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Endothelial Nitric Oxide Synthase Uncoupling: A Novel Pathway in OSA Induced Vascular Endothelial Dysfunction

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Abstract

The mechanism of vascular endothelial dysfunction (VED) and cardiovascular disease in obstructive sleep apnea (OSA) is unknown. We performed a comprehensive evaluation of endothelial nitric oxide synthase (eNOS) function directly in the microcirculatory endothelial tissue of OSA patients who have very low cardiovascular risk status. Nineteen OSA patients underwent gluteal biopsies before, and after effective treatment of OSA. We measured superoxide ($O_2^{\cdot -}$) and nitric oxide (NO) in the microcirculatory endothelium using confocal microscopy. We evaluated the effect of the NOS inhibitor L-Nitroarginine-Methyl-Ester (L-NAME) and the NOS cofactor tetrahydrobiopterin (BH4) on endothelial $O_2^{\cdot -}$ and NO in patient endothelial tissue before and after treatment. We found that eNOS is dysfunctional in OSA patients pre-treatment, and is a source of endothelial $O_2^{\cdot -}$ overproduction. eNOS dysfunction was reversible with the addition of BH4. These findings provide a new mechanism of endothelial dysfunction in OSA patients and a potentially targetable pathway for treatment of cardiovascular risk in OSA.

Keywords

Obstructive sleep apnoea; nitric oxide; endothelial dysfunction; hypertension

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1. INTRODUCTION

Obstructive sleep apnea (OSA) occurs in up to 24% of middle aged adults and is increasingly recognized as a modifiable cardiovascular risk factor (Marin et al., 2005; Peppard et al., 2000). The pathogenesis of cardiovascular disease (CVD) in OSA patients remains largely unknown. The high prevalence of hypertension, obesity, and aging upon diagnosis in OSA patients imposes considerable challenges to the study of OSA related cardiovascular risk directly in patients. Studies in children (Gozal et al., 2007), adults with low cardiovascular risk status (Ip et al., 2004; Kato et al., 2000), and animal models of intermittent hypoxia (Philippi et al., 2010; Phillips et al., 2004) have all confirmed that vascular endothelial dysfunction (VED) is the earliest vascular consequence of OSA preceding the occurrence of hypertension in models of OSA. VED is an established predictor of the progression of CVD in the general population (Clarkson et al., 1997; Suwaidi et al., 2000). Therefore, VED is the critical link between OSA and its cardiovascular consequences.

Nitric oxide (NO) production and availability in the vascular endothelium is the critical determinant of vascular endothelial function and reactivity (Arnold et al., 1977; Ignarro et al., 1987). Oxidant mediated dysfunction of endothelial nitric oxide synthase (eNOS) is a common cause of decreased NO availability in CVD (Ozaki et al., 2002; Shi et al., 2002). The mechanism of decreased NO availability in OSA is not known. There are conflicting reports regarding eNOS expressions in human studies of OSA patients. In OSA patients, VED was reversible with interventions targeting endothelial oxidants supporting oxidant mediated NOS dysfunction in the early reversible phase of OSA related VED (El Solh et al., 2006; Grebe et al., 2006). The sources and pathways of oxidant production in the human endothelium, and the expression and function of NOS in OSA remain largely unknown. Studies of eNOS expression in patients and animal models of OSA have yielded conflicting results (Arnet et al., 1996; Jelic and Le Jemtel, 2008; Kaczmarek et al., 2013; Takemoto et al., 2002). eNOS function, however, has never been evaluated directly OSA patients.

During oxidant stress, NO reacts with superoxide ($O_2^{\cdot-}$) forming the strong oxidant peroxynitrite, thus decreasing NO availability (Wang and Zweier, 1996). Furthermore, this oxidant stress can directly modify eNOS protein (Chen et al., 2010) or its cofactor, tetrahydrobiopterin (BH4) (Biondi et al., 2012; Kuzkaya et al., 2003) resulting in dysfunctional enzyme. In these conditions, eNOS shifts from NO production to overproducing $O_2^{\cdot-}$ and contributing to the oxidant stress within the endothelium (Antoniades et al., 2006). This dysfunction, termed “eNOS uncoupling”, has been described in several cardiovascular disorders including diabetes, hypertension, and heart failure.

We previously reported that in OSA patients who have very low cardiovascular risk status, decreased NO availability is associated with increased production of peroxynitrite in the microcirculatory endothelium (Patt et al., 2010). This overproduction of peroxynitrite was the first indirect evidence of local basal production of $O_2^{\cdot-}$ within the endothelium of OSA patients. From this background, we hypothesized that OSA related VED is associated with eNOS uncoupling. Given the affinity of peroxynitrite to oxidize the redox sensitive eNOS cofactor BH4, we hypothesized that decreased BH4 availability contributes to eNOS

dysfunction in OSA. To address these objectives, we developed a novel method for evaluating the human microcirculatory endothelium from freshly procured subcutaneous biopsy tissue in OSA patients. We aimed to confirm that $O_2^{\cdot -}$ overproduction occurs directly within the human endothelium of OSA patients and to determine the role of eNOS in this $O_2^{\cdot -}$ overproduction.

2. MATERIALS AND METHODS

2.1. Participants

2.1.1. Patients with OSA—Newly diagnosed OSA patients were recruited from the Ohio State University (OSU) Sleep Center within 4 weeks of their diagnostic polysomnography and prior to the initiation of continuous positive airway pressure (CPAP). OSA with an apnea hypopnea index (AHI) >15 events per hour of sleep was required for inclusion. Low cardiovascular risk status was required in all participants and was defined by a Framingham risk Score <5% (Wilson et al., 1998). In particular, exclusion criteria included any diagnosis or ongoing treatment of hypertension, hypercholesterolemia (Total cholesterol >200 mg/dl regardless of age), diabetes, or smoking. None of the participants were on any prescribed medications; and supplements were discontinued at least a week prior to participation. The purpose of the restrictive inclusion criteria is assure that VED in participants is due to OSA only and not to other subclinical cardiovascular risk factors.

2.1.1 Non-OSA Participants—Due to the lack of reference ranges for endothelial reactivity as well as the novel tissue measurements planned in the study, we enrolled body-mass index (BMI) and age matched healthy participants with no OSA or any other cardiovascular risk factor as a validation group. We planned to perform the eNOS function studies in the available tissue of these non-OSA validation participants to establish a reference range of human eNOS function ex-vivo. Non-OSA participants were recruited from patients of the sleep laboratory who had sleep complaints but negative polysomnography for OSA. Participants met the same exclusion criteria as the OSA patients.

2.2. Procedures

Participants with newly diagnosed OSA provided an initial visit within 4 weeks of initial OSA diagnosis, prior to starting CPAP; and a conclusion visit after 12 weeks of CPAP therapy. Adherence to CPAP was verified with device download during the conclusion visit. Only patients who used CPAP more than 4 hours per night underwent the conclusion biopsy. Each visit included endothelial reactivity study and gluteal subcutaneous biopsy. Non-OSA volunteers underwent the procedures once. The protocol was approved by the OSU Institutional Review Board (protocol number 2009H0212). The study was registered in the National Clinical Trials database (NCT01027078).

2.2.1. Endothelial reactivity—This was evaluated by measuring the flow mediated dilation (FMD) of the brachial artery with Doppler ultrasound. Image Acquisition was done with a linear array transducer (7 MHz frequency) and color spectral Doppler (GE Vivid 7).

Studies were performed according to published guidelines (Corretti et al., 2002) and our previously reported protocol (Patt et al., 2010).

2.2.2. Gluteal subcutaneous biopsy—Incisional skin biopsy techniques were used to obtain 2–3 cm³ of subcutaneous tissue from the lateral upper gluteal region of participants. The biopsy tissue was immediately sectioned and a portion frozen in liquid N₂ and kept in –80° for microscopy studies.

2.3. Measurements

2.3.1. NO Production in the Microcirculatory Endothelium—Transverse sections (8-μm) were prepared from optimal cutting temperature (OCT)-fixed tissue and incubated with the NO probe CuFL (500 μM) in the absence or presence of the NOS inhibitor: L-NG-Nitroarginine Methyl Ester (L-NAME) (1 mM). The specificity of CuFL fluorescence was confirmed by adding PTIO (NO scavenger, 50 μM) to the sections and confirmation of fluorescence quenching. The slides were viewed with Olympus Fluo-View 1000 confocal microscope at a magnification of 20X. The digital images were quantitatively analyzed for fluorescence intensities with the Olympus OIB software (FV10-ASW version 2.0). Intensity of the fluorescence was measured on a per pixel basis and this per pixel fluorescence intensity value was averaged for each contoured area of vascular endothelium. Measurements were obtained from multiple images of at least 3 sections. No significant differences were observed from different levels within a biopsy. The CuFL technique has been widely applied for cellular and tissue measurements of NO (Efremova et al., 2010; Schreiber et al., 2011). We have previously applied and validated the CuFL fluorescence along with the electron paramagnetic resonance (EPR) measurement of NO (Yang et al., 2013). Inhibition of the observed CuFL derived fluorescence by NOS inhibition with L-NAME provides additional confirmation of specificity.

2.3.2. O₂^{•-} Production in the Microcirculatory Endothelium—We determined O₂^{•-} in situ production using dihydroethidium (DHE) fluorescence microscopy techniques. The cell-permeable non-fluorescent DHE is oxidized to fluorescent hydroxyethidium. DHE is oxidized on reaction with O₂^{•-} to ethidium, which binds to DNA in the nucleus and fluoresces red. Sections (5 μm) of the subcutaneous tissue were incubated with DHE (10 μM) along with Hoescht (1 μM) in dark for 30 min (at 37 degrees C). The sections then were rinsed with Tris buffered saline (TBS) for 5 min, fixed with paraformaldehyde, and then mounted with the antifade mounting medium, Fluoromount-G, by overlaying the coverslip. In initial experiments, the superoxide dismutase (SOD) mimetic (MnTBAP) at 50 μM was added to the tissue sections and the resultant residual fluorescence values subtracted from the total fluorescence to determine the O₂^{•-} derived signal.

2.3.3. Determination of eNOS Uncoupling in the Microcirculatory Endothelium—We measured O₂^{•-} and NO production before and after the addition of L-NG-Nitroarginine Methyl Ester (L-NAME) to the subcutaneous tissue sections. L-NAME is an established NOS inhibitor that blocks both NO and O₂^{•-} formation at the oxygenase site of eNOS. Transverse sections (8-μm) were prepared from OCT-frozen tissues and were acutely

thawed and incubated with L-NAME (1 mM) for 1 hour 37°C prior to the addition of the probe solution containing DHE (10 µM) or the NO indicator CuFL (500 µM).

2.3.4. Effect of BH4 Limitation on eNOS Function in OSA—Transverse frozen sections (8-µm) were acutely thawed and incubated with BH4 (100 µM) for 1 hour at 37°C after the addition of the probe solution containing DHE (10 µM) along with the nuclear stain Hoescht 1 µM in the absence or presence of L-NAME (1 mM) and MnTBAP (50 µM). To detect NO generation, frozen sections were thawed and incubated with the NO indicator CuFL (500 µM) in the absence or presence of L-NAME (1 mM) before the addition of BH4.

2.3.5. Expression and Phosphorylation of eNOS in the Microcirculatory Endothelium—Frozen sections were thawed and incubated with primary mouse anti-phosphorylated eNOS (P-eNOS) antibodies Serine-1177 and rabbit anti-eNOS (BD Biosciences) and incubated with the respective secondary goat anti-rabbit, Alexa Fluor 488-conjugated and goat anti-mouse, Alexa Fluor 568-conjugated antibodies (Molecular Probes, Eugene, CA) and analyzed by Olympus Fluo-View 1000 confocal microscope with the 20x objective and with the 405 nm, 488 nm and 543 nm excitations for DAPI, green and red fluorescence, respectively.

2.4. Design and Analysis

We compared the outcomes within the same OSA patients before and after verified treatment with CPAP. We accepted that the only change between the baseline visit and the conclusion visit was the elimination of OSA by CPAP. Testing hypotheses within-patient eliminates any effect of age, obesity, or other cardiovascular risk factors that are not addressed by the strict inclusion and exclusion criteria. Comparisons between pre and post CPAP were done for the main hypotheses testing and measurements from the validation group were obtained for reference. For comparing pre- versus post-treatment outcomes, paired t-tests were used. Effects of L-NAME and BH4 additions were evaluated by paired t-tests; comparison of the L-NAME and BH4 effects in pre-CPAP versus post-CPAP tissues were evaluated by paired t-tests of the pre to post L-NAME/BH4 differences. Although the validation group was not generally used for hypothesis testing, we did compare pre-CPAP FMD with the BMI and age matched validation group to confirm that our patients have subclinical vascular abnormality. We also performed experiments in available tissue from the validation group to establish reference ranges for the novel measurements used in the hypothesis testing. Our primary hypothesis testing was the effect of L-NAME on $O_2^{\cdot -}$ in pre-CPAP patients. From our published studies of CPAP effect (Post-Pre CPAP) on peroxynitrite and assuming L-NAME will have a similar effect to CPAP (approximately 1 SD of change), we expected to need a sample size of 12 OSA patients for testing.

For all microscopy studies and measurements, localization of the signal to the endothelial layer of microvessels with diameter >30 µm was performed by an observer blinded to the OSA or treatment status of the tissue source. All experiments were done only on available tissue from the same patients in the pre and post CPAP states. All fluorescence and image quantification were done on all tissue per experiment in the same session.

3. RESULTS

3.1. Characteristics of Participants

Nineteen OSA patients and 12 validation volunteers participated in the study. Table-1 lists the characteristics of OSA patients. All OSA participants had total cholesterol <200 mg/dl as part of the inclusion criteria and were not on any medications. In addition to the characteristics listed in table-1, AHI ranged from 12.6 to 120 events/min and desaturation index (4%) ranged from 6 to 90 episodes/h. OSA patients used CPAP (mean $\pm$ standard deviation) 5.3 $\pm$ 1.2 hours per night, and had a post treatment AHI of 2.9 $\pm$ 2.6 events/hour on device download during the 12 week visit. Pre CPAP weight versus post CPAP weight was unchanged.

Non-OSA participants were 8 females and 4 males and had the following characteristics (mean $\pm$ SD): Age 39 $\pm$ 7 years; BMI: 32 $\pm$ 5 Kg/m²; Blood pressure 130 $\pm$ 14/76 $\pm$ 15 mmHg. There were no differences in age, BMI, blood pressure, or lipid profile between the validation group and the OSA participants.

3.2. Endothelial Reactivity in OSA patients and no cardiovascular risk factors

FMD was (mean (standard error of the mean) 5.2 % (0.6) in OSA patients (n=19) at baseline and increased significantly to 8 % (0.6) with CPAP (p<0.001). The BMI and age matched validation group (n=12) had an FMD of 7.8 % (0.7) which was significantly different from pre CPAP-OSA patients (p=0.01) but not post CPAP (0.84).

Baseline FMD correlated negatively with the baseline AHI in patients (r= -0.72, p = 0.001). There was no correlation between FMD and age, BMI or sex in the OSA patients. The findings confirm that OSA is associated with VED in patients who have no measurable cardiovascular risk factors (Figure-1).

3.3. Endothelial NO Production in OSA

NO production increased in the microcirculatory endothelium with CPAP from 63.7 (27.6) to 235 (71) fluorescence units, P=0.01 (Figures 2 and 4) (n=8).

3.4. Endothelial O₂^{-•} Overproduction in OSA Patients

Microcirculatory endothelial DHE-derived fluorescence decreased in patients from 352.7 (52.4) to 49.2 (8.9) fluorescence units after 12 weeks of CPAP (n=9), an 86% decrease with treatment (p<0.0001) (Figures 3 and 4). Non-OSA validation participants (n=8) had O₂^{-•} level of 29.3 (5.0) fluorescence units. O₂^{-•} was still higher in OSA patients post PAP than non-OSA participants (p = 0.08). There was a negative correlation between baseline •O₂^{-•} production and FMD in OSA patients (r= -0.68, p = 0.003).

3.5. eNOS Function in the Microcirculatory Endothelium of OSA Patients

3.5.1. NOS uncoupling in OSA—The addition of L-NAME resulted in a significant decrease in microcirculatory endothelial O₂^{-•} in pre-CPAP OSA patients (n=9) from 225.7 (37.9) to 59.6 (12.4); P=0.003. There was also a smaller but significant effect of L-NAME on decreasing microcirculatory endothelial O₂^{-•} in the post CPAP patients from 42.5 (7.4) to

24.3 (8.5); $P=0.04$ (Figure 5-left). This suggests a persistent role for eNOS in the production of $O_2^{\cdot -}$ 12 weeks after treatment with CPAP. In the paired test for L-NAME effects, L-NAME produced a greater decrease in $O_2^{\cdot -}$ in the pre-CPAP tissue than in post-CPAP ($p=0.01$).

The effect of L-NAME, a NOS inhibitor on NO production validates that NOS inhibition occurred with the technique used. In the pre CPAP state, only low endothelial NO levels were detected which decreased after L-NAME treatment with decrease in measured fluorescence from 49.1 (14.6) to 33.4 (10.1) units, $p=0.39$. In OSA patients post CPAP, much higher NO levels were seen that decreased significantly from 155.1 (19.8) to 71.1 (16.1) with the addition of L-NAME; $P=0.002$. In the paired test for L-NAME effects, L-NAME produced a greater decrease in NO post-CPAP than pre-CPAP ($p=0.07$). The decrease in NO confirmed achievement of the eNOS inhibitory effect of L-NAME in the tested tissue (Figure 5-right). The finding that L-NAME had more inhibitory effect on NO in the post CPAP than the pre-CPAP tissue support that CPAP restored the putative NO producing function of eNOS.

In the validation group ($n=6$), L-NAME decreased NO as expected with decrease I measured fluorescence from (58.7 (18.6) to 17.3 (7.5), $p=0.03$) but had no effect on $O_2^{\cdot -}$ (34.1 (9.8) vs. 34.7 (7.9)), suggesting that in non-OSA participants, eNOS does not contribute to $O_2^{\cdot -}$ production in the endothelium.

3.5.2. Role of BH4 Availability in OSA related VED—A profound effect of BH4 on $O_2^{\cdot -}$ occurred in pre-CPAP ($n=9$) patients with a decrease in $O_2^{\cdot -}$ as measured from DHE derived fluorescence from 68.5 (5.8) to 7.1 (1.2) units ($p<0.0001$); BH4 did not affect post-decreasing from 24.5 (4.3) to 21.4 (6.1), $p=0.41$. The CPAP patients significantly, with $O_2^{\cdot -}$ effect of BH4 on $O_2^{\cdot -}$ in the pre CPAP patients was significantly greater than the effect in the post CPAP state ($p=0.01$) (Figure 6-left). Note that microcirculatory endothelial NO levels prior to BH4 addition were significantly lower in pre CPAP patients than in the same patients post CPAP confirming the independent measurement of NO reported above.

The addition of BH4 to the microcirculatory tissues of pre CPAP patients resulted in a large increase from 6.0 (1.2) to 63.4 (30.0) units in microcirculatory endothelial NO production ($p<0.0001$). In the post-CPAP patients, the addition of BH4 resulted in a small but non-significant change in NO production from 83.1 (17.8) to 181.6 (47.4) $p=0.16$ (Figure 6-right). In the validation group ($n=4$), there was no effect for the addition of BH4 on either NO ($p=0.25$) or $\bullet O_2^{\cdot -}$ ($P=0.11$).

3.6. Microcirculatory eNOS Expression

PeNOS significantly increased in OSA patients ($n=8$) with CPAP treatment from 62.5 (13.2) to 188 (37.7) fluorescence units, $p=0.02$. Total eNOS only modestly increased with CPAP but this was not significant (190.5 (43) vs. 258 (70.5) $p=0.35$).

4. DISCUSSION

Conducted fully in freshly procured human endothelial tissue from OSA patients before and after CPAP, this study is the first to identify that eNOS uncoupling is the cause of decreased NO availability and a source of endothelial $O_2^{\cdot-}$ overproduction in OSA patients.

Furthermore, this study demonstrates that decreased BH4 availability is a pathway of eNOS uncoupling in OSA patients. These findings have therapeutic implications for the cardiovascular consequences of OSA. eNOS uncoupling can potentially be targeted pharmacologically to decrease VED and risk of subsequent development of cardiovascular disease in OSA, particularly in patients who do not use CPAP sufficiently.

4.1. VED in OSA

OSA is associated with several sleep, respiratory, and neurocirculatory perturbations. Of these perturbations, the nocturnal chronic intermittent hypoxia (CIH) pattern is of particular importance for the cardiovascular consequences of OSA (Fletcher et al., 1992c). Several human and animal studies have demonstrated that CIH alone, rather than hypercapnia or sleep disruption with arousals is the only abnormality required for the development of OSA related hypertension (Bao et al., 1997; Brooks et al., 1997; Xie et al., 2000). CIH causes prolonged post-stimulus upregulation of the carotid chemoreceptors and subsequent sustained activation of the sympathetic system (Fletcher et al., 1992a; Peng et al., 2003; Somers et al., 1995; Xie et al., 2000). Animal models of CIH develop hypertension that is dependent on intact sympathetic system and rennin angiotensin system (RAS) (Fletcher et al., 1999; Fletcher et al., 1992b). Taken together, it is likely that the cardiovascular consequences of OSA, and particularly hypertension, are mediated by CIH induced sympathetic and RAS activation.

The time course for the development of OSA related cardiovascular consequences was recently evaluated using mouse exposure to variable durations of CIH (Phillips et al., 2004) (Marcus et al., 2010). VED occurred in this CIH model prior to the development of sustained hypertension. Intact sympathetic system and RAS were also required for the development of VED in this model (Philippi et al., 2010). Our study demonstrates that OSA patients who have very low cardiovascular risk status have VED that is reversible with CPAP treatment (i.e. due to OSA). This finding confirms our previous findings (Patt et al., 2010) as well as others (Kato et al., 2000) that VED is the first cardiovascular abnormality detectable in OSA patients who have very low CVD risk status. Taken together, this study and the data from the CIH animal models strongly suggest that VED is the earliest cardiovascular abnormality in OSA and that VED contributes to the subsequent development or progression of OSA related CVD.

4.2. eNOS Uncoupling a Novel Pathway in OSA related VED

This study demonstrates that eNOS uncoupling is the critical mechanism for decreased NO availability and VED in OSA. Furthermore, the study identified decreased BH4 availability as critical for eNOS uncoupling in OSA patients. Animal models of CIH demonstrated that VED occurs early in the course of the CIH exposure without significant change in eNOS expression, and in association with increased peroxynitrite deposition in the

endothelium(Philippi et al., 2010). We have previously found that OSA patients who are free of CVD have deposition of peroxynitrite within the microcirculatory endothelium. These patients had VED without decrease in eNOS RNA transcription (Patt et al., 2010). The presence of peroxynitrite in the endothelium along with decreased NO availability suggested endothelial superoxide overproduction. This study demonstrates for the first time that eNOS uncoupling is the explanation for the endothelial superoxide overproduction and decreased NO availability.

We found decreased BH4 availability to be a cause for eNOS uncoupling in OSA patients. BH4 stabilizes and donates electrons to the ferrous-di-oxygen complex in the oxygenase domain of eNOS to initiate the oxidation of L-Arginine(Hurshman et al., 1999; Schmidt et al., 2001; Vasquez-Vivar et al., 2002). BH4 is redox sensitive and can be oxidized to dihydrobiopterin (BH2) under oxidative stress conditions resulting in decreased bioavailability and eNOS uncoupling (Antoniades et al., 2006; Dumitrescu et al., 2007). Increasing endothelial BH4 synthesis or availability for restoring eNOS function has been evaluated as a therapeutic target in CVD disease yielding mixed results (Antoniades et al., 2006; Cunningham et al., 2012). However, simple BH4 supplementation in disease states associated with increased endothelial ROS production could worsen outcome as a consequence of increased formation of the BH4 oxidation product (Cunningham et al., 2012; Worthley et al., 2007).

4.3. Role of endothelial $O_2^{\cdot -}$ overproduction in OSA induced VED

This study localized $O_2^{\cdot -}$ overproduction to the vascular endothelium of OSA patients and confirmed that eNOS is a source of endothelium-derived $O_2^{\cdot -}$ overproduction. eNOS uncoupling as a source of endothelial oxidant stress has not been demonstrated previously in OSA patients. Endothelial $O_2^{\cdot -}$ reacts with NO forming peroxynitrite within the endothelium, as we have already reported in OSA patients (Patt et al., 2010). Peroxynitrite readily oxidizes BH4(Kuzkaya et al., 2003) and results in eNOS uncoupling propagating the cycle of $O_2^{\cdot -}$ overproduction and NO deficiency.

Other possible sources of endothelial $O_2^{\cdot -}$ overproduction include the NADPH system and xanthine oxidase. The role of NADPH system is supported by evidence for RAS activation in CIH mediated VED (Fletcher et al., 1999; Marcus et al., 2010). It is well established that angiotensin II activates endothelial NADPH resulting in endothelial $O_2^{\cdot -}$ overproduction. Circulating angiotensin II levels are increased in OSA patients(Moller et al., 2003) and animal models of OSA.(Yuan et al., 2004). The role of angiotensin blocking agents in modifying OSA related VED has not been explored in patients. Human studies have demonstrated a role for xanthine oxidase in OSA related VED(El Solh et al., 2006). Taken together, it is likely that OSA is associated with activation of more than one source of endothelial $O_2^{\cdot -}$ overproduction.

4.4. Limitations and Technical Considerations

As discussed above this study established that the endothelium in OSA is a source of oxidant stress and that eNOS uncoupling is critical for the pathogenesis of OSA related VED. We determined decreased BH4 availability as a cause of eNOS uncoupling. Other possible

pathways of eNOS uncoupling in OSA include the recently described direct glutathionylation of eNOS (Chen et al., 2010) and increased methylarginines (Cardounel et al., 2007). We did not address all likely sources of $O_2^{\cdot -}$ overproduction in the endothelium. These pathways discussed above will need to be further investigated in specially designed and dedicated studies using the novel approach and techniques described in this study.

Decreased eNOS phosphorylation was reported previously (Jelic and Le Jemtel, 2008) in venous endothelial cells in a similar population of OSA patients as that we studied and this observation was further confirmed in our study. The presence of NOS dependent $O_2^{\cdot -}$ overproduction in the endothelium of OSA patients is confirmed in this study regardless of the underlying expression levels or phosphorylation patterns of eNOS. A role for inducible NOS was suggested previously in OSA (Jelic and Le Jemtel, 2008). We have previously found no change in iNOS mRNA expression in OSA patients with treatment. Even if iNOS upregulation is confirmed in the arteriolar endothelium, NOS uncoupling remains important regardless of the type of NOS protein.

The measurement of endothelial reactivity was done on the brachial artery, a mid-sized vessel; while the mechanistic studies were done on subcutaneous resistance arterioles. The choice of brachial artery FMD measurement was due to its established correlation with long term cardiovascular morbidity and mortality (Clarkson et al., 1997; Lind et al., 2011; Suwaidi et al., 2000). and to validate the population of this study against similar studies in OSA patients with very low CVD risk factors (Grebe et al., 2006; Ip et al., 2004). Correlation between endothelial dysfunction in the two vascular beds has been previously established (Agewall et al., 2006; Lind et al., 2011; Park et al., 2001). Importantly, this brachial FMD measurement is well correlated with important clinical cardiovascular outcomes allowing for generalizability of our findings to CVD risk in OSA patients.

Finally, this study utilized quantitative confocal microscopy techniques to test the primary hypotheses of this research. Other methods for quantifying $O_2^{\cdot -}$ and NO such as EPR are more specific but less sensitive. However, the limited amount of available tissue in these human studies and the current limitations in available in vivo EPR technology, with questions remaining regarding EPR probe safety in humans, precluded its application at this time. We used widely accepted and standardized confocal microscopy probes and techniques (Lim et al., 2006; Yang et al., 2013). We performed the microscopy analysis and quantification in a fully blinded fashion to improve the sensitivity of these techniques. Advantages of this approach included the immediate preservation of microcirculatory vascular tissue within the biopsy tissue no later than few minutes after the biopsy procedures. In addition, being able to localize the superoxide and NO produced directly to the endothelial layer of the microcirculatory vessels enhanced the sensitivity and specificity of the measurements. Only participants with very low cardiovascular risk status were included in this study. Therefore, the VED observed in OSA participants before treatment was attributable only to OSA. Furthermore, the primary comparisons were within subject before and after treatment eliminating any confounding effects of weight, age, or unidentified cardiovascular risk on the findings.

4.5. Conclusions and Clinical Implications

The techniques used in this study included novel functional and mechanistic measurements that have not been reported previously in OSA patients. These methods support the feasibility of performing randomized controlled trials addressing the effect of CPAP on novel mechanistic markers of cardiovascular disease directly in patients. The findings of persistent $O_2^{\cdot-}$ overproduction and eNOS uncoupling after 12 weeks of adherence to CPAP suggest that persistent cardiovascular risk may remain in adequately treated patients or that the effect of CPAP treatment may require longer than 3 months to reverse OSA related cardiovascular risk. Obviously, the average of 5 hours of use of CPAP in our population may simply be insufficient to reverse fully the effects of OSA within 12 weeks.

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Highlights

- OSA patients underwent endothelial reactivity studies and subcutaneous biopsies
- We evaluated endothelial nitric oxide synthase function in patient tissue before and after treatment
- eNOS uncoupling is the cause of endothelial dysfunction and $\bullet O_2^-$ overproduction in OSA patients
- Tetrahydrobiopterin restored eNOS function in OSA patients' tissue
- The findings provide a potential pharmacological target to decrease vascular disease in OSA

Figure 1. Effect of OSA Treatment with CPAP on Endothelial Reactivity

Endothelial reactivity measured by flow mediated dilation in OSA patients before and after treatment and in non-OSA validation group. Significant increase in FMD is noted with treatment in OSA patients ($p=0.001$). There was a significant difference between pre-CPAP and non-OSA age and BMI matched participants FMD ($p < 0.001$), and no difference between Post-CPAP and the non-OSA participants.

Figure 2. Endothelial Nitric Oxide Availability in OSA Patients before and after CPAP
Nitric oxide (NO) availability measured by CuFL fluorescence in the microcirculatory endothelium from OSA patients before and after CPAP. NO increased significantly with CPAP ($p < 0.001$).

Figure 3. Endothelial Superoxide Production in OSA patients before and after CPAP

Superoxide production was measured by DHE-derived fluorescence in patients before and after CPAP. Endothelial $\bullet O_2^-$ decreased after 12 weeks of CPAP ($p < 0.0001$).

Figure 4. Representative Confocal Imaging of NO and $O_2^{\cdot-}$ in the Microcirculatory Endothelium
 NO and $O_2^{\cdot-}$ detection by CuFL and DHE fluorescence respectively in the microcirculatory endothelium from human subcutaneous biopsy tissue (8 μ m sections). The images represent tissue from the same OSA patient before and 12 weeks after CPAP therapy. Magnification, 20X;

• $O_2^{\cdot-}$ detection by DHE fluorescence in the microcirculatory endothelium from human subcutaneous biopsy tissue (8 μ m sections) obtained from pre-CPAP, post-CPAP and control subjects. Magnification, 20X;

Figure 5. eNOS Uncoupling in OSA

Left Panel: Effect of L-NAME on $O_2^{\cdot -}$ production in OSA patients; L-NAME resulted in a profound decrease in $O_2^{\cdot -}$ in pre-CPAP patients ($P=0.003$) (left). A smaller decrease occurred in patients post CPAP ($P=0.04$) (right). There was no significant effect for L-NAME on $\bullet O_2^{\cdot -}$ production in non-OSA participants (not shown).

Right Panel: Effect of L-NAME on NO production in OSA patients; Note, the higher baseline levels of NO in tissue from Post-CPAP (left) patients than pre-CPAP (right); L-NAME resulted in a larger decrease in NO production in Post-CPAP tissue than in Pre-CPAP ($P=0.002$). There was also an expected significant effect for L-NAME in non-OSA participants ($P=0.002$) (not shown).

Figure 6. Role of Decreased BH4 in eNOS dysfunction in OSA

Left Panel: Effect of BH4 on $O_2^{\cdot -}$ Production; A profound decrease in $O_2^{\cdot -}$ production occurred with BH4 supplementation in OSA patients pre CPAP ($p=0.01$) (left). No significant effect for BH4 on $O_2^{\cdot -}$ was noted in the same patients post-CPAP (right).

Right Panel: Effect of BH4 on NO production; BH4 supplementation resulted in a significant increase in NO production in pre CPAP patients ($p<0.0001$) (left). There was no significant effect for BH4 supplementation on NO production after CPAP treatment (right).

Table 1

Baseline Characteristics of OSA Patients

Characteristics of OSA Patients (n=19)	Mean $\pm$ SD
Age (years)	44 $\pm$ 14
Female (n=5) (%)	26
Race (n,%)	
White	15, 79%
African American	3, 16%
Unknown	1, 5%
BMI Kg/m ²	36 $\pm$ 8
Blood Pressure mmHg	125 $\pm$ 5/79 $\pm$ 10
Epworth Sleepiness Scale	14 $\pm$ 4
Desaturation index (4%)	29 $\pm$ 36
Desaturation nadir	81 $\pm$ 7
AHI events/hour	43 $\pm$ 37
Total sleep time (min)	327 $\pm$ 87
REM percent/sleep time	17 $\pm$ 10
Sleep efficiency %	84 $\pm$ 9

BMI: Body mass index; Results are shown as mean $\pm$ SD. Note that all participants had total cholesterol <200 and Framingham score below 5%.