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Short communication

Effect of ovariectomy on inflammation induced by intermittent hypoxia in a mouse model of sleep apnea

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ABSTRACT

Patient data report marked gender and pre-vs-postmenopausal differences in obstructive sleep apnea (OSA). However, no experimental data are available on how sexual hormones modulate OSA consequences. Here we report novel results on estrogen-modulated heart and brain inflammation in female mice subjected to intermittent hypoxia, a major injurious challenge in OSA. C57BL/6J (14-week old) intact and ovariectomized mice ($n = 6$ each) were subjected to intermittent hypoxia (20 s at 5% and 40 s at 21%, 60 cycles/h; 6 h/day). Identical intact and ovariectomized groups breathing room air were controls. After 30 days, the gene expressions of interleukins 6 and 8 (IL-6, IL-8) in the brain and heart tissues were measured. Whereas, compared with normoxia, intermittent hypoxia considerably increased IL-6 and IL-8 gene expressions in intact females, no change was found in ovariectomized mice when comparing normoxia and intermittent hypoxia. These data suggest that estrogens modulate the inflammatory effects of intermittent hypoxia and point to further studies on the role played by sex hormones in OSA.
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1. Introduction

Obstructive sleep apnea (OSA) is a very prevalent disease characterized by recurrent events of upper airway obstruction, usually resolved by arousals, with a frequency of up to 60–80 events per hour in severely diseased patients. In addition to increased inspiratory efforts to breathe against a closed airway, with very negative intrathoracic pressure swings, patients with OSA experience disruption of sleep architecture, increased sympathetic activation and recurrent arterial oxygen desaturations. Among these injurious challenges, intermittent hypoxemia, and consequent hypoxia/reoxygenation at tissue level (Dalmases et al., 2014), has been identified as one of the major pathological causes of the mid- and long-term consequences of OSA, namely increased risk of cardiovascular, neurocognitive, metabolic and even neoplastic diseases.

It has been well established that OSA presents clear differences in men and women (Lin et al., 2008). Gender modifies the phenotype of this sleep breathing disorder since, as compared with men, women usually have milder OSA, shorter obstructive events, occurring mainly during rapid-eye-movement sleep, and in general notable differences in sleep architecture (Lin et al., 2008). Moreover, recent data seem to confirm previous studies suggesting that some consequences of OSA could be different in men and women (Campos-Rodriguez et al., 2014). Interestingly, whereas in young patients OSA is more frequent in men, at ages corresponding to postmenopause, prevalence tends to approach in both genders (Dancey et al., 2001). Even though the mechanisms determining all these differences are not well understood, they should be mostly attributed to the background determining gender differences, i.e. the function of sexual glands (Lin et al., 2008).

However, there are no experimental data available describing how sexual hormones, and specifically estrogen, could modulate the consequences of OSA. In fact, almost all data obtained from the widespread animal model of intermittent hypoxia (mimicking one of the main injuring factors in OSA patients) have been obtained in male, and no study has addressed the effect of menopause. Among the several pathophysiological processes

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altered by hypoxia/reoxygenation, inflammation has been identified as a major factor in driving the consequences of OSA. Interestingly, the role of estrogen in modulating the inflammatory cascade is still not clear since data in the literature report that, depending on the cause of inflammation and the target organ, estrogen could either enhance or attenuate inflammation (Straub, 2007).

In this context, the present study aimed at obtaining novel experimental insight into the potential effects of estrogen in the consequences of hypoxia/reoxygenation. To this end, we focused on the changes induced by chronic intermittent hypoxia mimicking OSA in the gene expression of two relevant inflammatory biomarkers – interleukins 6 and 8 (*IL-6*, *IL-8*) – in the heart and brain tissues of ovariectomized mice as compared with intact females.

2. Methods

The investigation – approved by the Ethical Committee for Animal Research of the University of Barcelona – was carried out on 24 C57BL/6J female mice (14-weeks old, 20.12 ± 0.17 g; m ± SEM) randomly distributed into two groups: ovariectomy (OVX) and sham-operated (SHAM) ($N = 12$ each).

Bilateral ovariectomy was carried out under intraperitoneal ketamine/xylazine (5/10 mg/kg) anesthesia in OVX mice. After shaving the lumbar area, a single short longitudinal skin incision and two bilateral abdominal muscle wall incisions were made to exteriorize both ovaries and oviducts. Each oviduct was bound near the ovary with sterile silk suture and the ovary was removed with a single cut, the remaining tissue was placed again into the peritoneal cavity and the skin was stitched with sterile silk suture. Buprenorphine (0.2 mg/kg) was subcutaneously administered as an analgesic for 2 days. Mice in the SHAM group were subjected to the same surgical procedure with the exception of oviduct bounding and ovary excision.

After 3 days of recovery from surgery, 6 OVX and 6 SHAM mice were randomly selected and subjected to a pattern of high-frequency intermittent hypoxia mimicking severe OSA (20 s at 5% and 40 s at 21%, 60 cycles/h; 6 h/day). The other 6 OVX and 6 SHAM mice were subjected to an identical experimental process but were breathing room air instead of intermittent hypoxic air (normoxia). After 30 days of intermittent hypoxia or normoxia, the mice were euthanized by exsanguination through the abdominal aorta under intraperitoneal urethane anesthesia (1 g/kg). Their hearts and brains were immediately excised and frozen in liquid nitrogen, and their uteri were excised and weighed.

Inflammation was assessed by measuring the gene expression of *IL-6* and *IL-8* in the brain and heart tissues. Total RNA was isolated using Ultraspec reagent (Biotecx, Houston, TX, USA). RNA samples were cleaned (NucleoSpin RNA II; Macherey-Nagel, Düren, Germany) and checked for integrity by agarose gel electrophoresis. The total RNA isolated by this method was undegraded and free of protein and DNA contamination. Relative levels of specific mRNAs were assessed by real-time reverse transcription-polymerase chain reaction (RT-PCR). Reverse transcription was performed from 0.5 μg total RNA using Oligo$(dT)_{23}$ and M-MLV Reverse Transcriptase (Life Technologies). The PCR reaction contained 10 ng of reverse-transcribed RNA, 2X IQ™ SYBRGreen Supermix (BioRad, Barcelona, Spain) and 900 nM of each primer. PCR assays were performed on a MiniOpticon™ Real-Time PCR system (Bio-Rad). Thermal cycling conditions were as follows: activation of Taq DNA polymerase at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 15 s and at 60 °C for 1 min. The primer sequences were: adenine phosphoribosyltransferase (*APRT*) forward: 5′-CAGCGGCAAGATCGACTACA-3′, reverse: 5′-AGCTAGG GAAGGGCCAAACA-3′, *IL-6* forward: 5′-ACACATGTTCTCTGGGAAAT CGT-3′, reverse: 5′-AAGTGCATCATCGTTGTTCATACA-3′ and *IL-8* forward: 5′-CTTGTCATTGCCAGCTGTGT-3′, reverse: 5′-TGAC TGTGGAGTTTTGGCTG-3′. Optimal primer amplification efficiency for each primer set was assessed and a dissociation protocol was carried out to ensure a single PCR product. The results for the expression of specific mRNAs were always presented relative to the expression of the control gene (*APRT*).

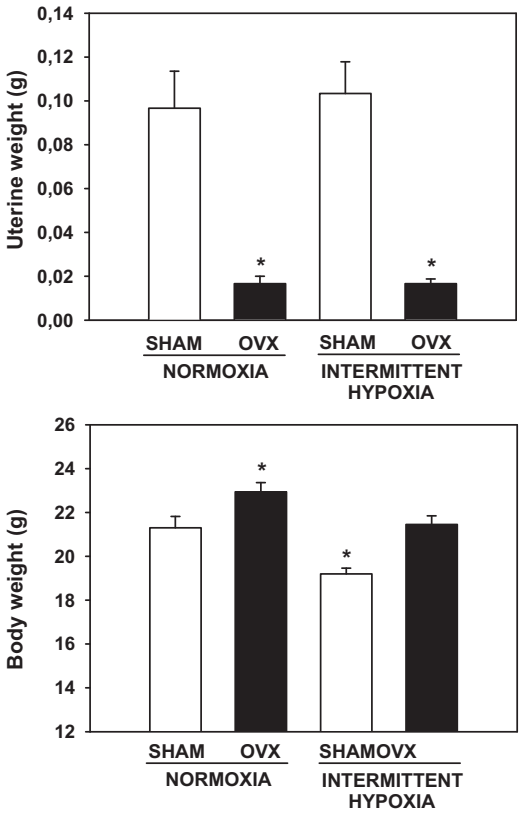

Fig. 1. Uterus and body weights in intact (SHAM) and ovariectomized (OVX) mice after 30 days of normoxia or intermittent hypoxia mimicking sleep apnea. *: $p < 0.001$ as compared with SHAM subjected to normoxia.

Group data were computed as mean ± SE. The differences in body and uterine weights, and in *IL-6* and *IL-8* gene expressions were assessed by two-way ANOVA considering group (OVX vs SHAM) and treatment (normoxia vs intermittent hypoxia) as factors, followed by Bonferroni post hoc analysis to determine the statistical significance ($p < 0.05$) of group differences as compared with the SHAM normoxia group.

3. Results

For both normoxia and intermittent hypoxia, ovariectomized mice showed a considerable 6-fold reduction in their uterine weight as compared with intact mice (Fig. 1). Both intermittent hypoxia and ovariectomy significantly modified body weight, as typically observed in these two animal models (Fig. 1). As compared with normoxic intact mice, body weight increased by 7.6% in ovariectomized normoxic mice, and decreased by 9.9% in intact mice subjected to intermittent hypoxia. As a result, the weight of ovariectomized mice subjected to intermittent hypoxia and of intact normoxic animals was similar.

The effects of intermittent hypoxia in the gene expression of *IL-6* was very different in intact and ovariectomized mice, as shown in Fig. 2, for both heart and brain tissues. In normoxic mice, ovariectomy did no significantly modify IL-6 gene expression. In intact mice, intermittent hypoxia induced a considerable increase in the

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