Article

J. Biol. Regul. Homeost. Agents. 2024; 38(5): 4453–4464
https://doi.org/10.23812/j.biol.regul.homeost.agents.20243805.354

Elevated Monocytic Angiopoietin-2 Expression under Intermittent Hypoxia and in Patients with Obstructive Sleep Apnea

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Published: 1 May 2024

Background: Angiopoietin-2 (Ang-2), a significant chemokine influencing monocyte chemotactic migration, was investigated under conditions of intermittent hypoxia and in obstructive sleep apnea (OSA) patients. This study aimed to elucidate the chemotactic impact of Ang-2 on monocytes during intermittent hypoxic conditions and to assess changes in the circadian concentration of Ang-2 in individuals with OSA.
Methods: The OSA dataset GSE135917 was downloaded, and the Gene Set Enrichment Analysis (GSEA) method was employed to investigate the association between Ang-2 expression and potential signaling pathways in OSA. Monocytic THP-1 cells were utilized to examine the modulation of Ang-2 under intermittent hypoxia. Subsequently, the chemotactic motility of THP-1 cells was evaluated using a Transwell migration assay, and the number of migrating cells was quantified through flow cytometry. Monocyte RNA was isolated from peripheral venous blood obtained from 60 adult OSA patients and 60 healthy controls to conduct an *Ang-2* mRNA expression study.
Results: Bioinformatic analysis indicated that pathways significantly associated with high Ang-2 expression were predominantly enriched in extracellular regulated protein kinases (ERKs), phosphatidylinositol 3 kinase/serine-threonine kinase (PI3K/AKT), and nuclear factor kappa-B (NF-κB) signaling pathways. Experimental results demonstrated that intermittent hypoxia actively enhanced the expression of Ang-2 in monocytic THP-1 cells and facilitated the migration of THP-1 cells. Evidence suggested intermittent hypoxia induced the upregulation of Ang-2 expression via PI3K, ERKs, and NF-κB pathways. Additionally, Ang-2 expression in peripheral blood mononuclear cells was elevated in OSA patients, correlating with disease severity. Furthermore, *Ang-2* mRNA expression in the OSA group was higher than in the control group.
Conclusion: Ang-2 levels are elevated in OSA patients and are correlated with disease severity. Increased monocytic expression of Ang-2 is closely associated with intermittent hypoxia induced by OSA.

Keywords: chemotaxis; angiopoietin-2; intermittent hypoxia; monocytes; obstructive sleep apnea

Introduction

Obstructive sleep apnea (OSA) is a prevalent clinical condition affecting over 10% of adults [1]. Its pathogenesis is characterized by recurrent partial or complete upper airway obstruction during sleep, resulting in sleep fragmentation and intermittent hypoxia [2]. Growing research indicates that OSA is a significant and independent risk factor for cardiovascular diseases (CVDs), including nocturnal arrhythmias, heart failure, myocardial infarction, and pulmonary hypertension [3]. Current treatments for OSA encompass drug therapy, oral appliances, nerve stimulation, oxygen therapy, and ventilator therapy. Surgical intervention becomes an option when patients are unsuitable or unresponsive to these treatments [4,5]. Pharyngoplasty, a surgical procedure tailored for OSA patients, has been shown to regulate the autonomic function of the heart, providing potential therapeutic benefits. Süslü *et al*. [6] reported that OSA patients undergoing pharyngoplasty exhibited significantly reduced sympathetic activity, correlating with improved surgical outcomes and lower apnea-hypopnea index (AHI).

Current evidence suggests that intermittent hypoxia activates inflammatory pathways in circulating monocytes, a pivotal step leading to endothelial damage [7,8]. During inflammation, the inflammatory region is infiltrated with mononuclear cells such as monocytes, macrophages, and lymphocytes, producing diverse inflammatory mediators, including pro-inflammatory cytokines [9]. One of the most extensively studied signaling pathways related to monocyte-endothelial adherence is the mitogen-activated protein kinase (MAPK) signaling pathway, which consistently triggers inflammatory reactions and the production of adhesion molecules. Oxidative stress activates the inflammatory gene promoter of monocytes through the nuclear factor κB pathway, activating related mRNA to produce inflammatory factors subsequently [10].

Numerous studies have reported elevated levels of angiopoietin-2 (Ang-2) in OSA patients, supporting the notion that endothelial dysfunction plays a pivotal role in OSA pathogenesis [11]. Ang-2 is primarily secreted by endothelial cells and plays a crucial role in vascular development, stabilization, and monocyte activation [12]. Acting as an inhibitor of Ang-1, Ang-2 competitively binds to the Tie-2 receptor, blocking the effects of Ang-1, thereby promoting inflammatory responses and capillary leakage [13]. While Ang-2 is typically expressed at lower levels in normal vessels, its expression is significantly upregulated in various inflammatory and angiogenic settings [14].

An increasing number of clinical studies have shown significantly elevated serum levels of inflammatory mediators in OSA patients, which positively correlate with OSA severity, indicating the presence of local and systemic inflammation [15]. Carvalheiro *et al*. [12] proposed that Ang-2 induces the synthesis of Interleukin-6 (IL-6) and Interleukin-8 (IL-8) in monocytes in patients with systemic sclerosis. Chuang *et al*. [16] revealed that intermittent hypoxia enhances the active expression of IL-8 by monocytic TPH-1 cells at the secreted protein and mRNA levels, subsequently increasing monocyte migration potential towards IL-8. Thus, it is pertinent to investigate whether Ang-2 can direct the chemotactic migration of monocytes.

While some literature indicates elevated circulating Ang-2 levels in OSA patients [17,18], the underlying reasons and mechanisms underlying this phenomenon remain elusive. Furthermore, no existing research has addressed whether "intermittent hypoxia" activates monocytes to produce more Ang-2, potentially promoting subsequent atherosclerosis formation. Therefore, we conducted this research to investigate the influence of intermittent hypoxia (IH) on Ang-2 expression in monocytes and the associated signaling pathways implicated in Ang-2 modulation. Additionally, to evaluate the diurnal variation of sleep apnea effects, we evaluated the concentration of Ang-2 in monocytes extracted from the blood of patients with OSA. We hypothesize that OSA patients exhibit elevated plasma Ang-2 levels due to intermittent hypoxia-induced activation of monocytes, leading to increased Ang-2 production.

Materials and Methods

Bioinformatics Analysis

Dataset Collection

The GSE135917 dataset, comprising mRNA expression profiles [19] and boasting the largest sample size, was obtained from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). This dataset offers insights into the dynamic nature of gene expression in OSA. Among the subcutaneous adipose tissue samples in the GSE135917 dataset, 66 were initially included. However, 24 patients treated with continuous positive airway pressure (CPAP) therapy, which might have influenced transcriptomic profiles, were excluded. Additionally, ten individuals with undetermined OSA severity were also excluded. Consequently, 24 patients with severe OSA and 8 healthy controls were retained for this study. The R software (Auckland, New Zealand, R Project for Statistical Computing, http://www.r-project.org/, version 3.6.1) was used to annotate probes with gene symbols based on the microarray platform (GPL6244). Subsequently, we employed the "normalizeBetweenArrays" command in the "limma" package to standardize the data following the first normalization of the expression profiles.

Gene Set Enrichment Analysis (GSEA)

Gene sets from GSEA and REACTOME (http://www.gsea-msigdb.org/gsea/msigdb/) were utilized to elucidate the molecular mechanisms underlying the relationship between Ang-2 expression and OSA. The median value of Ang-2 expression served as the cutoff point. The 24 patients with severe OSA were divided into two groups based on their Ang-2 expression levels (high- and low-expression). Given the evaluation of an entire gene set database, we adjusted the significance level estimation to accommodate multiple hypothesis testing [20]. A maximum of 1000 permutations was selected. The top five terms from pathway and REACTOME analyses were presented. Cutoff values were determined based on clinical relevance and previous studies. Gene sets with $|NES| > 1$, NOM $p < 0.05$, and false discovery rate (FDR) $q < 0.25$ were considered to be significantly enriched [21]. GSEA plots were generated using the "plyr", "ggplot2", and "grid" packages in R version 3.6.1.

Materials

The anti-Ang-2 blocking antibody used in this study was provided by Adipogen Life Science (Liestal, Switzerland). The human angiopoietin-2 enzyme-linked immunosorbent assay (ELISA) kit (ab99971, Range: 4.12 pg/mL–3000 pg/mL, Product specification: 1× 96 tests) and angiopoietin-2 antibody (ab155106) were procured from Abcam PLC (Cambridge, UK). Extracellular regulated protein kinases (ERKs) inhibitor PD98059, nuclear factor kappa-B (NF-κB) inhibitor Bay11-7082, and phosphatidylinositol 3 kinase (PI3K) inhibitor LY294002 were obtained from Sigma Inc. (St. Louis, MO, USA). For control experiments, the nonspecific antibody mouse IgG1, Kappa Monoclonal (NCG01)-Isotype Control-BSA and Azide free, was purchased from Abcam PLC (Cambridge, UK).

Monocyte Culture

THP-1, a human monocytic leukemia cell line, was procured from the American Type Culture Collection (Procell Life Science & Technology Co., Ltd., Wuhan, China) with the catalog number CL-0233. The cells were authenticated by short tandem repeat (STR) analysis, and mycoplasma testing yielded negative results. THP-1 cells were cultured in RPMI 1640 medium supplemented with antibiotics and 10% fetal bovine serum (FBS). Cultures were maintained in a humidified condition with 5% CO_2 and 95% air at 37 °C. Passaging was performed using fresh medium at a dilution ratio of 1:4.

Intermittent Hypoxic Incubation Conditions

THP-1 cells (1×10^6 cells/mL) were suspended in 5 mL of RPMI 1640 medium in a 5 cm diameter culture plate. Intermittent hypoxia was simulated using the OxyCycler C42 system (BioSpherix, New York, NY, USA). This system, equipped with a dual gas controller (OxyCycler C42, BioSpherix, New York, NY, USA) connected to a modular sub-chamber, allowed precise control of oxygen, nitrogen, and carbon dioxide levels. Oxygen and carbon dioxide concentrations were monitored using the sensors of the device, including fiber optic oxygen sensors employing the fluorescence quenching technique [22]. As previously described [23], the monocytic THP-1 cells were exposed to either normoxic conditions (21% oxygen, 5% carbon dioxide, with the remaining nitrogen) or intermittent hypoxic conditions (35 minutes of hypoxic exposure (0.1% oxygen, 5% carbon dioxide, with the remaining space filled with nitrogen) followed by 25 minutes of returned normoxia (21% oxygen, 5% carbon dioxide, and nitrogen) for one cycle) up to six cycles. Subsequently, cells subjected to intermittent hypoxia were transferred to a standard incubator (20220615, Thermo Inc., Waltham, OH, USA) (21% oxygen, 5% carbon dioxide, and nitrogen) at 37 °C for an additional 18 hours before subsequent analyses.

RNA Isolation and Real-Time PCR

Total cellular RNA was isolated by lysing the cells in a guanidinium isothiocyanate solution by a single-step extraction using phenol, chloroform, and isoamyl alcohol. M-MLV reverse transcriptase (20221215, USB Corporation, Cleveland, OH, USA) was used to transcribe total RNA into cDNA. The following sequences below were employed as PCR primers: Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) forward primer 5′-GAAGGTGAAGGTCGGAGTC-3′ and reverse primer 5′-GAAGATGGTGATGGGATTTC-3′ and *angiopoietin-2* forward primer 5′-GAGATGGACAACTGCCGCTCTTC-3′ and reverse primer 5′-GTTTGCTCCGCTGTTTGGTTCAAC-3′. Real-time quantitative PCR (RT-qPCR) was conducted using standard cycling conditions (95 °C pre-denaturation 30 s; 95 °C denaturation for 5 s; 60 °C annealing/extension for 30 s; a total of 40 cycles; followed by 1 min at 50 °C and 30 s at 70 °C). The MxPro program (WG23BK50, Agilent Technologies, Palo Alto, CA, USA) was utilized to calculate the cycle threshold (Ct) values. mRNA expressions were analyzed using the $2^{-\Delta\Delta Ct}$ calculation method after normalization with *GAPDH*.

Enzyme-Linked Immunosorbent Assay (ELISA)

A human angiopoietin-2 ELISA kit was utilized to assess the concentration of angiopoietin-2 in plasma and culture medium. The ELISA utilized a fixed plasma dilution of 1:200. The diluted capture antibody was added to a 96-well microplate and incubated overnight at room temperature. Subsequently, the microplate was washed, and blocking was performed using 300 µL of a diluent reagent for one hour. Specimens and standards, diluted with the diluent reagent, were then added to the microplate and incubated for two hours. After washing, 100 µL of the detection antibody was added, and the mixture was incubated for an additional two hours. Subsequently, 100 µL of streptavidin- horseradish peroxidase (HRP) was added, and the mixture was incubated for 20 minutes. The reaction was stopped by adding 50 µL of stop solution. The absorbance of the sample was measured at 450 nm using a microplate reader (20221230, Tecan Sunrise ELISA Reader, Morrisville, NC, USA) to determine the concentration of angiopoietin-2 present in the sample.

Western Blot Analysis

The Western blotting procedure was conducted following previously reported methods [24]. A Micro BCA protein assay reagent kit (Thermo Scientific, Cat No. 23235, Rockford, IL, USA) was used to measure protein content accurately. Subsequently, protein samples were electrophoresed on SD-PAGE to separate protein bands. The proteins were then transferred from the gel onto a PVDF membrane and blocked with 5% nonfat dry milk for 2 hours. The membrane was incubated overnight at 4 °C with a primary antibody (Rabbit monoclonal [EPR2891(2)] to Angiopoietin-2 (Ang-2), ab155106, dilution 1:2000; Abcam, Cambridge, UK), followed by incubation with a secondary antibody (Mouse monoclonal [SB62a] Anti-Rabbit IgG light chain, ab99697, dilution 1:1000; Abcam, Cambridge, UK) for 2 hours. Immunoreactive proteins were visualized using enhanced chemiluminescence (ECL; 16946863, GE Healthcare, Chicago, IL, USA) and detected with an ImageQuant 350 imager (20201230 ,GE Healthcare, Chicago, IL, USA). The relative grey value was calculated by visualizing the bands through the ECL method and quantifying the overall grey value of the protein bands (average grey value/grey value area). GAPDH (Rabbit polyclonal to GAPDH - Loading Control, ab9485, dilution 1:1000; Abcam, Cambridge, UK) served as an internal reference, with the target protein grey value normalized to the internal reference overall grey value.

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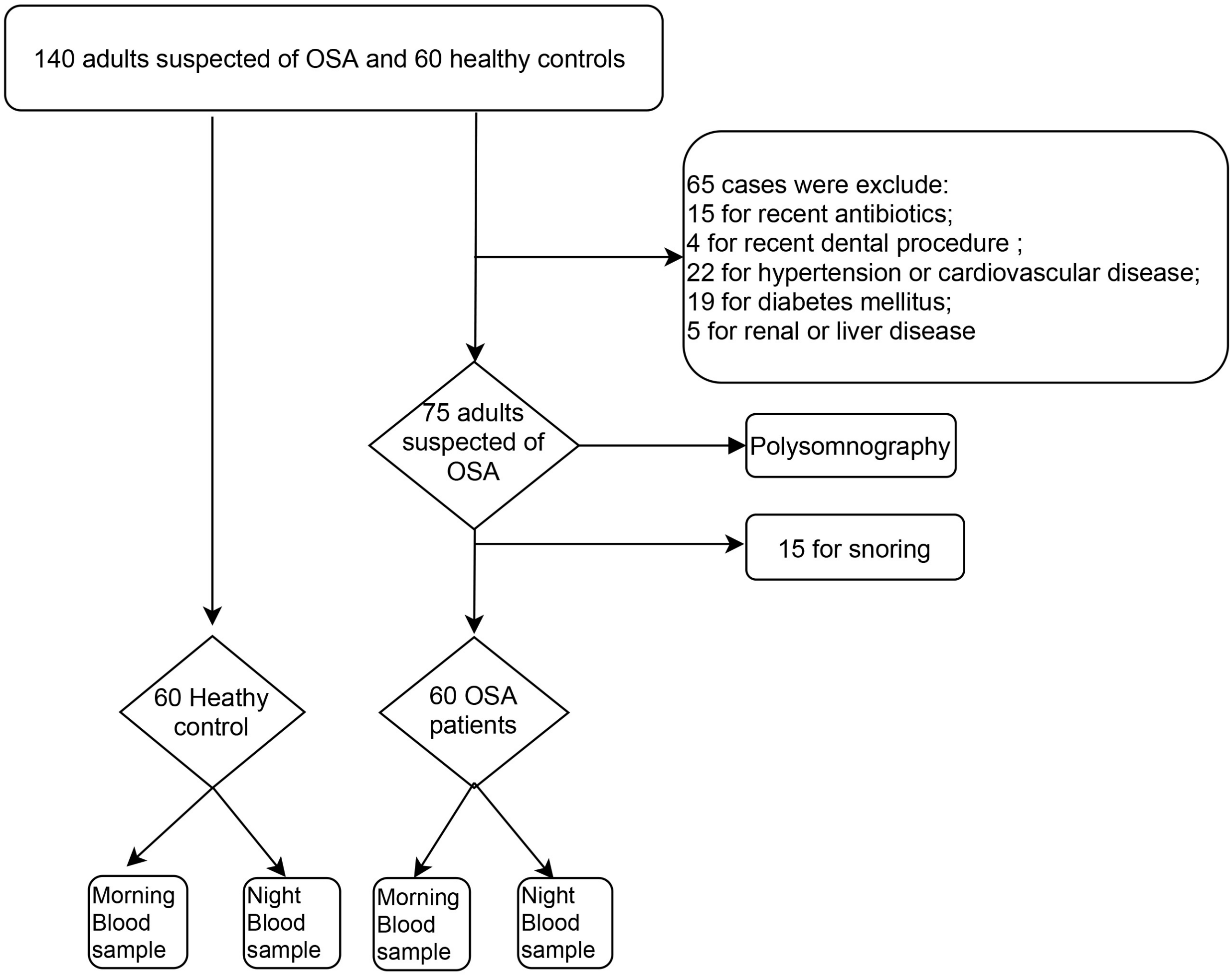

Fig. 1. The flowchart showing the patients screened and included in the study. OSA, obstructive sleep apnea.

siRNA Transfection

THP-1 cells were seeded in 6-well plates overnight, and the media were replaced with 1 mL of Opti-MEM before transfection. siRNA transfections were conducted using an Oligofectamine reagent (12252011, Invitrogen, Waltham, MA, USA). Mock control and Ang-2 siRNA duplexes (J0220, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were incubated with 5 µL of siRNA transfection reagent for 5 minutes at room temperature, and these mixtures were then added to THP-1 cells. The siRNA sequences are provided in **Supplementary Table 1**. After 12 hours of incubation, 1 mL of Opti-MEM containing 20% FBS was added to each well. At 48 hours after transfection with siRNA, the cells were treated with vehicle or CompC for 16 hours. The efficacy of gene silencing was evaluated using RT-qPCR for the specified time points and transfections. Each experiment was performed in triplicates and repeated three times.

Cell Migration Assay

A 24-well plate was equipped with Transwell inserts featuring a microporous membrane with an average pore size of 8 µm (202211062B, ThinCerts; Greiner, Kremsmünster, Austria). For the chemotaxis assay, Transwell inserts with microporous membranes were utilized; 2 $\times$ 10^5 THP-1 cells in 200 µL of RPMI 1640 were added to the upper chambers, while the lower chambers containing the conditioned medium obtained from the THP-1 cell cultures after different cycles of intermittent hypoxia were used as attractants. The Transwell plates were then incubated at 37 °C with 5% CO_2 for 6 hours. Subsequently, the cells in the lower chambers were centrifuged, fixed in 500 µL of Fixation Buffer (E22176-101, eBioscience, San Diego, CA, USA), and counted using a NovoCyte Flow Cytometer (E69512, NovoCyte, Agilent, CA, USA).

Flow Cytometry Assays

Migrating cells from the lower chamber were harvested and quantified using flow cytometry. The cells were collected via centrifugation at 4000 $\times$g for 5 minutes and subsequently resuspended in fresh media. The samples were then aliquoted into individual flow tubes, each containing 30 µL of the suspension. Careful pipetting was employed to ensure uniform mixing of cells within each tube.

Cell counting was conducted using the NovoCyte Flow Cytometry system. Acquisition settings were standardized with a fixed acquisition time of three accumulations, each lasting 30 seconds, totaling 90 seconds. Forward scatter (FSC) and side scatter (SSC) detectors were utilized to assess the light scatter characteristics of the cells. A gating strategy was applied, wherein SSC-H and FSC-A signals were plotted to exclude cellular debris from the analysis.

Clinical Study

In this study, we initially enrolled 140 patients (>18 years old) presenting at the Department of Respiratory and Critical Care Medicine, the First Affiliated Hospital of Chengdu Medical College, between December 2022 and June 2023, who were suspected of suffering from OSA. Inclusion criteria included a diagnosis of OSA confirmed by polysomnography (PSG) using the SOMNOtouch RESP system (20191015, SOMNOmedics, Randersacker, Germany), and an apnea-hypopnea index exceeding 5. Exclusion criteria encompassed acute (duration less than a month) or chronic (duration exceeding 1 month) severe inflammatory or infectious conditions, such as trauma or recent invasive medical/dental/surgical procedures, as well as comorbidities including ischemic heart disease, liver disease, cerebrovascular disease, hyperlipidemia, diabetes mellitus, hypertension, or kidney disease. Patients recently (within the last month) treated with anti-inflammatory drugs or antibiotics were also excluded. Ultimately, 60 participants were enrolled in the study. Additionally, 60 healthy donors matched by age and sex were included as the healthy control group. The patient selection process and research protocol are illustrated in Fig. 1. The Ethics Committee of the First Affiliated Hospital of Chengdu Medical College approved the study (No. 2022CYFYIRB-BA-Nov05), and all participants provided written informed consent before the commencement of the study. All procedures were conducted following the principles outlined in the Helsinki Declaration (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/).

Overnight Polysomnography (PSG)

All patients underwent evaluation via standard in-laboratory overnight polysomnography (PSG). The American Academy of Sleep Medicine (AASM) scoring manual version 2.4 was used to diagnose sleep apnea [25]. Specifically, data were recorded using four EEG channels (C3/A2, C4/A1, O1/A2, O2/A1), bilateral electrooculography, airflow (measured by thermistor and flow sensor), arterial oxygen saturation (SpO_2) (monitored by finger pulse oximetry), snoring (quantified via a neck microphone), and abdominal and chest wall movements (detected using inductive plethysmographic bands). DOMINO (20191020, SOMNOmedics, Randersacker, Germany) served as the computer-based sleep system for data capture. Apnea was defined as a cessation of airflow lasting at least ten seconds. Hypopnea was characterized by a decrease in airflow of at least 30% from baseline, lasting for a minimum of 10 seconds, accompanied by desaturation of at least 3% and/or arousal. The apnea-hyponea index (AHI) quantifies the total number of apnea and hypopnea events per hour of sleep. Oxygen desaturation index (ODI) was recorded as the frequency per hour at which blood oxygen level decreased by 3% or more from the baseline during sleep [26].

Methods for Isolating Monocytes from Blood

Under supine and fasting conditions, peripheral venous blood was drawn at a volume of 20 milliliters at 10 PM, shortly before the PSG test, and again at 6 AM the following morning, after patients had awakened following the completion of PSG. Peripheral blood specimens were collected in heparin-washed tubes and promptly centrifugated at 3000 $\times$g for 20 minutes. Plasma obtained from this process was utilized for the study of Ang-2. Subsequently, mononuclear cells in the blood were separated using Ficoll-Hypaque centrifugation, and CD14+ monocytes were sorted using an autoMACS magnetic cell sorting machine (5S2215E, Miltenyi Biotec, Bergisch Gladbach, Germany), as described in a previous study [27].

Statistical Analysis

Mean Ang-2 protein or gene expression levels were compared across the two groups using either a paired *t*-test (for comparing morning and night Ang-2 levels within the same group) or student's *t*-test (for comparing the OSA group with the control group). Differences in non-normally distributed numerical data were assessed using the Wilcoxon Rank sum test. The *p*-value reported is from the nonparametric Wilcoxon rank sum test based on Z statistic. Correlation between Ang-2 protein or gene expression and AHI was examined using Pearson correlation analysis. Statistical analyses were conducted using R software (Auckland, New Zealand, R Project for Statistical Computing, http://www.r-project.org/, version 3.6.1) and GraphPad Prism 8 (Prism 8, GraphPad Software, La Jolla, CA, USA). Statistical significance was considered at a probability value of <0.05 ($p < 0.05$).

Results

Gene Set Enrichment Analysis (GSEA)

Significant pathways for the Ang-2 high-expression group were predominantly enriched in the ERKs, PI3K/serine-threonine kinase (AKT), and NF-κB signaling pathways. Notable pathways with a false discovery rate (FDR)-corrected *p*-value less than 0.25 are shown in Fig. 2.

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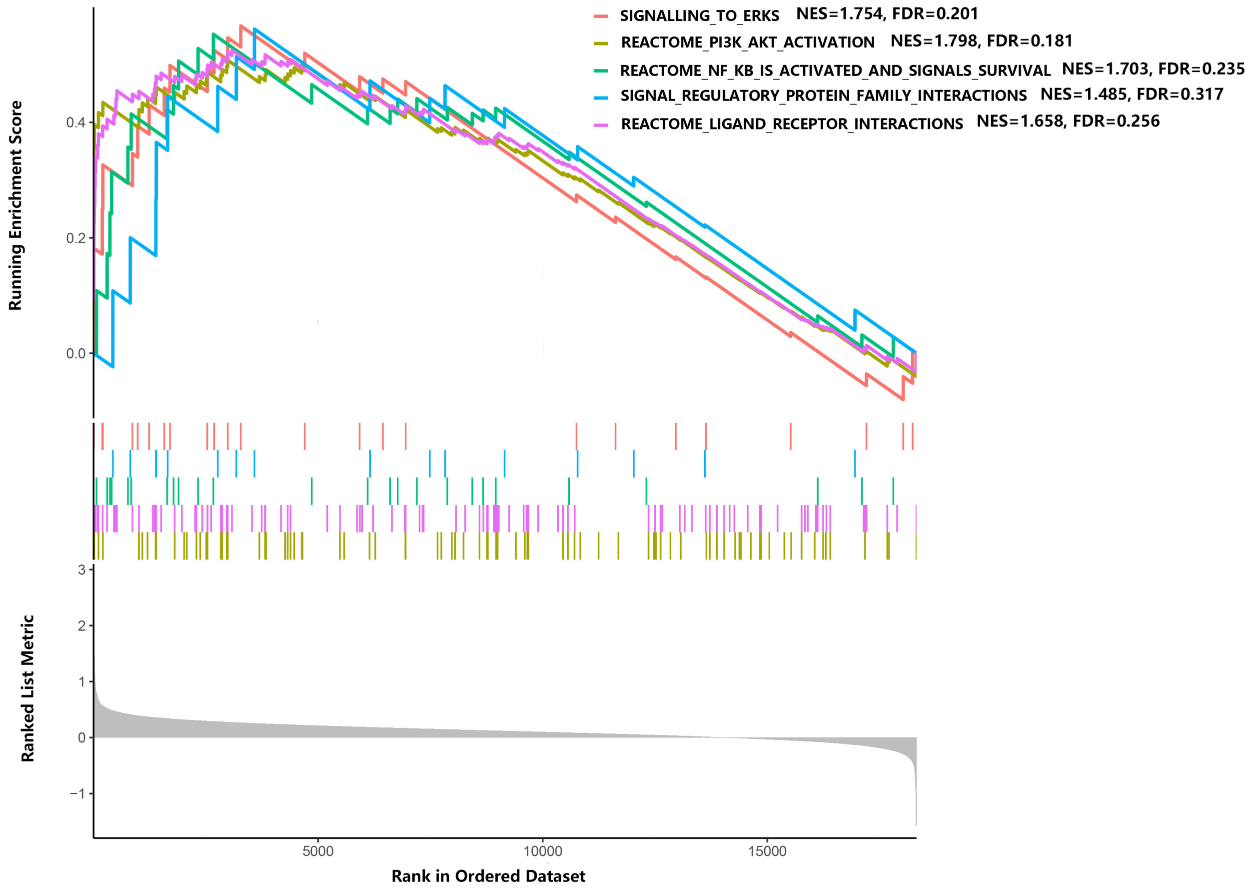

Fig. 2. Enrich plots from the Gene Set Enrichment Analysis (GSEA) in the high Angiopoietin-2 (Ang-2) group. PI3K/AKT, phosphatidylinositol 3 kinase/serine-threonine kinase; NF-κB, nuclear factor kappa-B; FDR, false discovery rate.

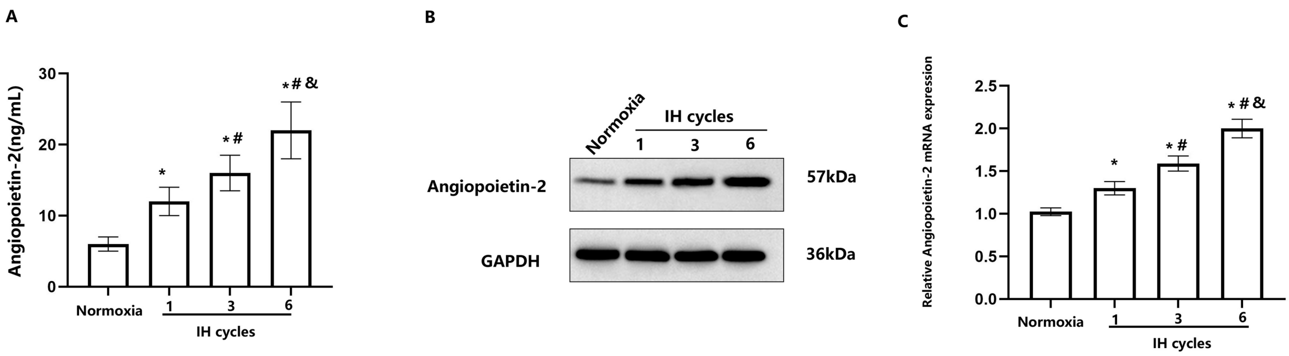

Fig. 3. Intermittent hypoxia affects the expression of Ang-2 in monocytes. (A) Ang-2 protein levels determined using enzyme-linked immunosorbent assay (ELISA). (B) Ang-2 protein levels evaluated through Western blot. (C) Ang-2 gene expression levels examined by real-time quantitative PCR (RT-qPCR) using the extracted RNA. Note: Results from three independent experiments are presented as means $\pm$ standard errors. *$p < 0.05$ vs. normoxia; #$p < 0.05$ vs. one IH cycle; & $p < 0.05$ vs. three IH cycles. Abbreviations: IH, intermittent hypoxia; Ang-2, angiopoietin-2; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase.

Intermittent Hypoxia Promotes Ang-2 Protein and mRNA Expression in Monocyte THP-1 Cells

Monocyte THP-1 cells were subjected to normoxic or intermittent hypoxia (IH) conditions for 1, 3, and 6 cycles. ELISA analysis of proteins in the culture supernatants of THP-1 cells under intermittent hypoxic and normoxic conditions indicated that IH elevated the level of angiopoietin-2 protein (Fig. 3A). Furthermore, Western blot (WB) results demonstrated that intermittent hypoxic conditions of THP-1 cells led to increased production of angiopoietin-2 protein (Fig. 3B). Additionally, as illustrated in Fig. 3C, mRNA expression of angiopoietin-2 was upregulated in monocyte

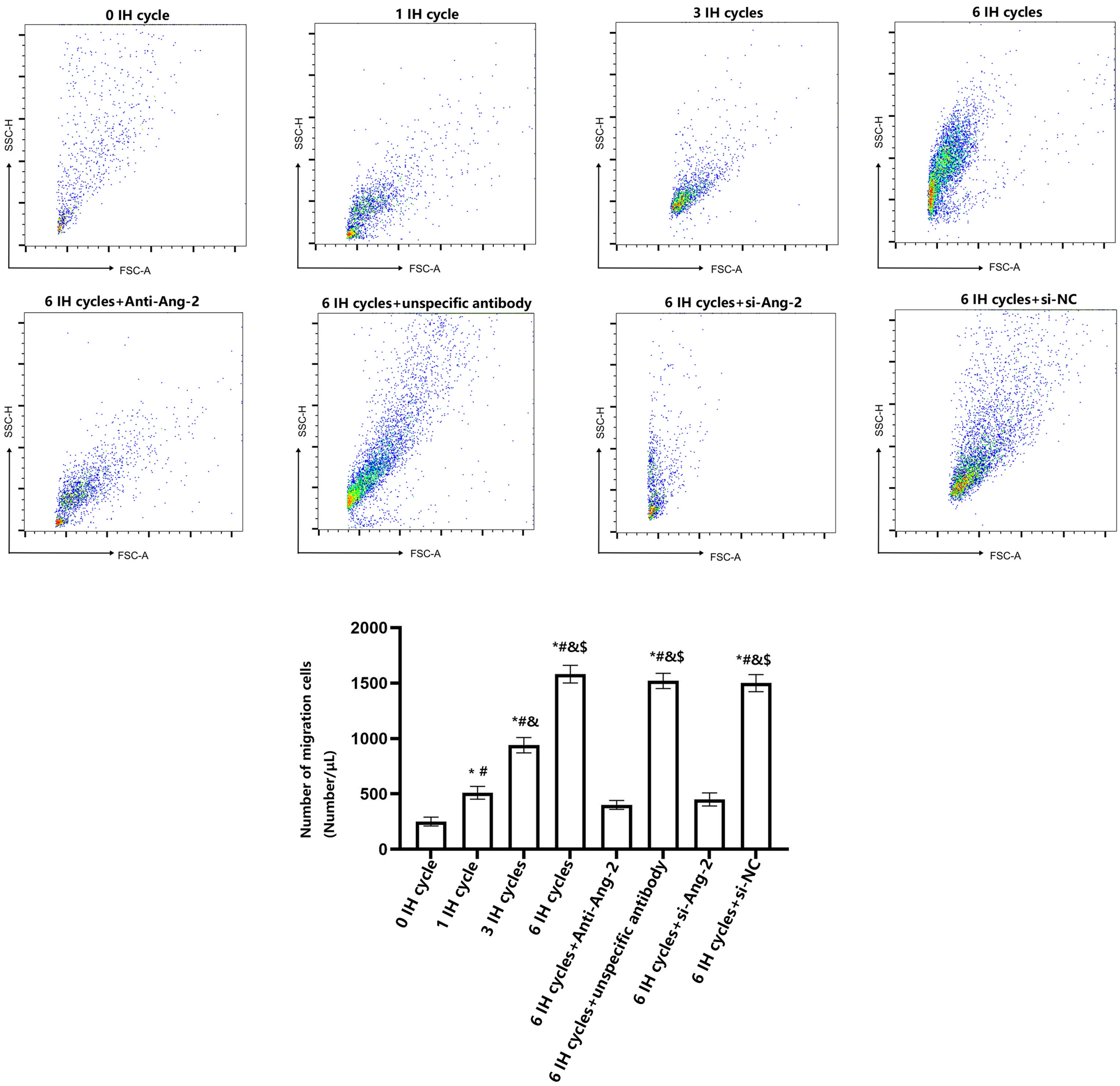

Fig. 4. A bar graph showing the number of cells migrating into the lower chamber. Note: Data are presented as the mean ± SEM of three independent experiments. * $p < 0.05$ vs. normoxia; # $p < 0.05$ vs. six IH cycles with Anti-Ang-2; & $p < 0.05$ vs. one IH cycle; \$ $p < 0.05$ vs. three IH cycles. Abbreviations: IH, intermittent hypoxia; Ang-2, angiopoietin-2.

THP-1 cells in response to intermittent hypoxia. The elevated expression of angiopoietin-2 protein and mRNA levels in monocyte THP-1 cells positively correlated with the number of intermittent hypoxic cycles.

Elevated Levels of Ang-2 Induced by Intermittent Hypoxia Promote Chemotactic Migration of Monocytes

Following exposure of THP-1 cells to normoxia or intermittent hypoxia for 1, 3, or 6 cycles, the culture media was collected for subsequent chemotactic migration assays. A 6-hour Transwell migration assay was conducted to examine the chemotactic migration of monocytic THP-1 cells towards the bottom chamber containing the conditioned media from the intermittent hypoxia-treated cells. Upon introduction of the media from cells exposed to intermittent hypoxia into the bottom chamber, a significant increase in the number of migrating THP-1 cells was observed, with this chemotactic response positively correlating with the number of IH cycles. Conversely, the impact of these stimuli on chemotactic migration was attenuated when Anti-Ang-2 was applied to the conditioned cultures. Additionally, the knockdown of the Ang-2 gene resulted in a marked reduction in THP-1 cell migration across the Transwell filter. The number of migrated cells in the various groups is shown in Fig. 4.

Intermittent Hypoxia Promotes Ang-2 Synthesis in THP-1 Cells via the PI3K, ERKs, and NF-κB Signaling Pathways

To further investigate the potential pathways through which intermittent hypoxia induces upregulated expression of Ang-2 in THP-1 cells, we treated THP-1 cells with Bay11-7082, LY294002, and PD98059 for one hour to inhibit NF-κB, PI3K, and ERKs, respectively, before subjecting them to intermittent hypoxia stimulation. Our findings indicated that treatment with 5 μM Bay11-7082, 20 μM LY294002, or 5 μM PD98059 substantially inhibited the increase in angiopoietin-2 production by THP-1 cells in response to intermittent hypoxia (Fig. 5).

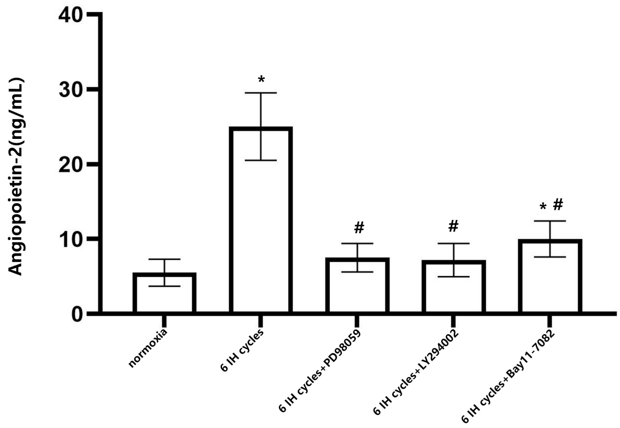

Fig. 5. Activation of extracellular regulated protein kinases (ERKs), phosphatidylinositol 3 kinase (PI3K), and NF-κB signaling pathways in THP-1 cells under intermittent hypoxia. The cell culture media was collected for enzyme-linked immunosorbent assay (ELISA) after exposure of THP-1 cells to either normoxia or six cycles of intermittent hypoxia. Monocytic THP-1 cells were pretreated with antagonists targeting the NF-κB, PI3K, and ERKs pathways, notably Bay11-7082, LY294002, and PD98059, respectively, for one hour before exposure to intermittent hypoxia. Note: Data are presented as the mean ± SEM of three independent experiments. * $p < 0.05$ compared to normoxia; # $p < 0.05$ compared to six IH cycles.

Ang-2 Expression and Protein Levels Increase in OSA Patients after One Night of Sleep

Table 1 presents demographic information for the 60 OSA patients and 60 healthy controls enrolled in this study. Blood samples were collected before and after overnight polysomnographic (PSG) testing for monocyte extraction. Fig. 6A illustrates Ang-2 levels in plasma samples from the observation group. The change in plasma Ang-2 levels (ΔAng-2) measured before and after sleep for a single night was significantly associated with the AHI ($p = 0.001$, r = 0.46) (Fig. 6B). Furthermore, monocytes *Ang-2* mRNA expression in the morning was found to increase with the severity of OSA ($p = 0.004$, r = 0.44) (Fig. 6C). The results indicate that in the morning, *Ang-2* mRNA expression in peripheral blood mononuclear cells (PBMCs) from the OSA group was higher than that in PBMCs from the control group (Fig. 6D). However, at night, the expression levels of *Ang-2* mRNA in PBMCs were not significantly different from those in the control group (Fig. 6E). Our findings suggest that Ang-2 is elevated in OSA patients and is associated with the severity of OSA.

Discussion

In this study, we demonstrated that intermittent hypoxia increases Ang-2 protein and mRNA levels in monocyte THP-1 cells, consequently enhancing Ang-2-mediated chemotactic migration. Furthermore, we identified the involvement of PI3K, ERKs, and NF-κB pathways in Ang-2 activation of THP-1 cells. Additionally, we observed elevated levels of monocyte Ang-2 protein and mRNA in the morning in OSA patients, positively correlated with disease severity.

Ang-2 is a glycoprotein with a relative molecular weight of approximately 70,000 Daltons, comprising 496 amino acids, with approximately 60% amino acid homology to Ang-1 [28]. Its amino terminus contains a coiled-coil domain (CC) mediating homopolymerization between ligands and monomers and a fibrinogen-like domain (FL) at the carboxyl terminus, which is responsible for signal transduction between ligand receptors [29]. Ang-2 is believed to act as a chemotactic agent for neutrophils, facilitating their migration and playing an active role in the primary defense layer of the immune system [30]. Lee *et al.* [31] demonstrated that endothelial and macrophage-derived Ang-2 persistently stimulates aberrant vascular remodeling and polarization of pro-inflammatory macrophages via integrin $\alpha 5\beta 1$ signaling, exacerbating cardiac hypoxia and the inflammatory response following myocardial infarction. Additionally, Ang-2 has been shown to recruit, adhere to, and facilitate the migration of monocytes, promoting the aggregation of M1-type macrophages within atherosclerotic plaques [32,33]. Furthermore, Ang-2 contributes to the initial interaction between monocytes and endothelial cells by synergizing with other chemokines. It stimulates endothelial expression of monocyte chemokines and adhesion molecules, leading to an increase in the proportion of Ly6C (low) macrophages in the aorta and an elevation in the production of the pro-fibrotic cytokine TGF-1 in aortic endothelial cells [34].

Coffelt *et al.* [35] illustrated that Ang-2 triggers the TIE2-expressing monocytes to inhibit T-cell activation and stimulate the growth of regulatory T-cells, while Lal *et al.* [36] demonstrated a substantial elevation in plasma Ang-2 levels in OSA patients through plasma protein profiling. Consistent with these findings, our study confirmed elevated plasma Ang-2 levels and Ang-2 mRNA expres-

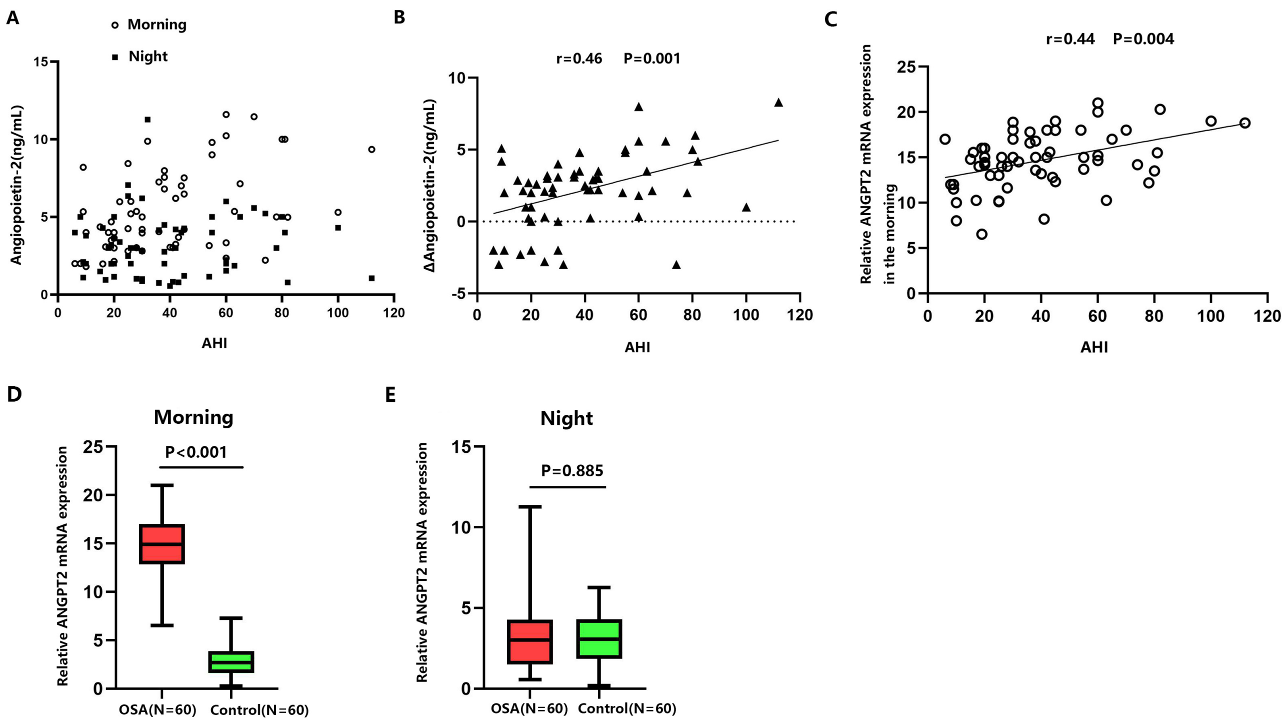

Fig. 6. Enhanced Ang-2 expression in plasma and monocytes of OSA patients. (A) Plasma Ang-2 levels of each patient before and after the night polysomnography (PSG) assessment. (B) Plasma Ang-2 levels measured before and after a single night of sleep, with the difference between the two represented as ΔAng-2. A significant association was observed between the AHI and the magnitude of the difference ($p = 0.004$, r = 0.46). (C) Correlation between AHI and Ang-2 mRNA expression in monocytes collected in the morning. (D) Differentially expressed *Ang-2* mRNA in peripheral blood mononuclear cells from OSA patients compared to healthy controls in the morning. (E) Differentially expressed *Ang-2* mRNA in peripheral blood mononuclear cells from OSA patients compared with healthy controls at night. Abbreviations: AHI, apnea-hypopnea index.

Table 1. Demographic data and polysomnography parameters of the enrolled OSA patients.

Variables	OSA group	Control group	χ^2/t	*p*-value
Number of subjects (male)	60 (38)	60 (30)	2.171	0.141
Age, years	46.9 ± 5.8	45.8 ± 4.7	1.414	0.256
BMI, kg/m²	27.4 ± 4.9	26.4 ± 4.3	1.188	0.237
AHI, events/h	36.2 (20.1–55.3)	2.1 (1.2–3.6)	9.535	<0.001
Sleep efficiency, %	71.4 ± 6.3	85.3 ± 7.8	–10.761	<0.001
ODI, events/h	39.7 (22.5–79.6)	1.9 (1.6–4.1)	9.476	<0.001
Mean SpO_2, %	85.8 ± 7.9	98.5 ± 5.3	–10.341	<0.001
Lowest SpO_2, %	80.1 ± 12.3	94.5 ± 4.1	–8.603	<0.001
Time with SpO_2 <85%, minutes	9.2 (3.5–12.0)	0.0 (0.0–0.0)	-	<0.001

Note: Normally distributed data are presented as means ± SD, and non-normally distributed data as medians with 25–75th percentiles. BMI, body mass index; AHI, apnea-hypopnea index; ODI, oxygen desaturation index; SpO_2, oxygen saturation.

sion in monocytes from OSA patients. Moreover, the use of Ang-2 inhibitors or knockdown of Ang-2 in our study attenuated the chemotactic migration of monocytes, a pathological feature of early atherosclerosis [37]. Therefore, we postulate that elevated Ang-2 levels may synergistically promote the chemotactic migration and adherence of monocytes to vascular endothelial cells, contributing to the progression of atherosclerosis in OSA patients.

While some research reports have indicated elevated levels of Ang-2 in the blood of OSA patients, the pathophysiological mechanisms underlying this phenomenon associated with intermittent hypoxia have yet to be fully elucidated. A previous study by Moreau *et al*. [38] observed upregulated Ang-2 levels in the early stages of intermittent hypoxemia by assessing the concentration of Ang-2 levels in cerebrospinal fluid samples obtained from individuals with sporadic amyotrophic lateral sclerosis and categorized according to the hypoxemic levels of those samples. Additionally, another study demonstrated that hypoxia induces increased secretion of Ang-2 by skin adipose stem cells [39]. Furthermore, recent research has shown elevated plasma Ang-2 production in children with severe OSA, presumably due to sleep-associated chronic intermittent hypoxia [17]. Our study is the first to demonstrate that intermittent hypoxia *in vitro* promotes increased protein and mRNA levels of Ang-2 expression in monocytes. Moreover, we investigated the promoting effect of intermittent hypoxia on atherosclerosis by examining Ang-2-induced monocyte chemotactic migration. Additionally, we explored diurnal fluctuations in Ang-2 expression in monocytes from OSA patients and discovered that nocturnal sleep apnea events might elevate Ang-2 expression in monocytes.

It has been established that signaling pathways, notably ERKs and PI3K, are necessary for Ang-2 activation in cells [40,41]. This was also confirmed in our bioinformatics analysis. The inhibition of PI3K and ERKs with LY294002 and PD98059, respectively, has been shown to reduce the expression of Ang-2 induced by different stimuli [42,43]. However, the pathways regulating Ang-2 expression in monocytes subjected to intermittent hypoxia have not been explored. In this study, monocytes exposed to intermittent hypoxia were treated with LY294002 and PD98059, inhibitors of the PI3K and ERKs pathways, respectively. The results illustrated that overexpression of Ang-2 in monocytes in response to intermittent hypoxia requires ERKs and PI3K activation.

Additionally, we observed that the NF-κB inhibitor Bay11-7082 significantly attenuated the increase in Ang-2 induced by IH, suggesting that NF-κB activation is necessary for intermittent hypoxia-induced upregulation of Ang-2. These results, combined with the activation of ERKs, suggest that oxidative stress in monocytes may be implicated in this process [44,45]. Patients with OSA experience alveolar hypoventilation between hypoxia/apnea events, leading to intermittent hypoxia, reduced arterial oxygen saturation, and subsequent oxidative imbalance. This imbalance results in lipid peroxidation, generation of reactive oxygen species, tumor necrosis factors, and inflammatory mediators such as IL-2, IL-4, IL-6, and IL-8. These molecules serve as markers to assess the severity of OSA [46]. Consistent with these observations, our study confirmed an increase in Ang-2 expression in monocytes from OSA patients as the disease worsened. Therefore, Ang-2 may serve as a crucial biological indicator for assessing OSA severity. Importantly, we collected and purified monocytes from freshly drawn blood immediately before and after PSG testing. Consequently, we hypothesized that one night of intermittent hypoxia during sleep may result in an excessive production of Ang-2 by monocytes.

However, there are several caveats that warrant attention in this research. Firstly, due to data limitations, our integrated bioinformatics analysis utilized data derived from adipose tissue of OSA patients. Secondly, due to the limited awareness of OSA and the lack of medical resources at the grassroots level, our study included only 120 OSA patients from Southwest China. It has been documented in the literature that ethnic differences can influence Ang-2 levels, with single nucleotide polymorphism alleles significantly correlating with overnight nocturnal oxygen saturation [47]. Therefore, future studies should encompass a broader spectrum of nations and races, including patients from more developed regions.

Nevertheless, confounding factors impacting Ang-2 expressions, such as ischemic heart disease and other inflammatory diseases, were excluded during enrollment. During an inflammatory response, the expression of Ang-2 and its release from Weibel Palade bodies are heightened, leading to an increased Ang-2:Ang-1 ratio [48]. Some studies have indicated a correlation between serum Ang-2 levels and the extent of myocardial damage, suggesting Ang-2 as a potential biomarker for disease severity [49]. The inclusion and exclusion criteria were stringent, and the study duration was brief, resulting in a relatively small sample size.

Thirdly, Chandel *et al*. [50] demonstrated that patients with hyperinsulinemia exhibit higher concentrations of Ang-2. Moreover, insulin has been implicated in inducing the synthesis and secretion of Ang-2 from human umbilical vein endothelial cells (HUVECs) via the p38 MAPK-cFOS pathway, promoting atherosclerosis and endothelial inflammation [50]. However, the precise mechanism by which Ang-2 contributes to atherosclerosis by promoting monocyte chemotactic migration in OSA patients requires further investigation.

Lastly, this study did not employ a blind method, potentially introducing bias into the research findings. Therefore, future research should incorporate multicenter, large samples, high-quality, double-blinded, randomized controlled trials to enhance the reliability of the findings.

Conclusion

In summary, this represents the first demonstration of the role of intermittent hypoxia in augmenting monocyte Ang-2 gene expression, protein production, and subsequent chemotactic migration. The upregulation of monocyte Ang-2 expression induced by intermittent hypoxia is mediated, at least partially, by the PI3K, ERKs, and NF-κB signaling pathways. Additionally, our results revealed that the expression of monocyte Ang-2 in OSA patients was elevated following a single night of sleep and positively correlated with AHI. These findings underscore the critical role of Ang-2 in facilitating the increased chemotactic migration of monocytes under intermittent hypoxic conditions.

Abbreviations

IH, intermittent hypoxia; OSA, obstructive sleep apnea; AHI, apnea-hypopnea index; Ang-2, angiopoietin-2; GSEA, Gene Set Enrichment Analysis; CC, coiled-coil domain; FL, fibrinogen-like domain; ODI, oxygen desaturation index; PSG, polysomnography; BMI, body mass index.

Availability of Data and Materials

The raw data supporting the conclusion of this study will be made available by the authors, without undue reservation.

Author Contributions

JH developed study design, processed data, wrote manuscript. LW and NH participated in study design, conducted research, worked with patients. JH processed data and made a revision of the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The Ethics Committee of the First Affiliated Hospital of Chengdu Medical College approved the study (No. 2022CYFYIRB-BA-Nov05), and all participants provided written informed consent before the commencement of the study. All procedures were conducted following the principles outlined in the Helsinki Declaration (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/).

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.23812/j.biol.regul.homeost.agents.20243805.354.

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