Original Article

Nocturnal Falls of Adiponectin Levels in Sleep Apnea with Abdominal Obesity and Impact of Hypoxia-Induced Dysregulated Adiponectin Production in Obese Murine Mesenteric Adipose Tissue

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***Aim*: Obstructive sleep apnea-hypopnea syndrome (OSAS) is associated with atherosclerotic cardiovascular disease. We reported recently daytime hypoadiponectinemia and nocturnal falls in circulating adiponectin concentrations (Δadiponectin) in OSAS patients, in part due to hypoxic stress. The present study investigated the association between Δadiponectin and fat distribution in OSAS males, and the effect of hypoxic stress on adiponectin production in obese yellow-KKAy mice.**

***Methods*: The participants in this study were 43 Japanese males who visited the clinic and were newly diagnosed with OSAS. Venous blood samples were collected before sleep and after wakingup. We investigated the effect of hypoxia on adiponectin expression in mesenteric and subcutaneous fat tissues of obese yellow-KKAy mice. We measured adiponectin secretion into media under hypoxic conditions in an *ex-vivo* model of yellow-KKAy mice.**

***Results*: In OSAS males with a relatively higher body mass index (BMI), Δadiponectin correlated inversely with the waist-hip ratio, but not with BMI, waist circumference or hip circumference. In obese yellow-KKAy mice, exposure to hypoxia for 2 days suppressed plasma adiponectin levels, with no apparent change in mesenteric and subcutaneous fat tissue adiponectin mRNA expression. In an *ex-vivo* study of obese yellow-KKAy mice, hypoxic stress reduced adiponectin in the supernatant of mesenteric fat tissues, but not subcutaneous fat tissues.**

***Conclusions*: These findings suggest that abdominal obesity, representing abundant mesenteric fat tissue susceptible to hypoxic stress, partly explains Δadiponectin in OSAS patients, and that reduction of visceral fat accumulation may combat OSAS-related atherosclerotic cardiovascular diseases in abdominal obesity.**

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Key words; Nocturnal falls of adiponectin, Body fat distribution, Waist-hip ratio, Mesenteric fat tissues, Obstructive sleep apnea-hypopnea syndrome

Introduction

Obstructive sleep apnea-hypopnea syndrome (OSAS) is associated in some patients with insulin resistance and hypertension, leading to atherosclerotic cardiovascular disease[1)]. Cardiovascular diseases are the most serious complications in OSAS[2)]. Previous studies indicated that people who died suddenly in the early hours of the morning from cardiac causes had significant sleep apnea compared to those who died suddenly from cardiac causes during other intervals[3)], and from angina attacks during night sleep (i.e. nocturnal cardiac events)[4, 5)].

Increasing evidence suggests that body fat accumulation, especially visceral fat accumulation, plays a role in the development of atherosclerosis. Recent

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studies have demonstrated that adipose tissue is not only a passive reservoir for energy storage but also produces and secretes a variety of bioactive molecules called adipocytokines, which are involved in energy metabolism, inflammatory response, and cardiovascular functions[6]. Adiponectin, as an adipocytokine, was identified by our group from the human adipose tissue cDNA library[7]. We demonstrated previously the association between visceral obesity and OSAS[8]. We recently reported daytime hypoadiponectinemia and nocturnal falls in circulating adiponectin concentrations (Δadiponectin) in severe OSAS patients, presumably due to hypoxic stress[9], possibly that might be associated with nocturnal cardiac events. The association between fat distribution and Δadiponectin in OSAS patients remains unclear.

Aim

The aim of the present study was to clarify the possible association between fat distribution and nocturnal falls in circulating adiponectin concentrations in male patients with OSAS, and the effect of hypoxic stress, seen partly in OSAS, on adiponectin production in the subcutaneous and mesenteric fat tissues of obese mice.

Materials and Methods

Human Studies

Participants

We studied 43 Japanese males (apnea hypopnea index (AHI) ≥5, age; 41.4±1.6 years, mean±SEM) between February 2006 and March 2007 who visited the clinic and were newly diagnosed with OSAS[8]. OSAS was diagnosed according to the guidelines of the American Academy of Sleep Medicine Task Force[10], as reported previously[8]. All recordings were scored manually by an experienced polysomnographic technologist, as described in detail previously[11]. Sleep duration was estimated using the self-reported sleep time and recorded data. The Medical Ethics Committee of Osaka University approved this study. All subjects enrolled in the study were Japanese and each gave written informed consent. This study (called The Osaka University Visceral Fat Study (O-VFStudy)) is registered under number UMIN 000002997 (https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=brows&action=brows&type=summary&recptno=R000003633&language=E).

Anthropometry and Laboratory Tests

Height, weight, waist circumference and hip circumference were measured in a standing position. Body mass index (BMI) was calculated using the formula [weight (kg)/height (m)^2]. Waist circumference (WC) at the umbilical level was measured with a non-stretchable tape in late expiration while standing (in cm). Hip circumference(HC) was measured horizontally at the level of the greater trochanter of the femur, also with the subjects standing[12]. The waist-to-hip ratio (WHR) was defined as WC divided by HC.

Blood pressure was measured with a standard mercury sphygmomanometer on the right arm after the subjects had rested in a sitting position for at least 10 minutes. Venous blood samples were collected to measure blood glucose, hemoglobin A1c, immunoreactive insulin (IRI), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C) after wakingup while the subject was in the supine position. Low-density lipoprotein-cholesterol (LDL-C) was calculated using the Friedewald formula[13]. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or treatment for hypertension. Diabetes mellitus was defined according to the World Health Organization criteria, and/or treatment for diabetes mellitus. Dyslipidemia was defined as an LDL-C concentration of >140 mg/dL, TG concentration >150 mg/dL, HDL-C concentration <40 mg/dL, and/or treatment for dyslipidemia. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: HOMA-IR (mU/L mg/dL)=[(fasting IRI×fasting glucose)/405]. In each sleep study that included adiponectin monitoring, venous blood samples were obtained before sleep and after wakingup while the subject was in the supine position. For the purpose of the present study, serum samples obtained at baseline from each study participant and stored at −20℃ were thawed and assayed for serum adiponectin concentrations using the sandwich enzyme-linked immunosorbent assay (ELISA) (Otsuka Pharmaceutical Co., Tokushima, Japan). No patients were on medications known to increase serum adiponectin levels, such as pioglitazone[14].

Animal and Cell Culture Studies

Animals and Exposure to Hypoxia

Male control KK and yellow-KKAy mice (each group; $n=6$) were obtained from Clea Japan (Tokyo, Japan) and housed under a 12-h dark-light cycle (lights on 8:00 A.M. to 8:00 P.M.) and constant temperature (22℃) with free access to food (Oriental Yeast, Osaka, Japan) and water. Male mice were housed in cages exposed to room air (ambient atmosphere) or sustained hypoxic chambers (Teijin Pharma, Osaka,

Japan, ~10% O_2), as we reported previously[9]. Both in control KK and obese yellow-KKAy mice, there were no significant differences in body weight, plasma adiponectin levels, and blood glucose levels at baseline between control (room air) and hypoxic (10% O_2 concentration) groups.

Measurement of Plasma Adiponectin Concentrations and Adipose Adiponectin mRNA Expression in Control KK and Obese Yellow-KKAy Mice

All experiments were conducted in mice at age 14 weeks. Mice were sacrificed under pentobarbital sodium anesthesia (50 mg/kg body weight) at the indicated times under each condition, and then the mesenteric and subcutaneous fat tissues and blood samples were collected. Each sample was subjected to measurement of plasma concentrations and mRNA expression levels (using real-time quantitative polymerase chain reaction; rt-PCR), as described previously[9]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

Measurement of Adiponectin Secretion into Media Under Hypoxic Conditions in an ex-vivo Model of Obese Yellow-KKAy Mice

Each pair of mesenteric fat tissue and subcutaneous fat tissue was obtained from 14-week-old male obese KKAy mice housed under room air. Each tissue was weighed and 250 to 300 mg were placed into a ϕ 3 cm-dish filled with 1 mL fetal calf serum (FCS)-free complete Dulbecco's modified Eagle's medium (DMEM), similar to the method described previously[15]. Next, the tissues were minced into small pieces, and the medium was removed and washed with 1 mL calcium- and magnesium-free PBS, incubated with 1 mL DMEM-free FCS for one hour under 1% O_2 hypoxia or control conditions (18-21% O_2 − 5% CO_2) (each group, $n=6$). An aliquot of the culture media was subjected to measurement of adiponectin in media using ELISA. We could not investigate non-obese KK mice because a sufficient amount of mesenteric fat tissue could not be obtained for the experiment.

Statistical Analysis

All values are expressed as the mean ± SEM. Relationships between two continuous variables were analyzed using scatter plots and Pearson's correlation coefficients. Differences among groups were compared by the unpaired Student's t-test for experiments involving only two groups. In all cases, p values <0.05 were considered significant. All statistical analyses were performed with StatView-J 5.0 (Statistical Analysis System Inc., Cary, NC).

Table 1. Clinical characteristics of male subjects with OSAS (AHI ≥5)

Number	43	
Age (years)	41.4 ± 1.6	(25-77)
Body weight (kg)	87.3 ± 2.7	(55.4-133.3)
Body mass index (BMI) (kg/m^2)	29.7 ± 0.7	(23.2-38.2)
Waist circumstance (cm)	98.9 ± 1.7	(82.0-126.0)
Hip circumstance (cm)	105.7 ± 1.3	(89.0-126.0)
Waist-hip ratio	0.93 ± 0.01	(0.85-1.03)
AHI (events/hour)	42.6 ± 4.1	(5.3-104.0)
ODI <4.0% (events/hour)	363.1 ± 35.6	(3.7-89.3)
SpO_2 <90% time rate (%)	22.1 ± 3.3	(0.1-67.0)
Baseline SpO_2 (%)	94.6 ± 0.3	(92-97)
Lowest SpO_2 (%)	71.9 ± 1.5	(54-87)
Systolic blood pressure (mmHg)	131 ± 2	(104-170)
Diastolic blood pressure (mmHg)	86 ± 2	(66-118)
Pulse rate (/min)	84 ± 2	(60-114)
Fasting glucose (mg/dL)	106 ± 2	(88-144)
Hemoglobin A1c (%)	5.1 ± 0.1	(4.5-7.3)
Immunoreactive insulin (mU/L)	16.5 ± 1.6	(3.0-44.0)
HOMA-IR (unit)	4.5 ± 0.5	(0.7-12.9)
Low density lipoprotein-cholesterol (mg/dL)	125 ± 4	(76.2-176.8)
Triglyceride (mg/dL)	158 ± 12	(70-444)
High density lipoprotein-choresterol (mg/dL)	44 ± 2	(27-88)
Diabetes mellitus (%)	14.0	
Dyslipidemia (%)	76.7	
Hypertension (%)	39.5	
Serum adiponectin before sleep (μg/mL)	6.0 ± 0.4	(1.7-17.2)
Serum adiponectin after wake-up (μg/mL)	5.2 ± 0.4	(1.2-13.5)
Δadiponectin (%)	− 13.1 ± 2.1	(− 36.5-16.6)

Data are presented as the mean ± SEM (range). OSAS: obstructive sleep apnea hypopnea syndrome, AHI: apnea hypopnea index, SpO_2: percentage of arterial O_2 saturation from pulse oximetry, ODI: oxygen desaturation index, 90% time: time at desaturation below 90% in minutes of total bedtime, HOMA-IR: homeostasis model assessment of insulin resistance, Δadiponetin = (serum adiponectin concentration after waking-up − before sleep)/after waking-up.

Results

Human Studies

The characteristics of the subjects enrolled in this study are presented in **Table 1**. The mean BMI was

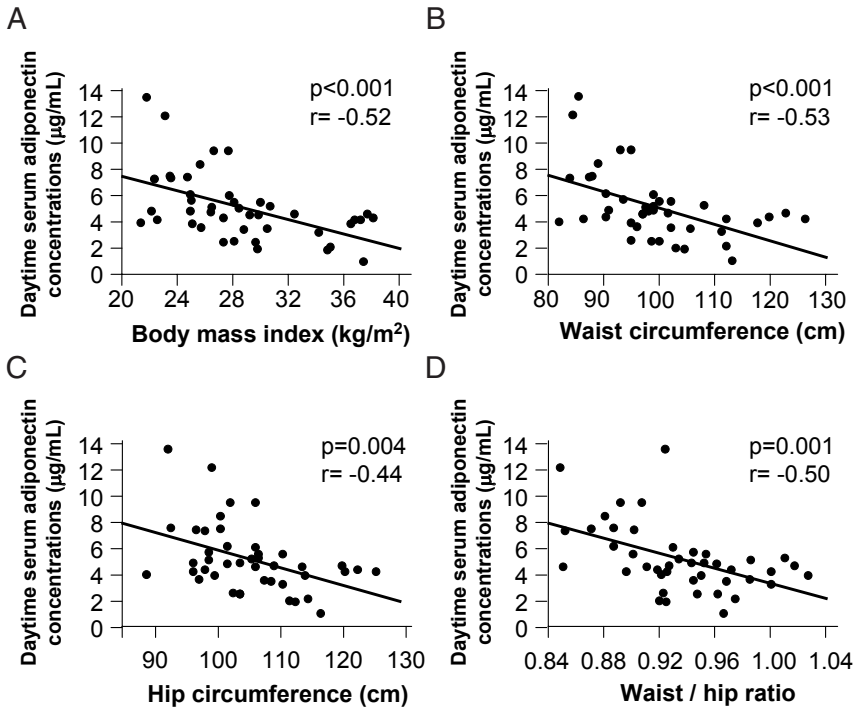

Fig. 1. Human studies.

Relationships between daytime serum adiponectin concentrations (in the morning) and body mass index (A), waist circumstance (B), hip circumstance (C), and waist-hip ratio (D) in patients with obstructive sleep apnea-hypopnea syndrome (OSAS).

29.7 kg/m^2 and mean WC was 98.9 cm. Our previous report showed daytime hypoadiponectinemia and Δadiponectin in severe OSAS patients, in part due to hypoxic stress[9]. First, we investigated the relationship between daytime circulating adiponectin concentrations and each of BMI and fat distribution in males with OSAS. Anthropometric measurements such as WHR, as an indicator of body fat distribution in obesity, are usually assessed using standard methods. In males with OSAS, serum adiponectin concentrations measured in the early morning correlated significantly with BMI, WC, HC, and WHR (**Fig. 1**), similar to a previous report[16]. Next, we analyzed the correlation between Δadiponectin and each of BMI and fat distribution in males with OSAS. Interestingly, Δadiponectin correlated inversely and significantly with WHR only ($p=0.026$, $r=-0.34$), but not with BMI, WC, or HC (**Fig. 2**), suggesting that fat distribution, such as visceral and subcutaneous fat tissue, is an important factor in Δadiponectin in OSAS.

AHI correlated significantly with BMI ($r=0.37$, $p=0.016$), WC ($r=0.36$, $p=0.019$), HC ($r=0.32$, $p=0.035$), and tended to correlate with WHR ($p=0.054$); however, there was no correlation between AHI and daytime serum adiponectin levels ($p=0.148$), Δadiponectin ($p=0.401$) (data not shown).

Animal Studies

Next, we studied the effect of sustained hypoxia on adiponectin expression in mesenteric and subcutaneous fat tissues of obese yellow-KKAy mice, as reported previously in lean mice[9]. Exposure to sustained hypoxia (10% O_2 concentration) for 2 days suppressed plasma adiponectin levels in obese yellow-KKAy mice (**Fig. 3A, left**), with no apparent change in mesenteric and subcutaneous fat tissue adiponectin mRNA expression in obese yellow-KKAy mice (**Fig. 3A, middle and right**). Exposure to sustained hypoxia for 2 days suppressed plasma adiponectin levels in control KK mice, with no apparent change in mesenteric and subcutaneous fat tissue adiponectin mRNA expression in obese yellow-KKAy mice (data not shown), compared with the control (room air), similar to our previous report on epididymal fat tissues of lean mice[9]. Exposure to sustained hypoxia for 4 days resulted in significant falls in plasma adiponectin concentrations of control KK and obese yellow-KKAy mice, and significant decreases in mesenteric

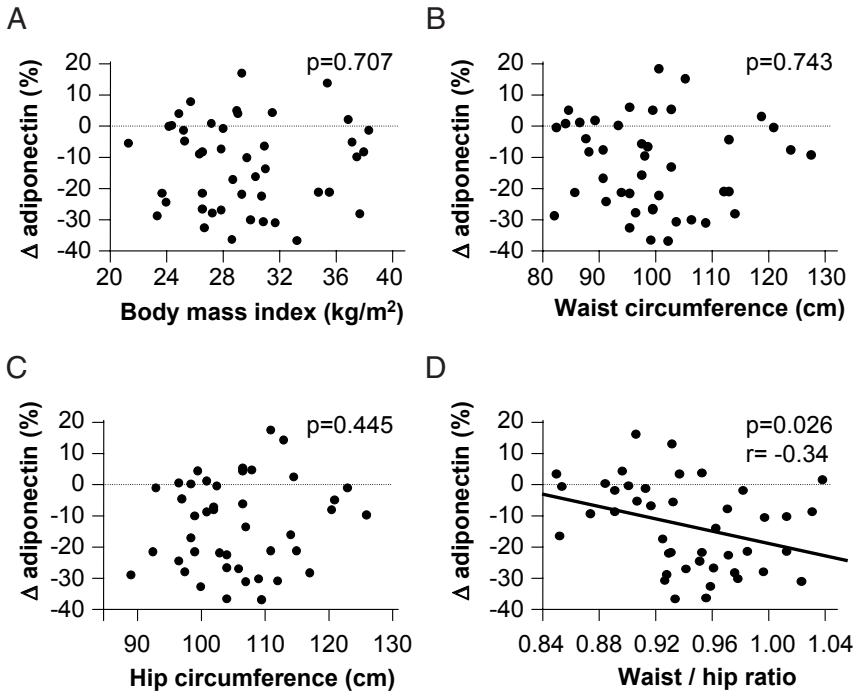

Fig. 2. Human studies.

Relationships between percent change in serum adiponectin level (Δadiponectin) and body mass index (A), waist circumstance (B), hip circumstance (C), and waist-hip ratio (D) in patients with obstructive sleep apnea-hypopnea syndrome (OSAS). Δadiponectin was calculated by the formula: [(serum adiponectin concentration after wake-up − before sleep) × 100/serum adiponectin concentration after wake-up].

and subcutaneous fat tissue adiponectin mRNA expression levels in obese yellow-KKAy mice (data not shown), compared with the control (room air), similar to our previous report on epididymal fat tissues of lean mice[9].

Animal *Ex-Vivo* Studies

Next, we performed an *ex-vivo* experiment to examine the effects of exposure to 1% O_2 on adiponectin concentration in media containing mesenteric and subcutaneous fat tissues of 14-week-old male yellow-KKAy mice. Adiponectin concentrations in these media increased linearly during the 2-hour hypoxia (data not shown); therefore, we conducted *ex-vivo* studies under each condition for 1 hour. Adiponectin concentrations in media cultures of mesenteric fat tissues were lower following 1-hour exposure to 1% O_2 (**Fig. 3B, left**) than the control condition, while those from subcutaneous fat tissues were not different (**Fig. 3B, right**) between control and hypoxic conditions. We also examined the adiponectin mRNA expression level in each *ex-vivo* fat tissue under control conditions and 1-hour exposure to 1% O_2. There were no significant changes in adiponectin mRNA expression levels in mesenteric and subcutaneous fat *ex-vivo* tissues between control and hypoxic conditions (date not shown). In addition, we examined the cytotoxicity of 1-hour exposure to 1% O_2 to mesenteric and subcutaneous *ex-vivo* fat tissues by measuring lactic dehydrogenase. No evidence of cytotoxicity was noted in both mesenteric and subcutaneous fat (data not shown). These results indicate that mesenteric fat tissue is susceptible to hypoxic stress with regard to adiponectin production, compared with subcutaneous fat.

Discussion

Two major findings of the present study were: 1) nocturnal falls in circulating adiponectin concentrations correlated negatively with WHR in male OSAS subjects with abdominal obesity; 2) in *ex-vivo* studies of obese mice, exposure of mesenteric fat tissue, but not subcutaneous fat tissue, to hypoxia resulted in the suppression of adiponectin level in the culture media.

Previous studies demonstrated the possible association between sleep apena and visceral obesity[8, 17-19]

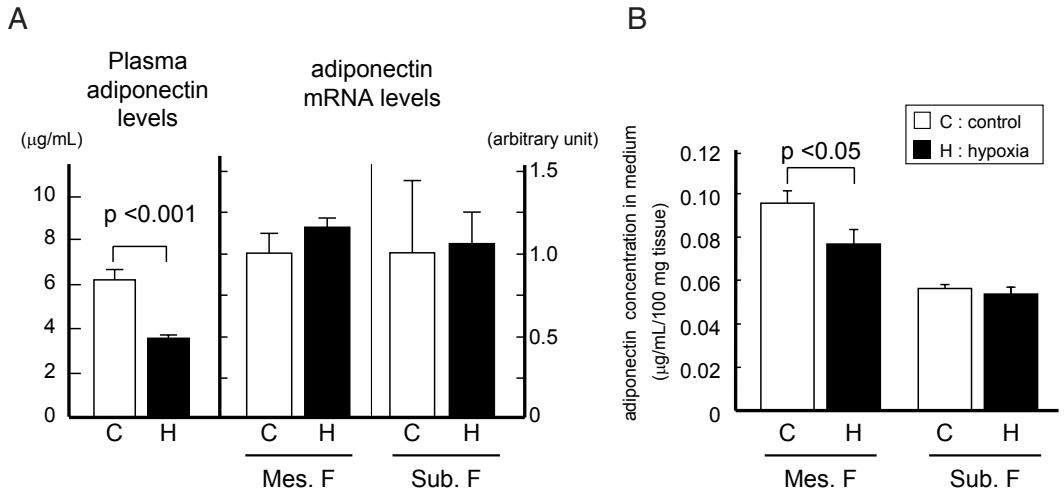

Fig. 3. Animal studies.

Effect of hypoxia on adiponectin in obese yellow-KKAy mice ($n=6$, each). A. plasma adiponectin concentrations in obese yellow-KKAy mice under control (20% O_2) and hypoxic exposure (10% O_2) for 2 days were measured by ELISA, as described in Materials and Methods. Expression levels of adiponectin mRNA in mesenteric fat tissue (mes. F), and subcutaneous fat tissue (sub. F) of obese yellow-KKAy mice under control (20% O_2) and hypoxic exposure (10% O_2) for 2 days. Data were normalized against 18s mRNA. Data are the mean ± SEM. Similar results were observed in other series of experiments. B. Fifteen-week-old yellow-KKAy mice were used in these ex-vivo experiments ($n=7$, each), as described in Materials and Methods. Mes. F: mesenteric fat ex-vivo tissues. Sub.F: subcutaneous fat ex-vivo tissues. C: control (18-21% O_2 − 5% CO_2), H: hypoxic stress (1% O_2) conditions. Data are the mean ± SEM. Similar results were observed in other series of experiments.

and metabolic syndrome[20], and recent studies reported that obese subjects with sleep apnea also suffer from daytime hypoadiponectinemia[21, 22]. Our recent study showed daytime hypoadiponectinemia and nocturnal falls in circulating adiponectin concentrations in patients with severe OSAS, in part due to hypoxic stress[9]. Previous studies also demonstrated that daytime circulating adiponectin levels correlated negatively with BMI, WC, HC and WHR in male patients with OSAS, although daytime circulating levels of leptin correlated positively with BMI, WC, or WHR[18, 23-26]. To our knowledge, the present study is the first to assess the relationship between nocturnal falls in circulating adiponectin concentrations and fat distribution in male subjects with OSAS. These results may partly relate to the characteristics of this study population, i.e. male OSAS subjects with obesity. It is known that fat distribution, such as WHR or the visceral fat area/subcutaneous fat area ratio obtained from CT cross-sectional images in the umbilical region[27], is useful in obese subjects, although WC is known to be a simple parameter of visceral fat accumulation in the general population. The current study showed that the nocturnal fall in circulating adiponectin correlated inversely with fat distribution, i.e.WHR, in male OSAS subjects with abdominal obesity (**Fig. 2**), suggesting that fat distribution matters.

The present study focused on the effect of hypoxia on adiponectin expression in mesenteric and subcutaneous fat tissues of obese yellow-KKAy mice. The results demonstrated that hypoxic stress resulted in the suppression of plasma adiponectin levels in obese yellow-KKAy mice, with no apparent change in mesenteric and subcutaneous fat tissue adiponectin mRNA expression in obese yellow-KKAy mice (**Fig. 3A**). For clarify the mechanism of their posttranscriptional dysregulation, we also investigated the effect of hypoxia on adiponectin production from mesenteric and subcutaneous fat *ex-vivo* tissues of obese mice. Exposure to hypoxia suppressed adiponectin secretion from mesenteric *ex-vivo* fat tissue from obese mice, but not from subcutaneous fat from the same mice (**Fig. 3B**). Based on these findings, we propose that visceral fat accumulation in obese subjects with OSAS might be associated with nocturnal falls in circulating adiponectin concentrations. The hypoxia-induced decrease of adiponectin production in mesenteric fat may be partly susceptible to decreased circulating adiponectin levels because subcutaneous fat is the largest proportion of total body fat content. Our previous works demonstrated that accumulated visceral fat associated strongly with the production of

reactive oxygen species (ROS), locally[28] and systemically[29]. Although not evaluated here, hypoxic stress might also occur more overtly in visceral fat tissue, related to hypoxia-induced ROS production, as reported previously in pulmonary vessels[30, 31] and cardiomyocytes[32]. Further studies are necessary to elucidate the precise mechanism in OSAS patients.

Conclusion

In conclusion, we demonstrated for the first time that exposure to hypoxia resulted in the dysregulation of adiponectin production from mesenteric fat tissues of obese mice. This association may explain the nocturnal falls in circulating adiponectin concentrations seen in OSAS patients with visceral obesity. The results suggest that reduction of visceral fat accumulation might be therapeutically beneficial in OSAS-related disease, especially OSAS-related nocturnal cardiac events in abdominal obesity.

Limitations of the Study

Our study had several limitations. First, the cross-sectional design makes it difficult to establish a cause-effect relationship. Second, the results may not be valid in non-Japanese populations. Third, it was difficult to recruit a sufficient number of subjects, and thus further multicenter studies of larger samples or controlled samples (non-OSAS versus OSAS, or non-obese versus obese) should be conducted in the future. Fourth, the present study used anthropometric measures, such as WC, HC, and WHR, to account for the effects of body fat distribution, because we could not measure visceral fat and subcutaneous fat areas using a computed tomography scan, as this clinic was unequipped. Further research on both visceral and subcutaneous fat areas measured by computed tomography scanning or magnetic resonance imaging is needed to delineate the effects of visceral and subcutaneous adiposity. Fifth, the present study lacks the clear advantage of diabetes and hypertension. Finally, although hypoxia (intermittent and sustained), reoxygenation, neuro-hormonal abnormality, abnormal metabolism, low sleep quality and other factors in OSAS during sleep could explain the nocturnal fall in circulating adiponectin levels, the current study used a sustained hypoxic model, as we reported previously[9].

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Disclosure Statement

The authors declare no conflict of interest.

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