_2 sat	96.9 ± 0.8	96.75 ± 0.5	NS
AHI, events/h	0.6 ± 0.7	50.25 ± 23.5	0.0001
Mean nocturnal O_2 sat	96.2 ± 1.02	93.75 ± 2.12	0.001
Lowest nocturnal O_2 sat	92.16 ± 3.68	59.5 ± 17.13	0.005
% of sleep time with Hb sat >90%	100	85.75 ± 10.24	0.03

and isoprostanes, failed to show evidence for increased ROS in OSA (22, 27, 44).

One possible explanation for the inconsistency of available data regarding oxidative stress in OSA is that OSA may also induce adaptive compensatory mechanisms that protect against oxidative stress, such that indices of systemic oxidative damage are not always evident in *in vivo* conditions. Therefore, in this study, using microarray techniques, we investigated whether OSA activates genomic adaptive regulatory responses that could modulate generation of ROS and induce mechanisms that may attenuate oxidative stress in blood cells. Since oxidative stress may affect various aspects of cellular growth and proliferation, we also evaluated changes in expression of genes involved in regulation of the cell cycle.

MATERIALS AND METHODS

Human subjects

We recruited 8 male subjects: 4 healthy controls and 4 patients with newly diagnosed, never-treated, severe OSA (AHI >30), without any other co-morbidities. None of the subjects was smoking or taking any medications. Patients and controls were of similar age and had similar body mass index (BMI) (Table 1). Subjects were admitted to the General Clinical

Table 2. Genes Involved in Modulation of Reactive Oxygen Species, and Function of the Coded Proteins

Name of gene	*Function of coded protein*	*Baseline OSA vs. control (at 9 P.M.)*	*Overnight % change OSA vs. control*
Heme oxygenase1 (HMOX1)	32-kDa heat shock protein, induced by stress and protects cell from oxidative damage (29)	↑, 0.01	↓, 0.02
Superoxide dismutase 1 (SOD1, Cu/Zn)	Endogenous antioxidant in mammalian cells, catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen (4)	↑, 0.008	↓, 0.02
Superoxide dismutase 2 (SOD2, Mn)	Involved in defense against toxic superoxide (O_2^-), protects macrophages from oxidative stress (31)	↓, NS, 0.38	↑, 0.008
Catalase	Antioxidant enzyme that detoxifies hydrogen peroxide by converting it to water and oxygen, thereby preventing cellular injury (33)	↑, NS, 0.53	↑, 0.10
Peroxiredoxin 5	Role in protecting the genome against oxidation (14)	NS, 0.11	NS, 0.97
Peroxiredoxin 4	Induces in a stress-specific fashion to protect human cells from oxidant injury (37)	NS, 0.37	NS, 0.39
Thioredoxin reductase	Member of a family of pyridine nucleotide oxidoreductases, plays a role in protection against oxidative stress (9)	NS, 0.67	NS, 0.16
Thioredoxin	Multiple functions in regulation of cell growth, apoptosis, and activation, constitutes an endogenous antioxidant system (30)	NS, 0.45	NS, 0.18
Thioredoxin interacting protein (TXNIP)	Inhibits antioxidative function by inhibition of the thioredoxin ROS-scavenging system (11)	NS, 0.83	NS, 0.23
Glutathione peroxidase 1	Member of the glutathione peroxidase family, functions in the detoxification of hydrogen peroxide, and is an important antioxidant enzyme in humans (1)	NS, 0.98	NS, 0.45

Numbers shown in right hand columns are *p* values.

Research Center unit at 5.30 P.M., and underwent full polysomnography. Blood was collected at two time points, before sleep (at 9 P.M., 4 h after the last meal), and then directly after waking from sleep (at 6 A.M.). Informed written consent was obtained from all subjects. The study was approved by the Institutional Human Subjects Review Committee.

Microarray experiment

The blood-containing tubes were initially incubated at room temperature for 3 h to stabilize cellular RNA, followed by its isolation using PAX Gene RNA isolation kit (Qiagen, Chatsworth, CA) according to manufacturer's instructions, and then processed for microarray experiments as previously described (41). Briefly, total RNA (2 μg) was converted to cDNA using the Superscript cDNA synthesis kit (Gibco-BRL, Gaithersberg, MD). Double-stranded cDNA was then purified by phase lock gel (Eppendorf, Westbury, NY) with phenol/chloroform extraction. The purified cDNA was used as a template for *in vitro* transcription reaction for the synthesis of biotinylated cRNA using RNA transcript labeling reagent (Affymetrix, Santa Clara, CA). These labeled cRNAs were then fragmented and hybridized onto the HG-U133A and B arrays (Affymetrix). Following hybridization, the solutions were removed, arrays were washed and stained with streptavidin–phycoerythrin (Molecular Probes, Eugene, OR). Following washes, arrays were scanned using GeneChip scanner 3000 made by Affymetrix.

Microarray data analysis

The microarray data were analyzed using Spotfire™ 7.2 -commercially available software (Spotfire Inc., Cambridge, MA). The level of gene expression for each subgroup was presented as an average with standard deviation.

Statistical analysis

The treatment comparison application using ANOVA was used in order to identify statistically significant differences in gene expression among the groups. Data are presented as mean ± SD for continuous variables, and as number and percentages for categorical variables. Paired and unpaired two-sample equal variance Students *t* test were used to determine statistical significance of differences and changes between and within the study groups, respectively. p Values < 0.05 were considered statistically significant.

RESULTS

The baseline characteristics and sleep profiles of the control subjects and OSA patients are shown in Table 1. Only sleep profiles (specifically nocturnal oxygen saturation and AHI) differed significantly between the two groups, indicating the presence of severe oxygen desaturations and apneic events in the OSA group.

Using ANOVA analysis of the microarray data before and after sleep, we identified significant differences in gene expression between subjects with and without OSA, as well as several sleep-induced changes in gene expression. The observed differences and changes were related to genes encoding antioxidant enzymes (Table 2), as well as proteins involved in cell cycle regulation and growth (Table 3).

TABLE 3. GENES MODULATING THE CELL CYCLE AND FUNCTION OF THE CODED PROTEINS

Name of gene	*Function of the coded protein*	*Baseline OSA vs. control (at 9 P.M.)*	*Overnight % change OSA vs. control*
Ribonucleotide reductase M1 polypeptide	Involved in DNA synthesis crucial for S phase of cell cycle (10)	NS, 0.17	NS 0.44
Cell division cycle 25 B (CDC25B)	Belongs to CDC25 phosphatases family, activates CDC2 thus enabling the cell to enter the mitotic phase (47)	↑, 0.01	↓, 0.01
Eukaryotic translation elongation factor2	Protein translation (7)	NS, 0.59	NS, 0.14
Signaling lymphocyte activating molecule (SLAM)	Co-stimulates T lymphocyte proliferation and IFN gamma synthesis (48)	↑, 0.10	↓, 0.03
Calgizzarin S100A11	Calcium induced growth inhibitor (35)	NS, 0.68	↑, 0.04
B-cell translocation gene	Inhibits cell cycle in G0/G1 phase (34)	NS, 0.61	NS, 0.43
Src-like adapter protein (SLAP)	Participates in T cell receptor signal transduction, negative mitosis regulator (32, 40)	NS, 0.88	↑, 0.05
Eukaryotic translation initiation factor 4E binding protein	Binds to eIF4E and inhibits protein translation (7)	↓, NS, 0.13	↑, 0.00007
Suppressor of Lin-12 *C. elegans*- like (Sel1)	Inhibits transcription of factors responsible for the cell growth (3)	NS, 0.97	NS, 0.18

Numbers shown in right hand columns are *p* values.

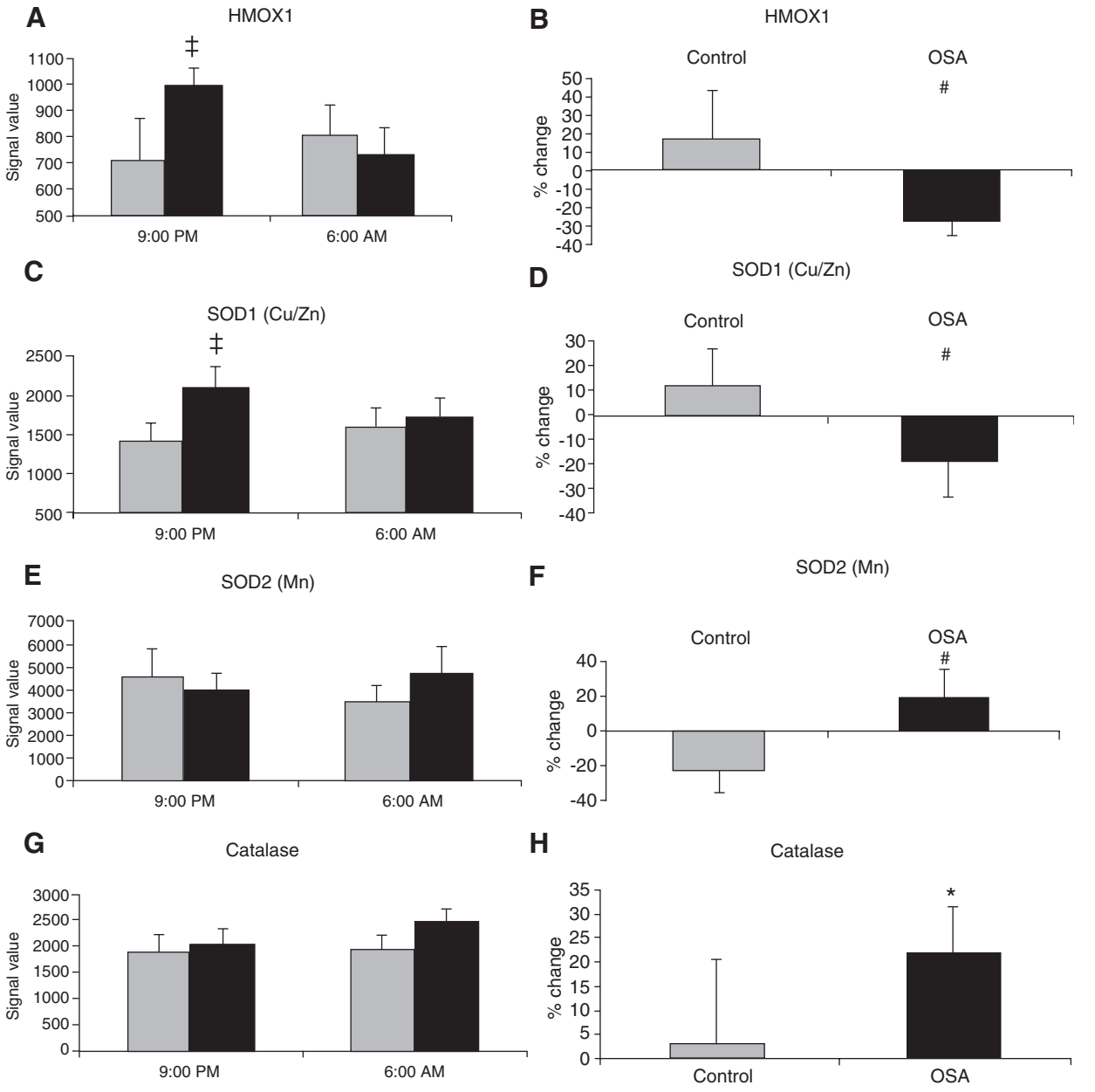

FIG. 1. Expression profiles of genes involved in ROS modulation in healthy controls (*gray bars*) and OSA subjects (*black bars*). *Left panels* show measures at 9 P.M. and 6 A.M. *Right panels* show overnight % changes. ‡$p < 0.05$ for OSA vs. controls at baseline (9 P.M.); #$p < 0.05$ for overnight % changes in OSA vs. controls; *$p > 0.05$ <0.10 for overnight % changes in OSA vs. controls.

Genes related to oxidative stress

The transcript levels of heme oxygenase 1(HMOX1) gene measured at night (9 P.M.) were higher in OSA patients than in healthy subjects ($p = 0.01$). However, HMOXI mRNA expression in OSA patients decreased during sleep to the same levels as observed in healthy controls (change in OSA vs. change in controls: $p = 0.021$) (Fig. 1A and B).

The gene transcript levels of superoxide dismutase 1 (cytoplasmic isoform: Cu/Zn, SOD1) at night were also much higher in OSA patients (OSA vs. controls, $p = 0.008$) than in healthy controls. SOD1 transcript levels also declined overnight in OSA patients, but remained unchanged in controls ($p = 0.02$) (Fig. 1C and D). Superoxide dismutase 2 (mitochondrial isoform: Mn, SOD2) had similar baseline activity in controls and OSA subjects. In the control group the gene transcript level decreased during sleep ($p = 0.06$), whereas SOD2 activity increased after repetitive nocturnal hypoxemia in OSA subjects (Fig. 1E). Consequently, overnight changes in SOD2 transcript levels in OSA vs. control subjects were significantly different ($p = 0.008$) (Fig. 1F). Although the transcript level of catalase gene at night before sleep was similar in OSA and control groups (Fig. 1G),

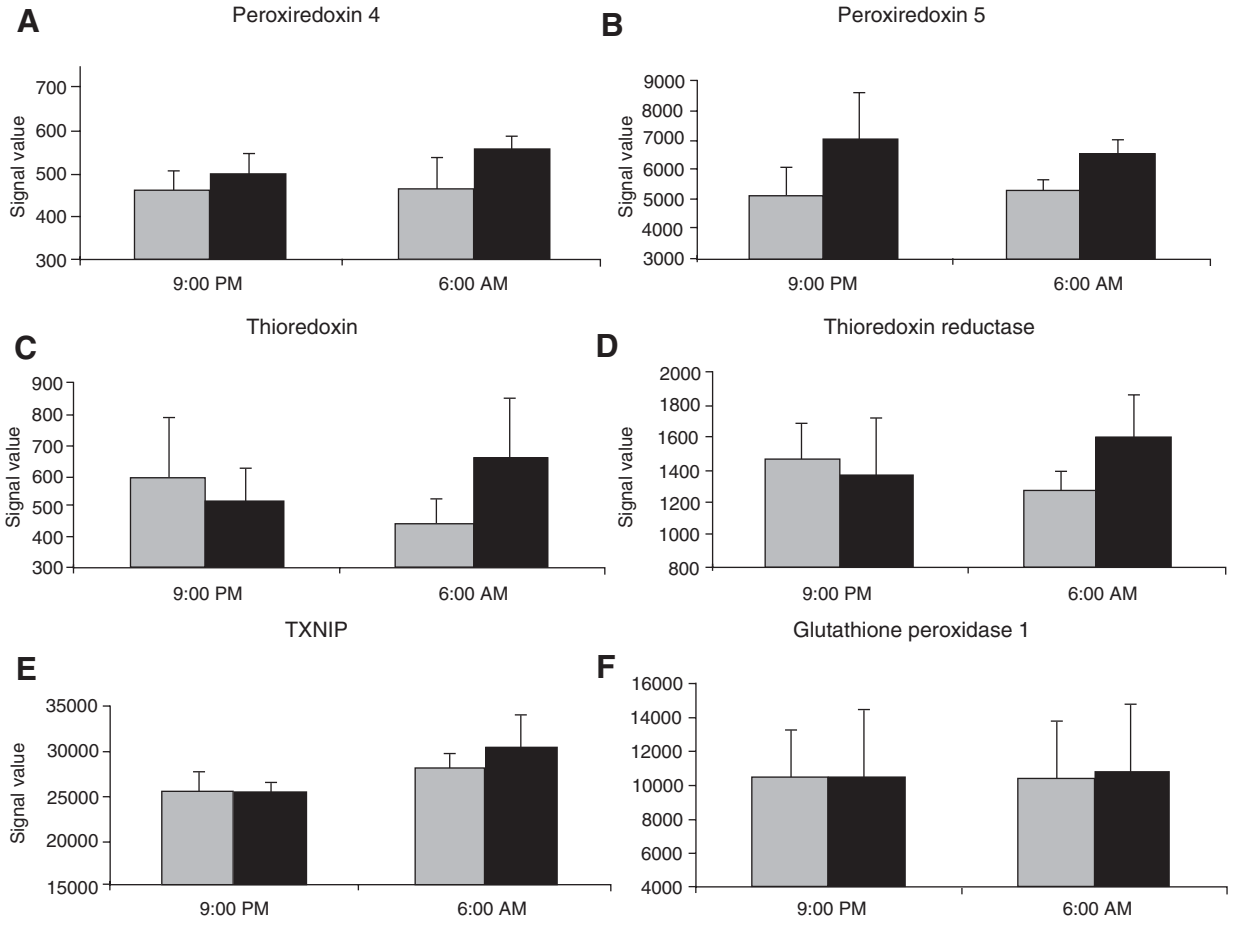

FIG. 2. Expression profiles of the genes involved in ROS modulation in healthy controls (*gray bars*) and OSA subjects (*black bars*).

catalase gene transcripts increased in response to overnight apneic sleep but did not change after a night of normal sleep (OSA vs. controls overnight % change: $p = 0.10$) (Fig. 1H)

Peroxiredoxin 4 (Fig. 2A) and peroxiredoxin 5 (Fig. 2B), thioredoxin (Fig. 2C), and thioredoxin reductase (Fig. 2D) gene transcript levels were not significantly different in OSA patients in comparison with controls at night. Even severe overnight hypoxemia in sleep apneics did not change these transcript levels significantly as compared to levels seen in control subjects after healthy normal sleep. We did not observe any significant differences in thioredoxin interacting protein (TXNIP), an endogenous inhibitor of thioredoxin (Fig. 2E), nor in glutathione peroxidase gene transcript levels (Fig. 2F) at any time point between the OSA and control groups.

Genes related to cell cycle and proliferation

Microarray measures of gene transcript levels of several proteins involved in cell proliferation, activation, and growth were also determined. Both cell division cycle 25B (Fig. 3A) and signaling lymphocyte activating molecule (SLAM) ($p = 0.10$) (Fig. 3C) were higher at baseline (9 P.M.) in OSA than in controls. However, after overnight hypoxemia in OSA patients, gene transcripts levels of both of these decreased significantly (Fig. 3B and D) in comparison to changes seen after overnight sleep in normal controls (Table 3). Significant changes after overnight sleep in OSA versus control subjects were also observed in genes coding for calgizzarin (Fig. 3E and F), Src-like adapter protein (SLAP) (Fig. 3G and H), and eukaryotic translation initiation factor 4E binding protein 2 (Fig. 3I and K). Changes observed in these gene transcript levels after overnight sleep in OSA patients showed significant differences as compared to control subjects. However, we did not observe any significant differences either at baseline measurements or overnight changes between control and OSA subjects in ribonucleotide reductase M1 polypeptide (Fig. 4A), eukaryotic translation elongation factor 2 (Fig. 4B), B-cell translocation gene (Fig. 4C), and suppressor of Lin-12 *C.elegans*-like (Sel1) (Fig, 4D).

DISCUSSION

Our novel findings include that several genes coding for enzymes involved in modulation of reactive oxygen species

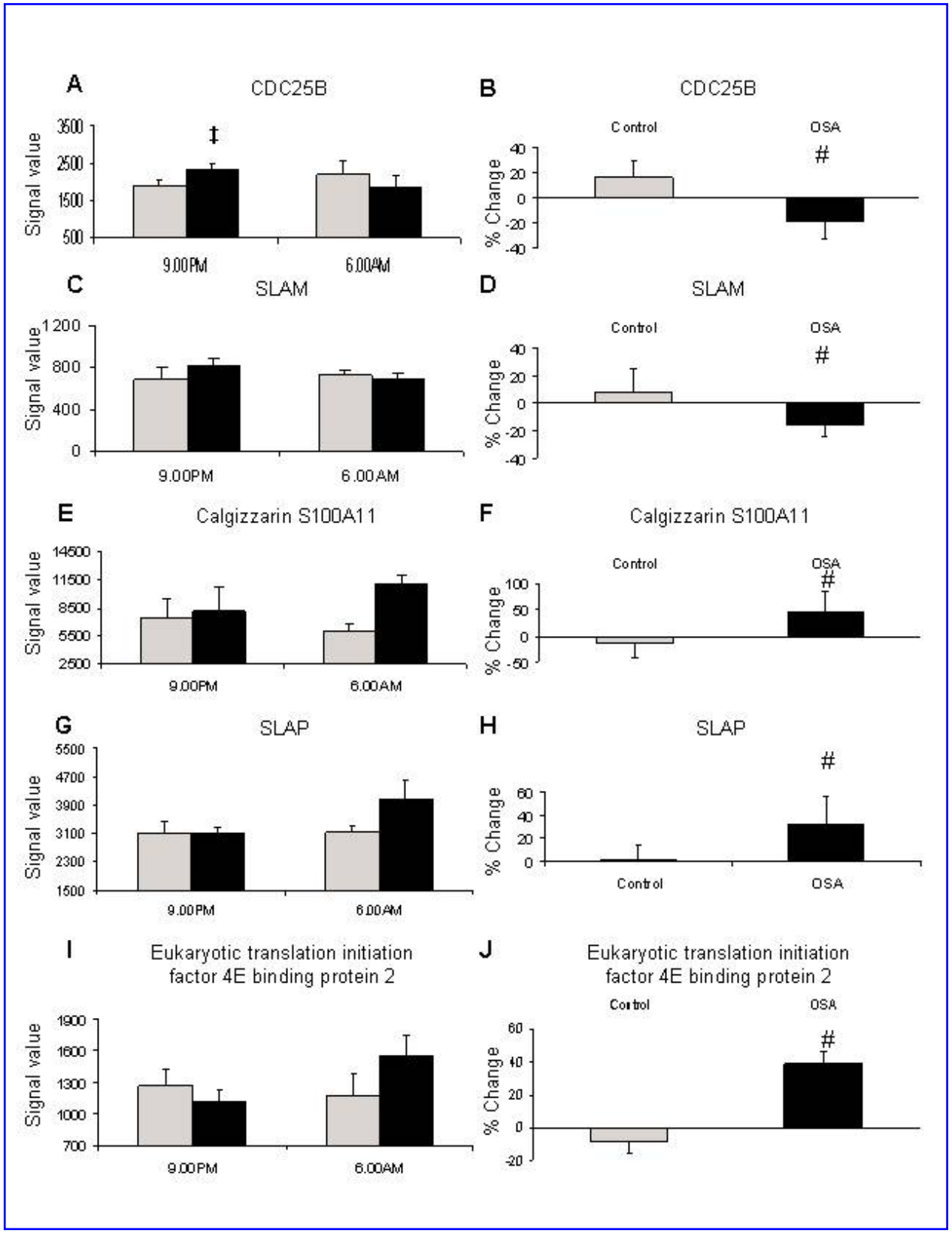

FIG. 3. Expression profiles of genes involved in cell growth, proliferation or cell cycle in healthy controls (*gray bars*) and OSA subjects (*black bars*). *Left panels* show measures at 9 P.M. and 6 A.M.. *Right panels* show overnight % changes. ‡$p < 0.05$ for OSA vs. controls at baseline (9 P.M.); #$p < 0.05$ for overnight % changes in OSA vs. controls.

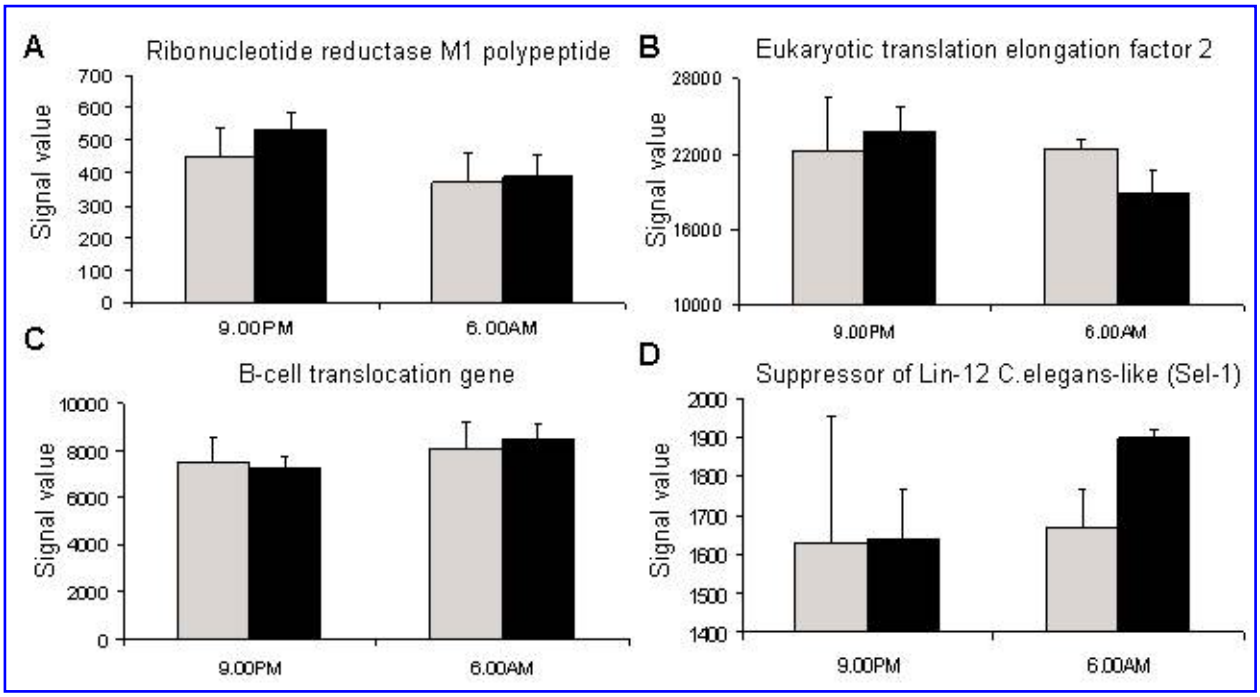

FIG. 4. Expression profiles of genes involved in cell growth proliferation or cell cycle in healthy controls (*gray bars*) and OSA subjects (*black bars*).

are differentially expressed in subjects with and without OSA and that the transcription of several genes may change acutely overnight during apneic sleep. These genes include those which are directly involved in lowering ROS levels, such as increased expression of catalase, and SOD2, along with increased basal expression of HMOX1. We further observed that genes that modulate the cell cycle are also altered in response to overnight apneic sleep, so as to potentially attenuate cell growth and proliferation. These may serve as further adaptive mechanisms to limit cell death and damage in response to oxidative stress.

Oxidative stress

White blood cells play an important role in the inflammatory process and can be a potent source of ROS. ROS are extremely reactive molecules that interact with cellular lipids, proteins, and nucleic acids, thus eliciting cellular oxidative stress (8, 12, 18). ROS may alter intracellular signaling and hence cellular functions including growth, cell division, and perhaps adaptive mechanisms (45, 46). Therefore, compensatory induction of antioxidant mechanisms, which can in turn modulate ROS generation and breakdown, may be of great importance for the maintenance of cell homeostasis.

After overnight apneic sleep, we noted activation of catalase and SOD2 gene transcripts, which would suggest activation of antioxidant mechanisms (24). These genes were not activated after a night of normal sleep in control subjects. Even though we observed a decrease in SOD1 and HMOX1 gene transcript levels after apenic sleep, their baseline activity before sleep was significantly elevated in OSA subjects as compared to control subjects. These changes in gene transcript levels observed specifically in OSA subjects are suggestive of responses generated so as to perhaps defend the cell against increased ROS during apneic sleep.

Thus, cellular mechanisms activated after overnight hypoxemia may act to reduce oxidative stress (increased catalase and SOD2 gene expression). Elevated SOD1 and HMOX1 even before sleep may also diminish oxygen radicals.

Cell cycle

Significant changes in another group of genes involved in cell cycle regulation and growth were observed in OSA patients after sleep. These included decreased gene transcript levels of cell division cycle 25B and SLAM, genes which promote cell division and proliferation. We also noted increased gene transcript levels after overnight apneic sleep in calgizzarinS100A11, SLAP, and eukaryotic translation initiation factor 4E binding protein. These genes are known to attenuate the cell cycle and are considered to be anti-proliferative (3, 17, 32, 34, 35, 40, 42). The most vulnerable phase during the cell cycle is DNA synthesis and mitosis during which excessive environmental stress can lead to cellular death. One of the mechanisms by which the cell may protect itself is by arresting or attenuating growth during such conditions.

SUMMARY AND IMPLICATIONS

Our data may provide some insight into inconsistencies in earlier studies, which are indicative either of increased ROS generation in OSA (2, 5, 49) or of no increase in ROS (22, 27, 44). Increased ROS generation during sleep may not be easily observed because of ROS modulation that may be dependent perhaps on duration of untreated OSA, existence of other comorbidities, medications, and other factors. Timing of blood sampling for oxidative stress measurements would also be important. Many of the differences between OSA and control

subjects that we observed were acute, evident only after overnight intermittent hypoxemia secondary to untreated OSA.

Limitations of our data include that our findings are dependent on mRNA transcripts and do not necessarily reflect protein expression and activity. The gene profiles we report would be indicative of cellular first responses to oxidative stress, which could be modulated in downstream processes. Hence, further studies are needed at the protein level. In addition, we studied only those OSA patients free of other comorbidities. While this allowed us to evaluate the effects of OSA per se, independent of medications or co-existing disease conditions, further investigation in larger sample sizes of OSA patients, including those with overt cardiovascular disease, are needed to verify whether the responses we describe are preserved or altered.

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ABBREVIATIONS

AHI, apnea hypopnea index; BMI, body mass index; CDC25B, cell division cycle 25B; cDNA, complementary deoxyribonucleic acid; HMOX1, heme oxygenase 1; IH, intermittent hypoxia; LDL, low density lipoprotein; OSA, obstructive sleep apnea; RNA, ribonucleic acid; ROS, reactive oxygen species; SD, standard deviation; Sel-1, suppressor of Lin-12 C.elegans- like 1; SLAM, signaling lymphocyte activating molecule; SLAP, Src-like adapter protein; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substance; TXNIP, thioredoxin interacting protein.

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