Leptin's Activity on the Hydroxyl Radical: A Possible Link to the Oxidative Stress-Related Endothelial Vasodilation in Patients with Obstructive Sleep Apnea

By: Madalina Macrea, Thomas Martin, Zhenquan Jia, Hara Misra

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Abstract:

Purpose: Obstructive sleep apnea (OSA) is associated with increased cardiovascular morbidity, whereas the underlying mechanism is still eluding, the thought participants are chronic intermittent hypoxia with consequent increase in the reactive oxygen species, leading to endothelial cell damage and dysfunction in these patients. As the hydroxyl radical (·OH) mediates the vascular smooth muscle relaxation, identification of its scavengers might reveal sentinel markers of decreased vascular responsiveness and worse long-term comorbid outcome. We therefore assessed leptin's scavenger effect on ·OH using the electronic paramagnetic resonance (EPR) method.

Methods: The ·OH was generated by the Fenton reaction in the presence of spin-trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DMPO) with various concentrations of leptin (0.25, 2.5, and 25 μg/ml) and without leptin. EPR spectrometer settings were: modulation frequency, 100 kHz; X band microwave frequency, 9.5 GHz; microwave power, 20 mW (milliwatts); modulation amplitude, 1.0 G (gauss); time constant, 160 s; scan time, 200 s; and receiver gain, $1 \times l0^5$. EPR signal intensity between 3,440 and 3,540 G of measurements taken in at least three separate experiments was reported. Mannitol, a known ·OH scavenger, at 100 mM significantly decreased the DMPO–OH adduct formation and was used as the active-control agent.

Results: Leptin added to aqueous solutions at all concentrations was associated with a statistically significant decrease in EPR signal compared with controls due to its scavenging activity towards the ·OH.

Conclusions: Leptin could be further investigated as a sentinel biomarker of decreased vascular responsiveness and future risk of atherosclerotic disease in obese OSA patients.

Abbreviations

ROS: Reactive oxygen species

·OH: Hydroxyl radical

OSA: Obstructive sleep apnea

EPR: Electron paramagnetic resonance

DMPO: 5-Diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide

M: Mannitol

NL: No leptin solution

LL: Low leptin concentration

IL: Intermediate leptin concentration

HL: High leptin concentration

Keywords: Leptin | Obstructive sleep apnea | Hydroxyl radical | Vasodilation

Article:

Introduction

Obstructive sleep apnea (OSA) is a common disorder characterized by repetitive collapse of the pharyngeal airway during sleep resulting in a myriad of adverse vascular risks from insulin resistance to dyslipidemia, elevated diastolic blood pressure and leptin levels, a 167-amino acids adipokine with a structure similar to cytokines. Clinically important, it is now accepted that untreated OSA disease is associated with increased cardiovascular morbidity and mortality [*1*], whereas the exact underlying mechanism is still eluding, the chronic intermittent hypoxia (CIH) [*2*, *3*] with increased oxidative stress (OS) and reactive oxygen species (ROS) [*4*] is thought to play a major role, leading to exaggerated endothelial cell damage and dysfunction in these patients. Having the endothelium play such a key role in the early development of the atherosclerotic process [*5*], it would be ideal to predict the future risk of cardiovascular disease (CAD) by identifying biomarkers that might translate indirectly the endothelium-related vasomotor properties.

As the hydroxyl radical (˙OH) mediates the vascular smooth muscle relaxation both directly (stimulating guanylate cyclase) [*6*] and indirectly (stimulating the synthesis/release of the endothelium-derived relaxing factor) [*7*], identification of its scavengers may clinically translate into the discovery of sentinel biomarkers of decreased vascular responsiveness and worse long-term cardiovascular comorbid outcome; furthermore, because leptin's role had been extended

into inducer of angiogenesis [*8*] and endothelial dysfunction [*9*] and its levels correlate both with worse cardiovascular outcomes [*10*] as well as several indices of OSA severity (such as degree of nocturnal hypoxia) [*11, 12*], we decided to assess leptin's scavenger effect on $^{\cdot}OH$ using the electronic paramagnetic resonance (EPR) spectroscopy in combination with spin trapping and mannitol, a known $^{\cdot}OH$ scavenger, as an active-control agent.

Materials and Methods

Chemicals and Materials

Purified 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO) was purchased from Alexis (Carlsbad, CA). Leptin, mannitol, and all other chemicals and reagents were obtained from Sigma Chemicals (St. Louis, MO).

EPR Study of the Scavenging Activity of Leptin Towards $^{\cdot}OH$

EPR is an excellent approach for the detection of radicals [*13*]. Briefly, the $^{\cdot}OH$ was generated by the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-}$) in the presence of spin-trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DMPO) with various concentrations of leptin: 0.25 μg/ml (low leptin concentration, LL); 2.5 μg/ml (intermediate leptin concentration, IL); and 25 μg/ml (high leptin concentration, HL); and without leptin (no leptin, NL). EPR spectrometer settings were: modulation frequency, 100 kHz; X band microwave frequency, 9.5 GHz; microwave power, 20 mW (milliwatts); modulation amplitude, 1.0 G (gauss); time constant, 160 s; scan time, 200 s; and receiver gain, $1 \times l0^5$. Reactants were mixed in test tubes in a final volume of 0.1 ml, and the reaction mixture was then transferred to a capillary tube for EPR spectral analysis at room temperature under conditions as described above. Mannitol (M) at 100 mM significantly decreased the DMPO–OH adduct formation and was used as the active-control agent.

Statistical Analysis

EPR signal intensity ($n = 12$) between 3,440 and 3,540 G of measurements taken in at least three separate experiments was expressed as standard mean ± SD. Unpaired *t* test was used for identifying the effect of various leptin concentrations (0.025, 0.25, and 25 μg/ml) compared with passive (no leptin) and active control (mannitol). Results were reported as *p* value and 95 % confidence interval (CI); $p < 0.05$ was considered statistically significant.

Results

At all tested concentrations, leptin added to aqueous solution resulted in a decrease in EPR signal. The EPR signal intensities of mannitol and leptin containing solutions compared with no leptin solutions were as follows: 87 % for the M solution; 68 % for the LL solution; 70 % for the IL solution; and 52 % for the HL solution. As shown in Fig. 1, the intensity of the signal

decreased, from top (NL) to bottom (HL), with increasing leptin concentrations of 0.025 μg/ml (Fig. 1, LL), 0.25 μg/ml (Fig. 1, IL), and 25 μg/ml (Fig. 1, HL).

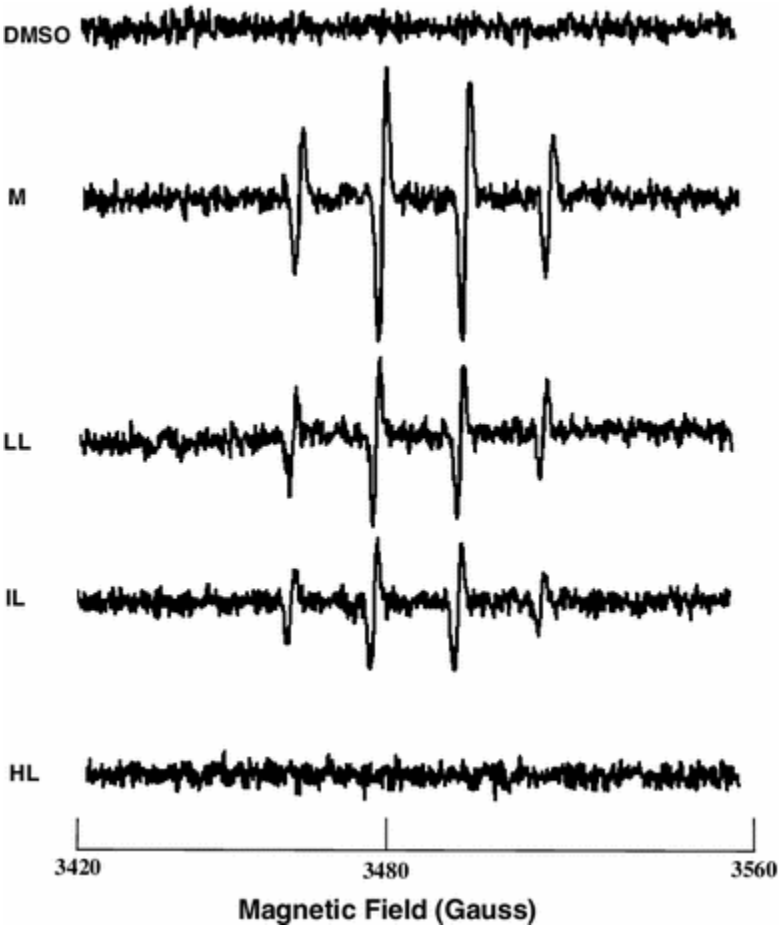

Fig. 1 EPR spectroscopy in combination with the spin probe DMPO was used to examine the hydroxyl radical-scavenging capability of leptin. From *top* to *bottom* DMPO; M, i.e., active control mannitol solution; LL, i.e., 0.025 μg/ml leptin concentration; IL, i.e., 0.25 μg/ml leptin concentration; and HL, i.e., 25 μg/ml leptin concentration

Compared with NL and M as controls, leptin in LL (p = 0.01, 95 % CI 19–136; and p = 0.0009, 95 % CI 33–106, respectively), IL (p = 0.001, 95 % CI 40–137; and p = 0.0006, 95 % CI 32–92, respectively), and HL (p = 0.0001, 95 % CI = 105–216; and p = 0.0009, 95 % CI 54–167, respectively) solutions was found to inhibit the ˙OH statistically significant (Fig. 2).

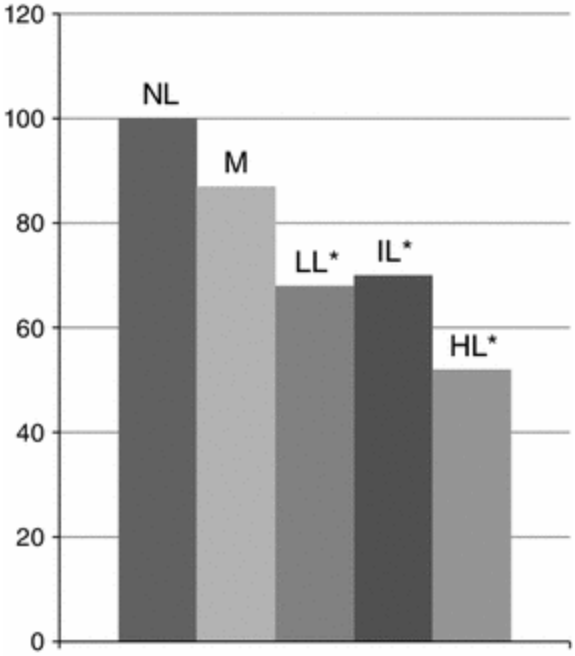

Fig. 2 Scavenging of the hydroxyl radical by leptin. Effect of leptin concentrations on the EPR signal corresponding to the ·OH adduct with DMPO. The 100 % reference value corresponds to the level of the DMPO–OH adduct produced in the corresponding control sample without leptin. *NL* no leptin; *M* active control, mannitol;*LL* low leptin concentration solution, i.e., 0.025 μg/ml; *IL* intermediate leptin concentration solution, i.e., 0.25 μg/ml; *HL* high leptin concentration solution, i.e., 25 μg/ml. Unpaired *t* test for each treatment showed significant differences (*$p < 0.0001$) compared with *M*

Discussion

Our results demonstrate that leptin added to aqueous solutions at 0.025, 0.25, and 25 μg/ml concentrations was associated with a decrease in the EPR signal due to leptin's scavenging activity towards the ·OH, highest activity being seen at the final concentration of 25 μg/ml.

Many ROS play central roles in the vascular physiology; we thought of analyzing ·OH due to its distinct role in obesity, an exceedingly prevalent condition in OSA disease, known to be associated with a state of chronic, low-grade inflammation, where both formation of ROS and alterations in local blood flow occur simultaneously [*14*]. More specifically, the adipose tissue is reported to modulate the sensitivity of the endothelium to the vasodilators effects of ·OH formation [*15*], enhancing the vascular relaxation to this oxygen radical, raising the question of a possible protective mechanism against the low-grade chronic inflammation, such as seen in obesity. Although several studies tend to disagree, reporting ·OH activity as neutral [*16*] or vasoconstricting [*17*] (likely as a result of the difficulties in accurately reproducing in vitro the normal physiologic conditions) [*18*], the concept of its activity as vasodilator seems to be

supported by a wide audience, making its analysis a logical step in understanding the link between inflammation-related ROS and endothelium-related CAD risk.

However, given the complexity and limitations of measuring ROS within integrating systems, such as humans [*19*], it seems convenient to have a particular ROS activity quantified indirectly, through their corresponding scavengers.

Leptin, an adipokine derived primarily from the white adipose tissue, and its elevated levels, as seen in obesity and OSA, have been reported to have pathologic consequences, particularly on the cardiovascular system; whereas the details of an exact mechanism are still lacking, several pathophysiologic pathways of leptin action on endothelium have been suggested, such as inhibition of NO synthase [*20*], augmented superoxide production [*21*] and/or increases in release of endothelin-1 [*22*]. In qualitative-oriented studies, increased leptin levels have been connected to hypertension [*23*] and increased CVD risk [*24*, *25*]; in a quantitative-oriented study, Knudson et al. [*26*] demonstrated that increased plasma leptin concentrations to levels similar to those observed in obese subjects (10–90 ng/ml) significantly impair the paracrine regulation of the coronary circulation. Our study, by uniquely demonstrating leptin's inhibitory action on the $^{\cdot}OH$ and implicitly its vasodilator activity, adds theoretical evidence of leptin use as sentinel biomarkers of future CVD risk in patients with major comorbidities, including obesity and OSA disease. Specifically, several other major conditions, such as chronic kidney disease [*27*], carotid artery and CAD with diabetes [*28*, *29*], metabolic syndrome [*30*], and its risk [*31*], are associated with a hyperleptinemic state that conceivably could be used as a sentinel plasma biomarker for clinical and, ideally, subclinical CVD. Equally important, our results could be expanded to a heterogeneous patient population, regardless of its ethnicity [*24*, *30*, *32*,*33*].

Although we have demonstrated leptin's scavenger action on $^{\cdot}OH$ at all concentrations, we failed to show concentration-dependent results for the two lowest concentrations, i.e., LL and IL. As we consider our experimental conditions to be rigorous, we attribute our findings not to be dissonant but rather in harmonious agreement with current data that support the hypothesis of two independent leptin receptor binding sites in the vascular endothelium: one of high affinity site (5 nM or 80 ng/ml), which couples to pathways that impair coronary endothelial function; and another of low affinity site (740 nM or 11,000 ng/ml), which couples to the NO-dependent vasodilation. Therefore, in our study, we tested leptin levels that would respect both the high, physiologically present (i.e., 0.025 μg/ml) as well as the low affinity site (i.e., 0.25 and 25 μg/ml), providing mutually confirmatory data to these established studies.

In conclusion, our study proposes a novel mechanism of leptin-induced endothelial dysfunction; given our findings, it could be suggested that hyperleptinemia may be of clinical significance as a biomarker able to translate indirectly the endothelium-related vasomotor properties. We therefore hope that further studies would extrapolate our results, given leptin's impact in several major comorbid conditions, including obesity and OSA, and its convenient and accurate measurement in both serum and urine.

Conflict of interest

None to declare for all authors.

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