Chronic Hypoxia and Cardiovascular Dysfunction in Sleep Apnea Syndrome

by

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(Abstract)

The purpose of the current study was to test the hypothesis that chronic hypoxia associated with sleep-disordered breathing relates to abnormal Nitric Oxide (NO) production and vascular endothelial growth factor (VEGF) expression patterns that contribute to aberrancy of specific determinates of cardiovascular and cardiopulmonary function before, during, and after graded exercise. These patterns may further reflect pathologic alteration of signaling within the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt-1) transduction network. To this end, 7 medically diagnosed OSA patients (3 male, 4 female), mean age 48 years and 7 apparently healthy control subjects (3 male, 4 female), mean age 42 years, underwent baseline venous blood draws and maximal bicycle ergometry. Mononuclear cells isolated from peripheral blood were utilized as reporter cells for measurement of VEGF, Akt-1, hypoxia inducible factor-1 alpha (HIF-1 alpha), and vascular endothelial growth factor receptor-2 (VEGFR2) gene expression by redundant oligonucleotide DNA microarray and real-time PCR technologies. Circulating angiogenic progenitor cells expressing VEGFR2 were profiled by flow cytometry. Plasma and serum concentrations of VEGF, nitrates/nitrites, catecholamines, and dopamine were measured by enzyme-linked immunosorbent assay (ELISA) and high performance liquid chromatography (HPLC). Arterial blood pressure, cardiac output, oxygen consumption and total

peripheral resistance were determined at Baseline, 100W, and peak ergometric stress by standard techniques. There were no apparent differences ($p \leq .05$) observed in biochemical markers relating to vascular function and adaptation including, serum nitrates/nitrites, norepinephrine, dopamine, and plasma VEGF. No differences were found relative to cardiac output, stroke volume, cardiopulmonary or myocardial oxygen consumption, expired ventilation, heart rate, arteriovenous oxygen difference, total peripheral resistance, and mean arterial pressure. Due to methodological issues related to the redundant oligonucleotide DNA microarray and real-time PCR gene expression analyses, results of these experiments were uninterpretable. Thus, the research hypothesis was rejected. Conversely, significant ($p \leq .05$) differences were observed in waist: hip ratios, recovery: peak systolic blood pressure ratio at 1 minute post-exercise and %VEGFR2 expression. OSA was associated with elevations in both waist: hip ratios and recovery: peak systolic blood pressure ratio at 1 minute post-exercise as well as significant depression of %VEGFR2 profiles. Moreover, significant negative correlations were found regarding waist: hip ratios and %VEGFR2 expression ($r = -.69; p = .005$) and recovery: peak systolic blood pressure ratio at 1 minute post-exercise and %VEGFR2 expression ($r = -.65; p = .01$). These findings did not provide evidence that NO-dependent vasoactive mechanisms are suppressed nor did they support the supposition that angiogenic mechanisms are pathologically activated in sleep-disordered breathing.

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To my parents, Tom and Roberta Chittenden. I feel genuinely blessed to have such loving parents. Thank you both for believing in me.

To Sara, a true confidant. Thank you for your love and friendship.

To my grandson, Dawson. I wish for you a spirit of inquiry.

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Chapter I

Introduction

Obstructive sleep apnea (OSA) is a chronic condition characterized by repetitive obstructions in the upper airway during sleep. Recent studies demonstrate a link between these repetitive occlusions in the upper airway and cardiovascular abnormalities. For example, significant correlations have been demonstrated between OSA and systemic and pulmonary hypertension [Henderson *et al*, 1999; Sajkov *et al*, 1994; Weitzenblum *et al*, 1988], cardiac ischemia and dysrhythmias [Henderson *et al*, 1999; Partinen *et al*, 1990], and cerebrovascular accidents [Henderson *et al*, 1999;Chittenden *et al*, 2002].

Systemic hypertension (HTN) is frequently associated with OSA and can be directly ascribed to the repeated and partial apneic events that disrupt autonomic cardiovascular reflexes during sleep [Young *et al*, 1997; Lies *et al*, 1996; Dempsey *et al*, 1997; Brooks *et al*, 1997]. The prevalence of HTN in OSA patients may be as high as 50% [Hall *et al*, 1995]. Diminished airflow during sleep results in decreased blood oxygen levels and increased carbon dioxide retention, both of which cause increased sympathetic nerve activity resulting in elevations in blood pressure [Somers *et al*, 1995]. Patients with OSA also exhibit increased sympathetic activity during the day [Hedner *et al*, 1988; Carlson *et al*, 1993]. These fluctuations in sympathetic nervous system activity may be involved in the pathogenesis of HTN. Furthermore, studies supporting this link between OSA and HTN have reported a decrease in resting sympathetic nervous system activity [Waradekar et al, 1996] and blood pressure [Wilcox *et al*, 1993; Suzuki *et al*, 1993] following nasal continuous positive airway pressure (nCPAP) treatment [Chittenden *et al*, 2002].

In addition, OSA is associated with both acute and chronic effects of sympathoexcitation, resulting in decreased vascular compliance [Chittenden *et al*, 2002]. It has long been postulated that the increased

vascular tone in OSA leads to abnormal diurnal blood pressure control and increases in the incidence of myocardial infarction and stroke. Therefore, it is likely that OSA adversely affects several different cardiovascular homeostatic mechanisms. Although these vascular regulatory mechanisms have not been thoroughly investigated in OSA patients, disease-related hypoxia might abate vascular function by depravation of tissue-derived or blood vessel-derived vasoactive substances such as adenosine, prostanoids, endothelin, and nitric oxide (NO) [Ni *et al*, 1998].

There is growing evidence of impaired endothelium-dependent vascular relaxation in OSA and that NO-dependent mechanisms are attenuated [Ip *et al*, 2000]. Because eNOS expression and subsequent endothelial generated NO production may be altered by conditions such as hypoxemia, hypoxia-reoxygenation, shear stress, and ischemia-reperfusion of the vascular wall, researchers are postulating that endothelial derived NO may be a major contributor to blood pressure regulation in OSA [Ip *et al*, 2000]. Interestingly, researchers have recently reported a negative correlation between NO levels and sleep apneic activity indicated by apnea-hypopnea indices and duration of sleep time with hypoxemia. This suggests that the more severe the sleep apnea, the greater the NO suppression. Moreover, these same researchers also reported a negative correlation between serum NO and systolic blood pressure, and that implementation of nCPAP therapy resulted in an increase of circulating serum NO concentrations to levels that were comparable with controls. This may indicate that hypoxia is the main mechanism for the suppressed NO levels reported in OSA [Ip *et al*, 2000].

In addition to the effects on vascular tone, there is also strong evidence linking NO release and the subsequent stimulation of blood vessel growth and development. This process, referred to as neovascularization, is thought to be controlled by two general mechanisms: 1) vascular shear stress and

2) hypoxia. In fact, it is now believed that the decrease in blood oxygen tension (hypoxia) is one of the major pathologic factors related to sleep-disordered breathing. Hypoxia has also been shown to stimulate the expression of many angiogenic peptides under both normal and pathologic conditions. One of the most studied proteins related to neovascularization is vascular endothelial growth factor (VEGF), an important mediator of vessel growth in a wide variety of tissues [Ware and Simons, 1999]. VEGF stimulates angiogenesis by activating receptors on endothelial cells of existing vessels. In concert with other growth/differentiation factors, VEGF stimulation via the PI3K/Akt-1 signal transduction network results in basement membrane breakdown, migration and proliferation of endothelial cells, and formation of functional blood-carrying structures. Acutely, VEGF signaling has vasodilatory activity mediated by NO [Ware and Simons, 1999].

Although NO production appears to be suppressed in OSA, studies have recently indicated that VEGF concentrations are elevated in sleep-disordered breathing [Gozal *et al*, 2001]. Although the exact mechanism for this phenomenon has not been determined, chronic hypoxia may serve to explain at least in part the pathologic VEGF protein expression associated with this disease state. Schulz *et al*. [2002] found that patients with severe nocturnal hypoxia had markedly increased serum VEGF levels when compared to patients presenting with moderate hypoxia and to control subjects. This study appears to support the findings of Gozal *et al*. [2001], which determined that elevated serum and plasma VEGF concentrations were predictive indicators of OSA. While both groups provided no definitive explanation for their findings, researchers believe the diminished inhibitory effect of nitric oxide suppression on VEGF gene induction may provide a plausible rationale for this pathophysiologic phenomenon [Schulz *et al*, 2000]. Studies have shown that NO production is not only regulated by hypoxia, but this molecule can also modify the effects of hypoxia on other genes. Liu *et al*. [1998] determined that NO inhibited the

hypoxic induction of VEGF at the transcriptional level. These researchers believe that this phenomenon was associated with decreased HIF-1 DNA binding activity, even in the presence of increased protein expression. To this end, Liu *et al*. [1998] concluded that NO controls induction of VEGF gene transcription via a hypoxic enhancer.

Attributed to these recent findings, researchers are now suggesting the processes of neovascularization and the resultant decrease in vascular compliance may explain the decline in morbidity and mortality associated with long-standing sleep apnea pathology. The Sleep Heart Health Study recently indicated that patients with mild OSA are at enhanced risk for occlusive vascular disease [Shahar *et al*, 2001]. However, these investigators also found that the risk did not rise further in patients with more severe OSA. This appears to support the findings of Lavie *et al* [2000]. These researchers witnessed decreases in cardiovascular morbidity and mortality in ~1500 men Israeli men after the eighth decade of life. These studies are supported by the clinical observations that, even in the presence of severe nocturnal oxygen desaturations, not all OSA patients suffer from cardiovascular co-morbidity [Schulz *et al*, 2002]. Consequently, it may be considered that certain as yet unrevealed mechanism(s) shield some OSA patients from developing cardiovascular complications.

In summary, decreased NO production with the subsequent hypoxic-release of inhibitory control mechanisms on VEGF gene induction in OSA may offer a plausible rationale for the elevated plasma and serum protein VEGF levels found in many OSA patients. In turn, the enhanced circulating VEGF concentrations may constitute an adaptive angiogenic mechanism to counterbalance the onset of cardiovascular co-morbidity. Hypothetically, this disease related phenomenon might provide a feasible

explanation for the reported decrease in morbidity and mortality associated with long-standing sleep-disordered breathing [Lavie *et al*, 2000].

Statement of the Problem

OSA predisposes individuals to increased cardiovascular morbidity and mortality [Hall *et al*, 1995]. This increased pathological risk is exacerbated by factors of age, body mass index (BMI), elevated blood pressure, and aberrant polysomnography indices [Laks *et al*, 1995; Ancoli-Israel *et al,* 1989]. Thus, the importance of early detection and treatment cannot be understated. Adequate intervention has been shown to improve daytime alertness and quality of life, daytime hypertension, neuropsychiatric performance, and reduce nocturnal oxyhemoglobin desaturation and respiratory arousals [Henderson *et al*, 1999;Strollo *et al*, 1998]. Therefore, in an attempt to develop improved early detection and treatment protocols, researchers have recently focused their attention on determining the complex pathogenic mechanisms of sleep-disordered breathing. These efforts have lead to the realization that cardiovascular dysfunction plays a major role in patient morbidity and mortality. Thus, the present study systematically evaluated the intricate relationship between aberrant nitric oxide production and vascular endothelial growth factor expression on indicators of disease severity and cardiovascular function. The experimental design incorporated molecular analysis of gene expression within the PI3K/Akt-1 signal transduction network, evaluation of circulating vasoactive molecules, and clinical measurement of specific determinates related to cardiovascular and cardiopulmonary performance before, during, and after graded exercise in both OSA patients and non-experimental controls.

Significance of Study

Obstructive sleep apnea (OSA) is a serious disorder caused by repetitive obstructions in the upper airway during sleep. This sleep-mediated disturbance in airflow constitutes the primary pathophysiological event in OSA. Although this common condition is still under-recognized by many in the health care profession, epidemiological studies have identified this syndrome in 2% to 4% of middle-aged adults. A substantial number of individuals may go undiagnosed, which suggests an even higher prevalence, particularly given the trend toward rising obesity, a common co-morbidity of OSA [Skomro *et al,* 1999; Redline *et al*, 1993]. Other clinical research has shown a link between OSA and cardiovascular abnormalities. Significant correlations have been shown between OSA and systemic and pulmonary hypertension [Henderson *et al*, 1999; Sajkov *et al*, 1994; Weitzenblum *et al*, 1988], cardiac ischemia and dysrhythmias [Henderson *et al*, 1999; Partinen *et al*, 1990], and cerebrovascular accidents [Henderson *et al*, 1999;Chittenden *et al*, 2002]. Therefore, the importance of early detection and treatment of the underlying pathology is vital. Treatment of sleep-disordered breathing has revealed improvements in daytime alertness and quality of life, daytime hypertension, neuropsychiatric performance, and reductions in nocturnal oxyhemoglobin desaturation and respiratory arousals [Henderson *et al*, 1999;Strollo *et al*, 1998].

According to Agency for Health Care Policy and Research (AHCPR), adjunctive screening tools are needed to increase the current diagnostic and prognostic potential of polysomnography. Given the high rate of cardiovascular co-morbidity witnessed in sleep disordered breathing, cardiopulmonary stress testing could be used for this purpose.

Cardiopulmonary stress testing has been shown to be an important diagnostic and prognostic tool in the evaluation of patients with hypertension for a number of years. In fact, an exacerbated blood pressure response to exercise in normotensive subjects has been shown to be one of the best predictors of future hypertension. In support of this notion, Lim *et al.* [1996] preformed a meta-analysis over a 10-year period from 1985 to 1995 that evaluated information on exercise testing in hypertensive patients and persons at risk for developing hypertension. Data on hemodynamic responses in hypertensive patients and persons at risk for developing hypertension and correlations to end-organ damage, mortality, and exercise tolerance were analyzed. Their analysis was aimed at determining whether this type of methodology is a valuable screening procedure for the diagnosis, prognosis, or assessment of the effect of therapy. They found that the exercise capacity of hypertensive patients was reduced by as much as 30% compared with age-matched controls. In addition, this exercise impairment increases with age and end-organ damage, and its origin can be traced back to adolescence. Total peripheral resistance also progressively increases. These researchers believe that the associated changes are caused by functional and structural involvement of the cardiovascular system and that abnormal blood pressure responses to exercise have prognostic value for the future development of hypertension, end-organ damage, and death.

In summary, cardiopulmonary exercise testing, in combination with polysomnography and various molecular and biochemical assessment techniques, may prove to be viable means of not only determining disease severity but also assessing the degree of cardiovascular dysfunction in OSA patients. These measures may eventually provide insight into the underlying pathology of this condition, which could ultimately lead to superior treatment regimes and improved quality of life for many OSA patients.

Research Hypothesis

The chronic hypoxia associated with sleep-disordered breathing relates to abnormal NO production and VEGF expression patterns that contribute to aberrancy of specific determinates of cardiovascular and cardiopulmonary function before, during, and after graded exercise. These patterns may further reflect pathologic alteration of signaling within the PI3K/Akt-1 transduction network.

Research Aims

The major aim of this study was to determine the relationship between chronic hypoxia and vascular pathogenic mechanisms in humans. To this end, the intricate association linking NO release and VEGF expression was investigated in an attempt to establish a connection between cardiovascular dysfunction and disease severity.

1.) Specific Aim 1: To evaluate specific biomarkers related to endothelial (re) generation in study subjects. Mononuclear cells isolated from peripheral blood were utilized as reporter cells for measurement of VEGF, HIF-1 alpha, Akt-1, and VEGFR2 gene expression by redundant oligonucleotide DNA microarray and real time PCR technologies. Circulating angiogenic progenitor cells expressing VEGFR2 were profiled by flow cytometry.

2.) Specific Aim 2: To profile vasoactive molecules associated with neovascularization in study subjects. Plasma and serum concentrations of VEGF, nitrates, catecholamines, and dopamine were measured by enzyme-linked immunosorbent assay (ELISA) and high performance liquid chromatography (HPLC).

3.) Specific Aim 3: To profile cardiovascular/cardiopulmonary function in study subjects. Arterial blood pressure, cardiac output, oxygen consumption and total peripheral resistance were determined at Baseline, 100W, and peak ergometric stress by standard techniques.

Assumptions

1. Subjects accurately answered the medical/health history questionnaire, thus allowing for implementation of co-morbid exclusion criteria.
2. Control subjects did not experience nighttime hypoxia.
3. Subjects correctly reported their physical activity levels.
4. Subjects exerted maximal effort during cycle ergometry.
5. Cycle ergometer was accurately calibrated and maintained.
6. Metabolic cart accurately measured all cardiopulmonary variables.

Limitations

1. Cardiac output measures were not obtained at peak exercise.
2. Because of logistical limitations, exercise testing and blood draw procedures were not limited to a specific time of day.
3. Because of funding constraints, polysomnography was not performed on control subjects.
4. Purification procedures yielded lower than expected amounts of RNA from reporter cells. To this end, experimental and control subjects' RNAs were pooled into their respective groups.
5. The experimental design included seven experimental subjects and seven control subjects. Sample size estimates for the major variables of interest are included in Appendix B.

Delimitations

1. Experimental subjects were volunteers who were referred to the Allergy and Sleep Disorder Center in Christiansburg, VA for evaluation of a suspected sleeping disorder.
2. Control subject were volunteers from local communities.
3. Both experimental and control subjects had not been involved in any cardiovascular training defined as (> 2day/wk) for at least 6 months prior to participation in the study.
4. Both experimental and control subjects did not have preexisting conditions such as congestive heart failure and/or moderate to severe chronic obstructive pulmonary disease.
5. Both experimental and control subjects were not taking cardiovascular medications.

Summary

OSA is a serious condition caused by repetitive obstructions in the upper airway during sleep. Studies have now shown that this disease associates with many cardiovascular aberrations. Many of these pathological abnormalities can be ascribed to both acute and chronic effects of sympathoexcitation, resulting in decreased vascular compliance. Often, this leads to aberrant diurnal blood pressure control and increases in the incidence of myocardial infarction and stroke. Suppressed NO production and subsequent hypoxic-release of inhibitory control mechanisms on VEGF gene induction may enhance circulating VEGF concentrations. It is quite plausible that this may constitute an adaptive mechanism to counterbalance the onset of cardiovascular co-morbidity and explain the reported decrease in morbidity and mortality associated with long-standing sleep apnea syndrome.

Furthermore, enhanced understanding of the relationship between cardiovascular dysfunction and sleep-disordered breathing might eventually lead to superior treatment regimes and improved quality of life

for many OSA patients. To this end, the current study scientifically evaluated the relationship between aberrant nitric oxide production and vascular endothelial growth factor expression on indicators of disease severity and cardiovascular function. The experimental design included molecular analysis of gene expression within the PI3K/Akt-1 signal transduction network, evaluation of circulating vasoactive molecules, and clinical measurement of specific determinates related to cardiovascular and cardiopulmonary performance before, during, and after graded exercise in both OSA patients and non-experimental controls.

Chapter II

Review of the Literature

Obstructive sleep apnea (OSA) is a serious disorder caused by repetitive obstructions in the upper airway during sleep. This sleep-mediated disturbance in airflow constitutes the primary pathophysiological event in OSA. Although this common condition is still under-recognized by many in the health care profession, epidemiological studies have identified this syndrome in 2% to 4% of middle-aged adults. A substantial number of individuals may go undiagnosed, which suggests an even higher prevalence, particularly given the trend toward rising obesity, a common co-morbidity of OSA [Skomro *et al,* 1999; Redline *et al*, 1993]. Research has also shown a link between OSA and cardiovascular abnormalities. Significant correlations have been observed between OSA and systemic and pulmonary hypertension [Henderson *et al*, 1999; Sajkov *et al*, 1994; Weitzenblum *et al*, 1988], cardiac ischemia and dysrhythmias [Henderson *et al*, 1999; Partinen *et al*, 1990], and cerebrovascular accidents [Henderson *et al*, 1999;Chittenden *et al*, 2002].

OSA predisposes individuals to increased mortality and this increased risk is exacerbated by age, body mass index (BMI), hypertension, and a high apnea index as determined through a polysomnography study [Laks *et al*, 1995; Ancoli-Israel *et al,* 1989]. Thus, the importance of early detection and treatment cannot be understated. Adequate intervention has been shown to improve daytime alertness and quality of life, daytime hypertension, neuropsychiatric performance, and reduce nocturnal oxyhemoglobin desaturation and respiratory arousals [Henderson *et al*, 1999;Strollo *et al*, 1998].

Excessive daytime sleepiness (EDS) is the most commonly reported symptom in these patients, characterized by impairments in concentration, memory, and cognitive function. This can result in

irritability, poor school or work performance, and mood disorders [Chittenden *et al*, 2002]. Sufferers often change lifestyle patterns by napping, ingesting large amounts of caffeine, and sleeping in on non-work days. Excessive fatigue along with the cognitive and affective limitations of excessive daytime sleepiness are often what prompt many patients to seek medical attention [Day *et al*, 1999]. While presentation of OSA in the obese, male snorer with excessive daytime sleepiness is commonly seen; other less specific signs and symptoms may be consistent with this diagnosis such as mild fatigue, decreased energy, difficulty concentrating, and inadequacies in emotional or social interaction [Skomro *et al*, 1999]. Patients with these less specific symptoms are often misdiagnosed and treated for the wrong condition when OSA is not considered [Chittenden *et al*, 2002].

One important clinical consequence for those suffering from excessive daytime sleepiness caused by OSA is a dramatic increase in the risk for automobile accidents [Wu *et al*, 1996]. In recent clinical trials, George *et al.* [1996] reported lower simulated driving scores in OSA patients. Of these subjects, approximately 25% reported falling asleep at least once a week while driving. In 1987, these researchers found 90% of sleep apnea patients had been involved in at least one automobile accident [George *et al,* 1987]. Other research has reported that OSA patients are seven times as likely to be involved in a car accident as subjects without the sleep disorder [George *et al,* 1987]. In fact, falling asleep behind the wheel is the number one cause of lethal automobile accidents in Germany and the estimated yearly cost of this type of mishap in the United States has been reported to exceed $40 million [Peter *et al,* 1995; Chittenden *et al*, 2002]. Much of this could be avoided through the proper diagnosis and treatment for those suffering from sleep-related illness such as OSA. A recent report in the New England Journal of Medicine showed that proper medical intervention for OSA patients reduced the risk of automobile accidents for those individuals [Teran-Santos *et al,* 1999]. Although there appears to be

an increased risk of motor vehicle accidents in this population, presently there are no assessment measures to accurately predict driving performance in OSA patients.

Nature of Obstructive Sleep Apnea

Sleep apnea syndromes are characterized by periodic cessations of breathing during sleep. The classifications of these disordered breathing patterns include: 1) OSA, defined as oronasal blockage of airflow during normal respiratory effort; 2) Central Sleep Apnea, defined as the complete cessation of respiratory muscle contraction; and 3) Mixed Sleep Apnea, defined as presentation of the features encompassing both obstructive and central apnea [Guilleminault *et al*, 1998; Halvorson *et al*, 1998; Lalloo e*t al*, 1998; Kyzer *et al*, 1998; Sin *et al*, 1999]. Clinical diagnoses of these sleep disorders are confirmed by polysomnography (PSG), which is completed via overnight observation in a sleep laboratory. During the observation period, the patient's sleep stages are monitored by the electroencephalogram (EEG), electrooculogram (EOG), electromyogram (EMG), and electrocardiogram (ECG). The monitoring period also includes the evaluation of respiratory effort, airflow, oxygen saturation, and body position and movements [Skomro *et al*, 1999; Yamashiro *et al*, 1995; Yamashiro *et al*, 1995; Sanders *et al,* 1993; Chittenden *et al,* 2002].

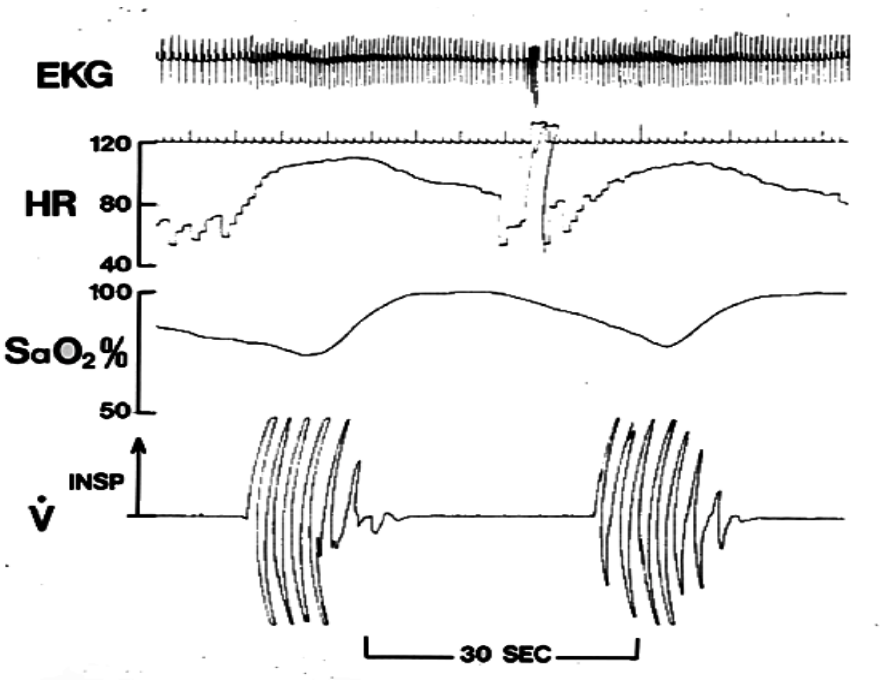

Figure 1. An example of the effects of an apneic event on various cardiovascular and neurological measures, as indicated by a simplified polysomnographic record. Apnea is occurring in the 30 sec designation. EKG = electrocardiograph, HR = heart rate, SaO_2% = percentage oxygen saturation of the blood, V_{INSP} = inspiratory airflow. Illustration provided by ResMed Inc., San Diego, CA.

The polysomnographic findings indicative of OSA include the appearance of five or more apneas or hypopneas per hour of sleep. Apnea is defined as the cessation of airflow for at least 10 seconds; where as obstructive hypopnea is defined as a decrease of 30% to 50% in airflow for 10 seconds in the presence of respiratory effort [Skomro *et al*, 1999]. These events are frequently accompanied by arterial oxygen desaturation (levels <90%). The severity of OSA is categorized by the apnea-hypopnea index (AHI), also known as the respiratory disturbance index (RDI) [Chittenden *et al,* 2002]. The respiratory disturbance index is determined by combining the number of apneas and hypopneas and dividing by the total time of sleep in hours [Skomro, 1999].

Predisposing Factors

Predisposing factors for OSA in men and women are presented in Table 1. Although the vast majority of medically diagnosed OSA patients are obese male snorers between 30 to 60 years of age, a growing number of non-obese women are now being identified with this condition. The underestimated prevalence of OSA in women remains unclear [Redline *et al,* 1994]. However, studies have shown that women tend to underreport typical conditional symptoms such as snoring, gasping, and apnea. Some clinicians have indicated that this may be caused by a reduced ability for women to generate orononasal tissue vibrations due to smaller anatomical structure [Chittenden *et al*, 2002]. In turn, their snoring or apneic episodes may go unnoticed by bed partners. In addition, the majority of women afflicted with sleep-disordered breathing are non-obese and do not fit the typical patient profile (Table 1). These circumstances make recognition difficult and many women are often undiagnosed [Redline e*t al,* 1994]. The typical female OSA patient often presents with dysmenorrhea or amenorrhea, abnormal craniofacial features, and small neck circumferences. In addition, the incidence of OSA in the female population has been shown to increase following menopause [Redline *et al*, 1994; Skomro *et al*, 1999; Sin *et al*, 1999]. Besides the typical physical and physiological manifestations of OSA, recent studies suggest that family history is an established risk factor that increases the likelihood of OSA two to fourfold [Redline *et al*, 1994; Skomro *et al*, 1999; Sin *et al*, 1999; Redline *et al*, 1995]. Moreover, certain types of endocrinological conditions such as hypothyroidism, acromegaly and various genetic disorders including Marfan's syndrome have also been implicated as predisposing factors in OSA.

Table 1. Some characteristics of OSA patients

Characteristic	Men	Women
Obesity	BMI >28 kg/m^2	BMI >28 kg/m^2
Gender	**More Prevalent**	Less Prevalent
Age	>40 years	>40 years
Neck Circumference	>17 inches	>16 inches
Witnessed Events	Snoring/Apneic Events/ Nocturnal Choking	Snoring/Apneic Events/ Nocturnal Choking
Blood Pressure	Hypertensive	Hypertensive
Craniofacial Abnormalities	Increased likelihood	Increased likelihood

Several assessment tools are now available to aid in the diagnosis of sleep-related disorders. The Pittsburgh Sleep Quality Index (PSQI) and the Epworth Sleepiness questionnaire are two such tools. These inventories attempt to assess a person's sleep quality and sleep quantity. Patients who present with an Epworth Sleepiness score >12 and/or a Pittsburgh Sleep Quality Index global score >5, along with a body mass index >28 kg/m^2 should be considered for further evaluation [Pouliot *et al*, 1997].

Pathogenesis

OSA is associated with loss of upper-airway (UA) patency during sleep. This narrowing or closure of the upper-airway that generally occurs during rapid-eye-movement (REM) sleep is caused by the interaction of multiple anatomic and physiological abnormalities. In addition, genetic and environmental influences may also play a role in the determination of airway size. The increased occurrence of OSA in some families cannot be fully explained by obesity alone. Although the proposed inherited association is still not fully understood, genetically determined craniofacial abnormalities and/or ventilatory control dysfunction may account for this pattern of familial apnea [Guilleminault *et al*, 1995; Redline *et al*, 1995].

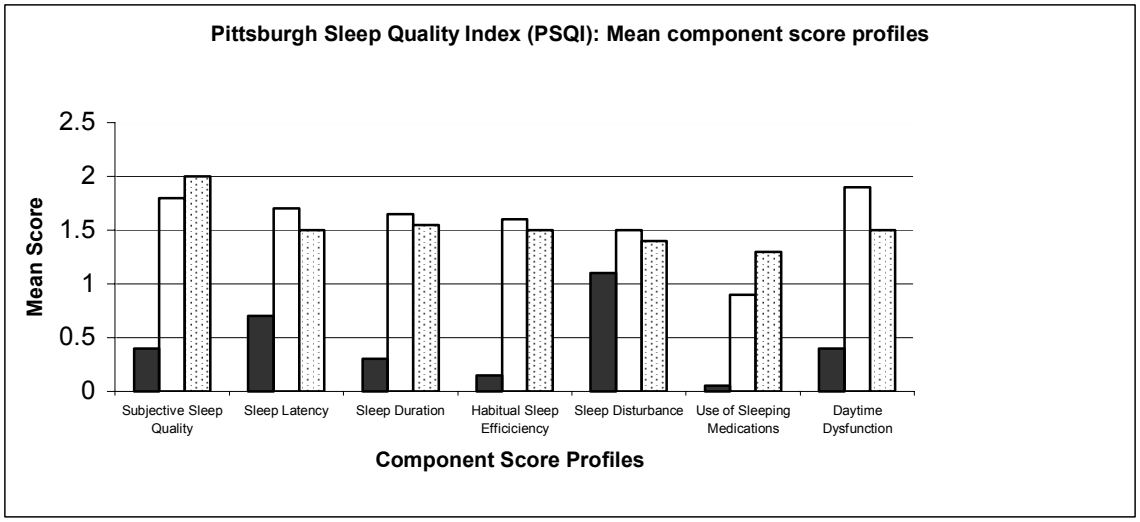

Figure 2. The Pittsburgh Sleep Quality Index (PSQI) – Score summaries from a sleep disturbance questionnaire used to evaluate quality of sleep. Scores are tabulated to yield seven components, ranging from Subjective Sleep Quality to Daytime Dysfunction. The result includes a Global Score, with a range of 0-21 points. A Global Score >5 is a basis for further evaluation relative to possible causes related to sleep-disordered breathing. The box shows Global Scores for individuals with psychological depression, those with inability to maintain sleep, and controls.

Anatomically, upper-airway size is determined by soft-tissue involvement, adipose tissue deposition, and craniofacial factors such as maxillomandibular positioning [Strollo *et al*, 1996]. Repeated exposure of the soft palate to vibratory trauma such as snoring and collapsing transmural pressure during inspiration can cause lengthening of this tissue, due to stretching and thickening caused by edema [Woodson *et al*, 1991]. In obese patients, increased adipose tissue deposition in the neck is related to stenosis of the upper-airway. However, the development of OSA in non-obese individuals may arise from tonsillar hypertrophy and/or craniofacial abnormalities that predispose the upper-airway to narrowing or closure during sleep [Partinen *et al*, 1988].

Physiological factors related to upper-airway patency during sleep include: dilation of the upper-airway due to increased muscle activity; reduced transmural pressure during inspiration; changes in caudal traction; increased vasomotor tone; and mucosal adhesive forces [Badr *et al*, 1999]. Physiological

dysfunction generally occurs during rapid-eye-movement sleep. This stage of sleep is associated with hypotonia of the upper-airway muscles [Strollo *et al*, 1996]. However, the majority of data on sleep-disordered breathing has been derived from studies that involve non-rapid-eye-movement (NREM) sleep, due to the difficulty of achieving rapid-eye-movement sleep during invasive procedures in a laboratory setting [Badr *et al*, 1999; Chittenden *et al*, 2002].

The absence of upper-airway narrowing in the OSA patient during wakefulness points to the obligatory removal of wakeful stimuli during sleep as the key factor underlying sleep disordered breathing. Rapid-eye-movement sleep causes hypotonia of the musculature in the upper-airway [Strollo *et al*, 1996]. This phenomenon is linked to reduced upper-airway caliber and increased pharyngeal wall compliance in snorers that is manifested by decreased airflow during inspiration. This corollary of increased resistance and inspiratory airflow limitation causes increased respiratory work, hypoventilation, and sleep arousals that lead to excessive daytime sleepiness [Shepard *et al*, 1996; Badr *et al*, 1999; Chittenden *et al*, 2002]. The capacity of the ventilatory control system to compensate for the increased workload in the OSA patient is lessened during non-rapid-eye-movement sleep. Thus, this pathophysiological state is associated with decreased tidal volume and minute ventilation. These events eventually lead to alveolar hypoventilation and an increase in arterial P_aCO_2. Non-rapid-eye-movement sleep also reduces the ability of upper-airway muscles to respond to increased negative transmural pressure during inspiration. During wakefulness, this negative inspiratory pressure gradient evokes activation of the genioglossus or the tensor palatinii muscle. This wakeful reflex response dilates the upper-airway, whereas during non-rapid-eye-movement sleep this activation mechanism is not present, suggesting sleep abolishes a protective mechanism that maintains upper-airway patency during deformation [Wheatley *et al*, 1993; Badr *et al*, 1999; Chittenden *et al*, 2002]. The failure of the ventilatory control system to adequately

compensate for the relative increase in workload results in hypoventilation and increased respiratory effort. This may explain why OSA patients have abnormal nocturnal CO_2 retention in the presence of increased respiratory muscle activity [Badr *et al*, 1991; Badr e*t al*, 1999].

Central apnea occurs when P_aCO_2 falls below the hypocapnic apneic threshold during non-rapid-eye-movement sleep and represents the major inhibitory factor during this stage of sleep. The development of this type of apnea may have ramifications for the development of upper-airway obstruction. Researchers now believe that central apnea causes pharyngeal occlusion, which in turn requires greater neural stimulation to resume ventilation [Badr *et al*, 1991; Badr *et al*, 1999]. In addition, the ventilatory control system requires the elevation of P_aCO_2 above normal or eupneic levels to resume rhythmic breathing. The result of elevated P_aCO_2 and upper-airway obstruction causes arousal and hyperventilation, which generates the concomitant apnea [Badr *et al*, 1999].

Although the exact mechanisms of upper-airway obstruction during sleep are still not fully understood, Badr *et al*. [1999] believe that the underlying defect in the development of OSA is a small pharynx vulnerable to collapse. Their research has also led them to believe that reduced ventilatory drive and decreased neural output to the upper-airway dilating musculature sets off a chain of events that eventually leads to sleep related pharyngeal obstruction. This narrowing of the pharyngeal airway that is caused in part by a collapsing transmural pressure leads to increased velocity of airflow. The consequent diminution in intramural pressure promotes increased pharyngeal narrowing that ultimately advances to total upper-airway obstruction. After complete obstruction transpires, the combination of gravity and mucosal adhesion promotes continuance of the apneic event. In most instances, arousal from sleep is needed to achieve pharyngeal opening. This event leads to hyperpnea, hypocapnia, and as

restoration of sleep occurs, a decrease in ventilatory drive [Badr *et al*, 1991; Badr *et al,* 1999; Chittenden *et al*, 2002].

Obstructive Sleep Apnea and Related Morbidity

Patients with OSA are at increased risk for systemic hypertension [Silverberg *et al,* 1997; Young *et al*, 1997], myocardial infarction, stroke, and nocturnal arrhythmias [Henderson *et al*, 1999; Olson *et al*, 1995; Yamashiro *et al*, 1994].

Systemic hypertension is frequently associated with OSA and can be directly attributed to the repeated and partial apneic events that disrupt autonomic cardiovascular reflexes during sleep [Young *et al*, 1997; Lies *et al*, 1996; Dempsey *et al*, 1997; Brooks *et al*, 1997]. The prevalence of hypertension in OSA patients is approximately 50% [Hall *et al*, 1995]. Apneic events during sleep result in decreased blood oxygen levels and increased carbon dioxide retention, both of which cause increased sympathetic nerve activity resulting in elevations in blood pressure [Somers *et al*, 1995]. Studies have also shown that patients with OSA exhibit increased sympathetic activity during the day [Hedner *et al*, 1988; Carlson *et al*, 1993]. These fluctuations in sympathetic nervous system activity may be involved in the pathogenesis of hypertension. Studies supporting this link between OSA and hypertension have shown a decrease in resting sympathetic nervous system activity [Waradekar et al, 1996] and blood pressure [Wilcox *et al*, 1993; Suzuki *et al*, 1993] following nasal continuous positive airway pressure (NCPAP) treatment [Chittenden *et al*, 2002].

OSA is associated with acute and long-term effects of sympathoexcitation, resulting in increased vascular tone. It has long been postulated that the increased vascular tone in OSA leads to disturbed diurnal blood pressure control and increases in the incidence of myocardial infarction and stroke.

Therefore, it is likely that OSA adversely affects several different cardiovascular homeostatic mechanisms. In OSA, the renin-angiotensin-aldosterone system seems to be suppressed, suggesting that this system is responding to central volume loading. These vascular regulatory mechanisms have not been thoroughly investigated in sleep apneics. There is also growing evidence of impaired endothelium-dependent vascular relaxation in OSA and that nitric oxide (NO)-dependent mechanisms are attenuated. Although not fully investigated, modified activity in the neurogenic, hormonal, and local regulatory systems seem to be poorly correlated with the hypertension frequently found in sleep apneics [Chittenden *et al*, 2002].

In addition to the effects on blood pressure regulation, there is also strong evidence linking NO release and the subsequent stimulation of blood vessel growth and development. This process, referred to as angiogenesis, is governed by two proposed mechanisms: 1) vascular shear stress and 2) hypoxia. Hypoxia is one of the major pathologic mechanisms related to obstructive sleep apnea. Hypoxia has also been shown to stimulate the expression of many angiogenic proteins. Vascular endothelial growth factor (VEGF) is an important mediator of neovascularization in a wide variety of tissues [Ware and Simons, 1999]. VEGF stimulates new vessel growth by activating receptors on endothelial cells of existing vessels. In concert with other growth/differentiation factors, VEGF stimulation results in basement membrane breakdown, migration and proliferation of endothelial cells, and formation of functional blood-carrying structures. Acutely, VEGF has vasodilatory activity mediated by NO [Ware and Simons, 1999].

Elevated serum and plasma VEGF levels are positively correlated to total sleep time below 90% SaO_2 during sleep [Gozal *et al,* 2002; Levy *et al,* 2000]. Furthermore, angiogenic processes may account for

the decreased morbidity and mortality related to long standing OSA reported in the literature [Levy *et al*, 2000].

Outlined below is a detailed review of currently proposed mechanisms associated with neovascularization.

Angiogenesis

Cell survival depends on a continuous supply of oxygen and nutrients carried by the blood. Therefore, every cell in the body must be sufficiently close to a blood capillary to allow for efficient nutrient diffusion. A tissue cannot grow beyond ~1mm in diameter before requiting new blood vessels to invade and nourish it [Rubanyi, 2000]. Over the past decade, the elucidation of the molecular mechanisms associated with both tumor (pathologic) and physiologic blood vessel formation has become the focus of an intense worldwide research effort to develop treatment regimes for various pathologic states such as cardiovascular disease and cancer [Carmeliet, 2000]. Discerning these mechanisms may lead to therapeutic options to improve or perhaps cure these biologic disorders that are now leading causes of morbidity and mortality in industrialized societies. This review attempts to provide the reader with an understanding of the systems biology implicated in the development of lumen containing blood vessels, as a basis for future (anti)-angiogenic management.

The term angiogenesis broadly refers to formation of new blood vessels. Several distinct processes can contribute to new vessel formation including true angiogenesis (formation of new capillaries from pre-existing vessels), arteriogenesis (formation of muscular arteries either de novo or from pre-existing collaterals) and vasculogenesis (formation of new vasculature from circulating vascular precursor cells)

(Fig.4). All of these processes are strictly controlled, both spatially and temporally, under normal physiologic conditions. Angiogenic factors, such as vascular endothelial growth factors (VEGFs) and fibroblast growth factors (FGFs) (Fig.3), promote endothelial cells (ECs) to secrete several proteases and plasminogen activators, which eventually leads to the degradation of the basement membrane. This in turn allows endothelial cells to invade the surrounding extracellular matrix. ECs migrate, proliferate and eventually differentiate to form a new, lumen-containing vessel. Finally, the ECs deposit a new basement membrane and secrete growth factors, such as platelet-derived growth factor (PDGF), which attract cells such as pericytes, ensuring the stability of the new vessel [Carmeliet, 2000]. Overall, this is a complex process that involves the combined action of several other factors, such as hepatocyte growth factor (HGF), angiopoietins, and ephrins, which act on specific receptors to regulate vessel integrity [Yancopoulos *et al*, 2000].

The serine/threonine protein kinase Akt/PKB signaling network and angiogenesis

In addition to growth factors, recent studies have suggested that insulin, cholesterol-lowering agents known as statins, and physical forces such as shear stress, can stimulate angiogenesis through activation of the PI3-K/Akt-1 signal transduction network. Induction of this biochemical network produces nitric oxide (NO) from endothelial NO synthase (eNOS). The subsequent production of NO leads to increased angiogenesis both *in vitro* and *in vivo* [Simons, 2000].

In addition to activation of eNOS, Akt-1 is intricately involved in a number of cellular events including promotion of endothelial cell survival and inhibition of Raf-1-MEK-ERK pathway [Simons, 2000; Davignon *et al*, 1999]. Akt-1 regulation is controlled by phosphoinositide-dependent kinase-1 (PDK-1)

activation in the presence of the cofactor, phosphatidylinositol 3,4,5-trisphosphate (PIP3), which is phosphorylated by PI3-K (Fig.3) [Simons, 2000].

Kureishi *et al*. [2000] recently demonstrated that an adenoviral vector expressing constitutively active Akt-1 stimulated both tube formation in an *in vitro* Matrigel assay and angiogenesis in a rabbit ischemic hind-limb model. This study also revealed that dominant-negative Akt-1 blocked simvastatin-induced angiogenesis in the same Matrigel model. Others have also demonstrated that the expression of constitutively active or dominant-negative forms of PI3-K and Akt-1 can either produce and inhibit vascular development in chick chorioallantoic membrane, respectively [Jiang *et al,* 2000]. In addition, Akt-1 may also be necessary for endothelial cell migration towards VEGF. The capacity of Akt-1 to mediate VEGF-induced endothelial cell survival, migration, and NO production indicates that Akt-1 signaling may mediate endothelial response to angiogenic stimuli [Morales-Ruiz *et al*, 2000]. Although these finding suggest a central role for Akt-1 in regulation of angiogenesis, evidence that Akt-1 activation is required for all angiogenic signaling networks requires demonstration of its involvement in signaling of other angiogenic growth factors as well as presentation that a dominant-negative form of this enzyme inhibits vessel growth in a physiological model *in vivo*. It would also be very important to distinguish Akt-1 involvement in true angiogenic vs. arteriogenic responses. If a dominant-negative Akt-1 construct were to attenuate either arteriogenesis (collateral formation) or true angiogenesis (capillary development in the ischemic foot) in an ischemic hindlimb model, it could be inferred that Akt-1 plays a central role in these processes. Conversely, unsuccessful dominant-negative Akt-1 angiogenic inhibition would point to the subsistence of other angiogenic signaling networks [Simons, 2000].

Currently, evidence for this postulate has not yet surfaced in the literature; however, Endres *et al*. [1998] revealed that while both FGF2 and VEGF induced Src activation in avian endothelial cells, a dominant-negative construct inhibited VEGF but not FGF-stimulated vessel growth. This leaves many unanswered questions concerning the nature of this protein kinase as a central mediator in angiogenic signaling. Further elucidation of the PI3K–Akt–eNOS signaling network may prove advantageous in the development of pro-angiogenic and anti-apoptotic therapeutic interventions for patients with advanced ischemic limb or obstructive coronary disease.

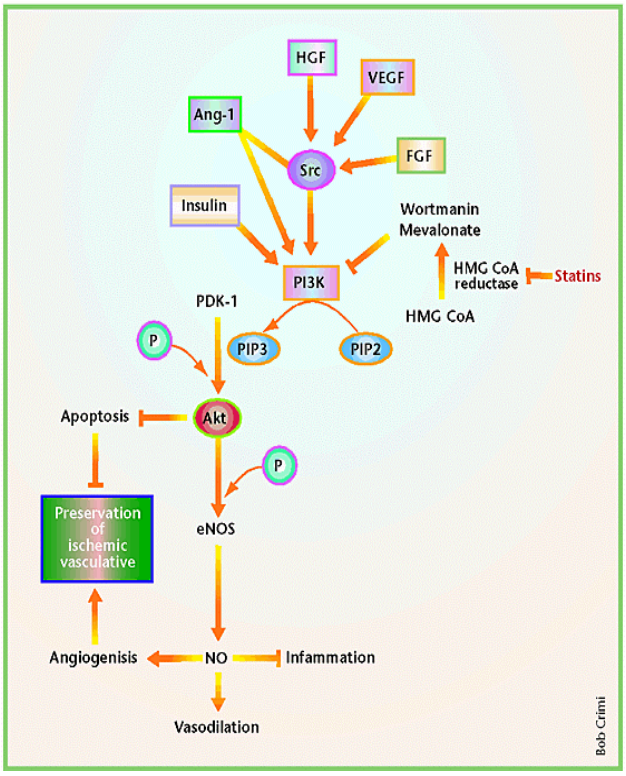

Figure 3. Proposed Akt-1 signaling network in angiogenesis. Ang-1, HGF, VEGF, FGF and insulin act either directly or through Src activation to induce phosphorylation of PIP2 to PIP3 by PI3K. PIP3 formation is necessary for phosphorylation of Akt-1 by PDK-1. Statins may also increase Akt-1 phosphorylation. Subsequent Akt-1 activation eventually leads to increased endothelial nitric oxide synthase (eNOS) activity, nitric oxide (NO) synthesis, and a variety of physiological effects including angiogenesis. Akt-1 also prevents apoptosis in endothelial cells. These biochemical events can eventually lead to the conservation of ischemic tissues. [Simons, 2000].

VEGF ligands, Receptors and Signaling

VEGF was originally named vascular permeability factor (VPF) because of its ability to induce vascular leakage [Senger *et al*, 1983]. The VEGF family currently comprises six members: VEGF-A, placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and the orf parapox virus VEGF (VEGF-E). There are 5 molecular variants that differ in total amino acid number. In humans, these represent VEGF-A_{121},

VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{189} and VEGF-A_{206}. VEGF-A_{165} is secreted by a broad variety of cells and is a heparin-binding disulfide-linked homodimeric molecule [DiSalvo *et al*, 1995]. VEGF expression is regulated by hypoxia, which occurs during tumour expansion and ischemia [Minchenko *et al*, 1994] (Fig.6).

The biological effects of VEGFs are regulated by three cell receptors: VEGFR-1 (Flt-1), VEGFR-2 (KDR or Flk-1), and VEGFR-3 (Flt-4). All three have of an extracellular domain consisting of seven Ig-like domains, a transmembrane domain, and a kinase domain that is divided in two parts by the insertion of a non-catalytic 100-amino-acid residue sequence, and a C-terminal tail. VEGFR-1 and VEGFR-2 are mainly expressed on endothelial cells [Cross *et al*, 2001; Shibuya *et al*, 1999]. VEGFR-3 is found mainly in the lymphatic endothelium. In addition, hypoxia has been shown to regulate both VEGFR-1 and VEGFR-2 expression, however, hypoxia-responsive elements have only been determined for the VEGFR-1 promoter [Li *et al*, 1996; Gerber *et al*, 1997].

VEGF receptor signal transduction still remains mostly undetermined. VEGFR-1 is a weak kinase, at least in tissue culture cell lines, while VEGFR-2 exhibits a strong induction in kinase activity to VEGF-A. However, the location of all the autophosphorylated tyrosine residues has not been determined [Dougher-Vermazen *et al*, 1994]. Researchers now believe that VEGFR-2 associates with the integrin $\alpha_v\beta_3$. This complex interaction may allow VEGF transduction effects on cell-matrix interaction [Cross *et al,*2001].

Blood Vessel Development

Embryonic blood vessels develop by a process, historically, referred to as vasculogenesis. This occurs by *in situ* differentiation of undifferentiated progenitor cells (angioblasts) to endothelial cells that eventually form a primitive vascular lumen (Fig.4). Traditionally, the term angiogenesis described the growth of endothelial sprouts from preexisting post-capillary venules. However, this term is now used to indicate the growth and remodeling process of primitive networks into intricate vascular systems. This involves the enlargement of venules, which sprout or become divided by pillars of periendothelial cells or by transendothelial cell bridges, which then split into individual capillaries [Risau, 1997]. *De novo* adult blood vessels arise primarily through angiogenesis; however, recent studies now support the contention that vasculogenesis also contributes to the development of mature vascular networks [Shi *et al*, 1998; Asahara *et al*, 1999].

The smooth muscle/endothelial cell relationship

Endothelial cells can initiate the angiogenic process, however, periendothelial cell involvement is needed for vascular maturation. During "vascular myogenesis", mural cells strengthen immature vessel lumens by restraining endothelial proliferation and migration. These cells also help stimulate production of the extracellular matrix [Carmeliet, 2000]. These cells also act to protect new lumen containing vessels against rupture and/or regression. Recent research has found that vessels are less likely to regress if covered by smooth muscle cells [Benjamin *et al*, 1998). During arteriogenesis, vessels become inundated with pericytes and smooth muscle cells, thus providing blood vessels with vasomotor tone that is essential for adequate tissue perfusion [Risau, 1997].

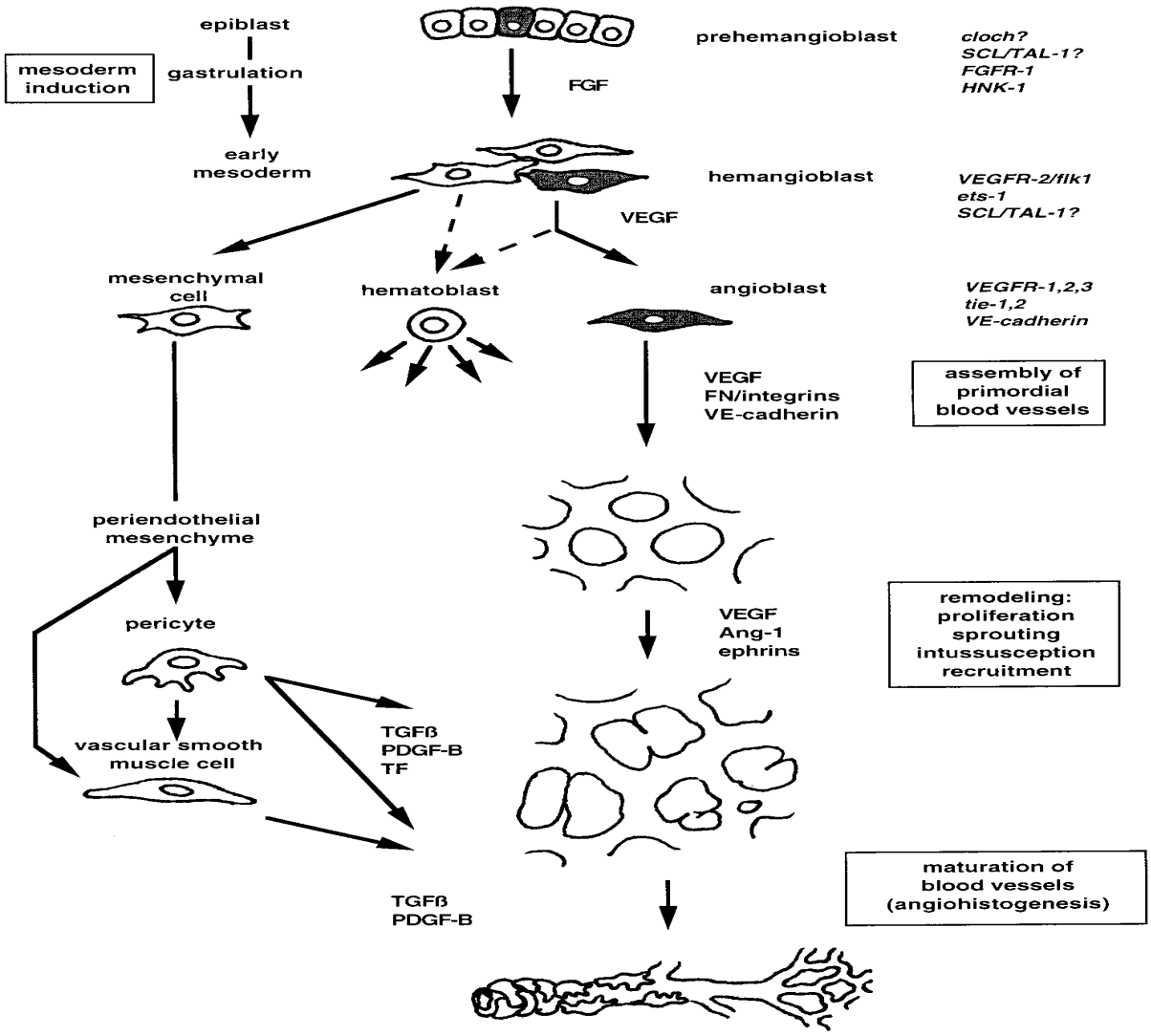

Figure 4. Schematic outline of the steps of vasculogenesis and early angiogenesis. The prehemangioblastic and hemangioblastic cells are still hypothetical. Factors relevant for the individual steps of vascular development are indicated next to the arrows. Molecules characteristic of prehemangioblastic, hemangioblastic, and angioblastic cells are indicated in italics [Rubanyi, 2000].

Vasculogenesis

The hemangioblast serves as a progenitor for both endothelial and hematopoietic cells (Fig.4). In embryonic development, hemangioblasts develop aggregates in which the inner cells develop into hematopoietic precursors and the outer population into endothelial cells [Shi *et al*, 1998]. VEGF, VEGF receptor 2 (VEGFR2) and basic fibroblast growth factor (FGF2) control angioblastic differentiation (Fig.4) [Ferrara *et al*, 1999; Shalaby *et al*, 1997]. However, The molecular mechanisms involved with transforming growth factor Beta-1 (TGFB-1) and its receptor (TGFB-1R2) in the vasculogenic process remain unresolved [Dickson *et al*, 1995]. Molecules mediating interactions between endothelial cells and the extracellular matrix, fibronectin or matrix receptors such as the alpha 5 integrin, also modulate vasculogenesis [Risau, 1997; Carmeliet, 2000].

During pathologic conditions, endothelial cells can differentiate and become angiogenic, thus integrating into existing arteries or veins. This phenomenon is referred to as the angiogenic switch [Carmeliet, 1999] and is proposed to endow the tumor with the ability to recruit blood vessels, either by down regulation of natural angiogenesis inhibitors or by inducing angiogenic factors. This is thought to explain the phenomenon of tumor dormancy (i.e., when the tumor remains in its avascular phase). While little is known about the mechanisms associated with this type of endothelial cell differentiation, *Ets-1*, *Hex*, *Vezf1*, *Hox* and *GATA* family members, and basic helix–loop–helix factors may be involved [Lyden *et al*, 1999]. Integration of endothelial cells into arteries or veins is mediated by the bHLH transcription factor gridlock at the angioblast stage, and by members of the ephrin family [Gale *et al*, 1999] (Fig.4). It was once thought that endothelial progenitors were only involved with fetal vessel development. However, as stated previously, endothelial precursor cells have now been isolated in bone marrow and found circulating in peripheral blood of adult animals and humans [Shi *et al*, 1998; Asahara

et al, 1999]. Granulocyte–monocyte colony-stimulating factor, VEGF, FGF2, and insulin-like growth factor (IGF)-1 activate cellular differentiation and mobilization of these early cells [Takahashi *et al*, 1999]. Furthermore, such precursors home to sites of angiogenesis and may hold promise for future therapy [Kureishi *et al*, 2000; Carmeliet, 2000].

Angiogenesis

Although the molecular mechanisms of angiogenesis have not been fully delineated, researchers have now determined six distinct steps involved with this complex biologic process. These steps include: 1.) Vasodilation, endothelial permeability and periendothelial support; 2.) Endothelial cell proliferation and migration; 3.) Lumen formation; 4.) Endothelial cell survival; 5.) Endothelial cell differentiation; 6.) Vessel remodeling [Carmeliet, 2000]. The following narrative incorporates a brief description of proposed mechanisms and players involved with the individual steps of angiogenesis [Carmeliet, 2000].

Vasodilation, endothelial permeability and periendothelial support

Angiogenesis is a concerted process that begins with NO-induced vasodilation (Fig.3). This promotes vessel permeability in response to VEGF, and thus allows plasma proteins to lay down the framework for migrating endothelial cells. Researchers now believe the increased permeability is caused by the formation of fenestrations, vacuolar organelles and the redeployment of PECAM-1 and VE–cadherin [Eliceiri *et al*, 1999]. Destabilization of the mature vessel is then required for endothelial cell emigration. Ang2, an inhibitor of Tie2 signaling, may be involved in shedding smooth muscle cells and loosening the extracellular matrix [Gale *et al*, 1999]. Plasminogen activator proteinases, matrix metalloproteinase (MMP), chymase or heparanase families control vessel formation via degradation of

matrix molecules and by activating growth factors, sequestered within the extracellular matrix [Carmeliet, 2000; Coussens *et al*, 1999].

Endothelial cell proliferation and migration

Once degradation of the extracellular matrix has occurred, endothelial cells migrate to sites of vessel formation. Although many of the mechanisms are still undetermined, VEGF, PLGF, VEGF-B, VEGF-C, VEGF-D and their receptors VEGFR2, VEGFR3, Ang1, and neuropilin-1 act in a coordinated fashion to promote collateralization. VEGF and its receptor VEGFR2 are involved with embryonic (Fig. 4), neonatal and pathological angiogenesis and are currently the focus of many therapeutic Strategies. VEGFR3 is also associated with fetal angiogenesis and is expressed in pathological angiogenesis [Dumont *et al*, 1998]. However, VEGF-C is strictly angiogenic in adult pathology [Ferrara *et al*, 1999]. Researchers recently discovered that Ang1 (Fig. 3) phosphorylates specific tyrosine residues in Tie2. It also induces vessel sprouting and amplifies VEGF function [Suri *et al*, 1998].

Fibroblast growth factor and platelet-derived growth factor (PDGF) members may affect angiogenesis by recruiting mesenchymal or inflammatory cells. Conversely, TGF-β1 and tumor necrosis factor (TNF)-α can either stimulate or inhibit endothelial growth, and may be involved in tumor dormancy [Gohongi *et al*, 1999]. Specific molecules involved in cell–cell or cell–matrix interactions (integrins) have been found to localize MMP-2 at the endothelial cell surface as well as mediate endothelial cell spreading. In addition, NO (Fig.3), TGFβ-1 and other angiogenic factors affect pathological angiogenesis and improve the re-vascularization of denuded vessels [Carmeliet, 2000; Murohara *et al*, 1998].

Lumen formation

Endothelial cells aggregate in solid cords that eventually differentiate into a lumen-containing vessel. Unlike physiologic vasculature, tumor vessels are unusually distended; however, blood flow in these biologic structures is generally not adequate or effective enough to meet metabolic demands [Helmlinger *et al*, 1997]. The VEGF variants have differing effects on lumen formation. $VEGF_{189}$ has been shown to decrease lumen diameter, while $VEGF_{121}$, $VEGF_{165}$ increase lumen formation. In addition, Ang1 in conjunction with VEGF also increases lumen diameter [Suri *et al*, 1998]. Other molecules affecting lumen formation are integrins ($\alpha_v\beta_3$ or α_5), myocyte enhancer binding factor 2C (MEF2C) transcription factor, and TSP-1 [Carmeliet *et al*, 2000].

Endothelial survival

Once integrated into new vessels, endothelial cells become quiescent and survive for years. However, endothelial apoptosis can be induced through deficiency of nutrients or survival signals [Gerber *et al*, 1999; Jain *et al*, 1998]. The survival function of VEGF depends on the interaction between VEGFR2, β-catenin and vascular endothelial (VE)–cadherin [Carmeliet *et al*, 1999]. Ang1 and various integrins such as $\alpha_v\beta_3$ also promote endothelial survival. Research has now shown that disruption of the interaction with matrix macromolecules results in endothelial apoptosis [Varner *et al,* 1995]. As described earlier, physiologic shear stress is essential for vascular maintenance, as it reduces endothelial turnover and abolishes endothelial apoptosis [Carmeliet *et al*, 1999]. Endothelial apoptosis can be also activated by NO, reactive oxygen species, angiostatin, TSP-1, the metallospondin METH-1, interferon-γ, tissue factor $\alpha_v\beta_3$ pathway inhibitor and vascular endothelial growth inhibitor (VEGI) [Gerber *et al*, 1999; Jain *et al*, 1998]. However, VEGF, Ang1, and $\alpha_v\beta_3$ have been shown to suppress p16, p21, p27,

p53, and *Bax*. These molecules also serve to activate the PI3-kinase/Akt (Fig.3), p42/44 mitogen-activated protein kinase, Bcl-2, A1 and survivin pathways [Carmeliet, 2000].

Endothelial differentiation

Research has shown that endothelial cells obtain specialized characteristics that are organ/tissue specific [Risau, 1998]. Endothelial cells associated with a tumor endothelium appear to differ in a number of ways from their physiologic counterparts. While the mechanisms of growth are still unknown, these cells are multilayered and associated to a higher degree with bridging and splitting vessels (Fig.5). They also contain intercellular and transcellular holes and undergo constant remodeling. Recent findings have shown that tumor vessels are "mosaic" and lined with both endothelial cells and malignant "vasculogenic" tumor cells. These findings may have significant consequences for anti-angiogenesis tumor therapy [Maniotis *et al*, 1999]. In addition, tumor endothelial cell specific epitopes are currently being evaluated for the "homing" of pro-apoptotic or thrombotic molecules in anti-cancer therapy [Ellerby *et al*, 1999; Carmeliet *et al*, 1999; Carmeliet, 2000].

Remodeling

Vessel maturation involves remodeling and "pruning" capillary-like vessels to uniform size. To date, little is understood about the mechanisms involved with the differential angiogenic growth patterns (Fig.5) of either pathologic or physiologic collateralization. However, gene inactivation studies indicate the involvement of VEGF isoforms and VEGFR3 [Dumont *et al*, 1998], the endothelial 'orphan' receptor Tie1 [Patan, 1998], the T-cell-leukemia protein stem cell leukemia factor/tal-1, *TEL*, the GTP-binding protein $G\alpha_{13}$, Jagged, chemokine receptor 4, vascular cell adhesion molecule 1, α_4 integrin and

fibronectin [Carmeliet *et al*, 1999]. The elucidation of the mechanisms involved with remodeling has become a goal for therapeutic angiogenesis [Carmeliet, 2000].

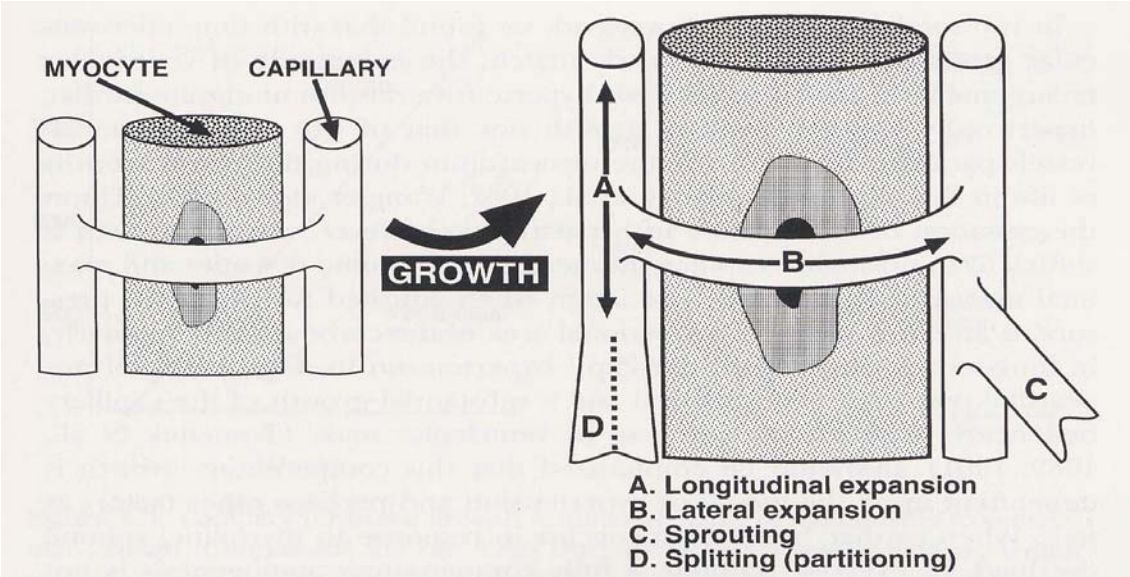

Figure 5. Differential angiogenic growth patterns that associate with both physiologic and pathologic vessel formation [Ware and Simons, 1999]

Vascular myogenesis

Smooth muscle cells can trans-differentiate from endothelial cells, differentiate from mesenchymal cells *in situ*, or from bone marrow precursors or macrophages. The fate of these cells is under transcriptional regulation. These factors include: capsulin, *Prx-1* and *Prx-2*, *CRP2/SmLIM*, serum response factor, and members of the *Hox*, *MEF2* and *GATA* family [Creazzo *et al*, 1998; Dettman *et al*, 1998; Carmeliet *et al*, 1996].

Smooth muscle cell recruitment and growth

PDGF has been shown to chemo-attract for smooth muscle cells in coordination with VEGF [Lindahl *et al*, 1998]. Ang1 and Tie2 also provide vessel stabilization by the interaction of mural cells with nascent endothelial channels. This allows for growth and maintenance by inducing branching and remodeling [Zhou *et al*, 1998; Maisonpierre *et al*, 1997]. TGF-β1, TGF-βR2, endoglin and Smad5 are also involved in vessel maturation. These molecules have been found to restrain endothelial proliferation and migration, promote smooth muscle differentiation, and activate extracellular matrix production [Dickson *et al*, 1995]. In addition, N-cadherin appears to adhere endothelial and mural cells in close proximity. Recently, tissue factor of coagulation promotes pericyte mobilization, possibly through the generation of thrombin and/or a fibrin-rich scaffold [Lin *et al*, 1998,;Carmeliet, 2000].

Arteriogenesis

Once smooth muscle cells are mobilized to the sites of active collateralization, they inundate the vessel and provide contractility for the developing vasculature. It is now believed that signals regulating mural cell involvement in vascular myogenesis are also implicated in arteriogenesis [Carmeliet, 2000].

Smooth muscle cell differentiation

Mural cells develop specialized characteristics that provide or allow for the maintenance of vascular tone. These contractile proteins and interstitial matrix components include: the intermediate filament desmin, MEF2C, elastin, fibrillin-2, collagen, and fibrillin-1. During pathological conditions involving inflammation, these muscle cells may "de-differentiate" from a "contractile" to a "synthetic" phenotype [Li *et al*, 1998; Carmeliet, 2000].

Modulation of vascular growth by hypoxia

Ischemic-driven angiogenesis is a feedback response by which a normal tissue matches its energy supply to its metabolic demands. The ischemic tissue senses insufficient oxygen (hypoxia) or inadequate glucose (hypoglycemia) and responds by inducing the production of angiogenic factors that attract new blood vessels to the oxygen deprived area [Rubanyi, 2000]. Hypoxia inducible factor 1 (HIFI-1) has emerged as key mediator to hypoxia and probably to hypoglycemia as well [Maltepe *et al*, 1997]. HIF1 is a heterodimeric Transcription factor comprised of two subunits (HIFI-1 alpha and beta), both of which participate in recognizing the hypoxia response element (HRE) on target genes. The beta subunit (also known as ARNT) is a dimerization partner for several other transcription factors and is found in large excess in the cell. The alpha subunit is solely associated with the HIFI-1 complex, and its level determines HIFI-1 transcriptional activity [Carmeliet, 1998].

Hypoxia leads to HIFI-1 alpha posttranscriptional regulation that includes stabilization of the protein, nuclear translocation, and activation of its transcription activation domain. Under normoxic conditions, the alpha subunit is degraded by the ubiquintin/proteasome system (Fig.6). Upon hypoxic exposure, degradation is markedly inhibited thus allowing activation of its respective target genes. HIFI-1 alpha is translocates to the nucleus via a hypoxia-inducible nuclear translocation signal located in its carboxy terminus. Once in the nucleus, the alpha and beta subunits form a heterodimer that recognizes HRE on target genes [Rubanyi, 2000].

Transcriptional activity under hypoxia also involves the recruitment of the transcriptional coactivator CREB-binding protein CBP/p300, which is thought to modify chromatin structure and enable transcription [Semenza, 1998]. Once activated, HIF-1 induces transcription of a wide range of target

genes, all which contain at least one HRE [core sequence 5'-(A/G) CGTG-3'] at the promoter or 3'enhancer regions [Maltepe *et al*, 1997]. These target genes include: 1) Erythropoietin and Transferrin that help improve systemic oxygen delivery by increasing erythropoiesis; 2) Inducible nitric oxide synthase and hemoxygenase 1, which promote vasodilation through the production of nitric oxide and carbon monoxide, respectively; 3) Glut-1 and most glycolytic enzymes that promote anaerobic metabolism and glucose storage (glycogen); and 4) Although targets have not been identified, HIF1 appears to play a role in cell cycle arrest and apoptosis through the downregualtion of Bc12, induction of p53, and induction of the cyclin Kinase inhibitor p21. Tumor cells may escape from this by mutating p53, thus gaining growth advantage in hypoxic niches of the tumor (Fig.6) [Rubanyi, 2000; Semenza, 1998; Maltepe *et al*, 1997; Carmeliet, 1998].

Furthermore, *in vitro* studies have uncovered three levels of control for hypoxia-induced VEGF expression: 1) VEGF transcription is increased three-to four fold during hypoxia (and hypoglycemia) by direct binding of HIFI-1 to the HRE present in the VEGF promoter [Semenza *et al,* 1994]; 2) Both hypoxia and hypoglycemia lead to posttranscriptional stabilization of VEGF mRNA. This is presumed to occur through hypoxia-augmented binding of proteins to the 3'-untranslated region (UTR) [Ikeda *et al*. 1995]; and 3) Production is also regulated at the translational level; the 5'-UTR of VEGF contains an internal ribosome entry site (IRES) that allows efficient translation during stress conditions, where conventional cap-dependent translation is compromised [Stein *et al*, 1998].

Modulation of vascular growth by mechanical factors

Angiogenesis is also stimulated by shear stress related to blood flow. High blood pressure associated with increased flow in the capillaries leads to the incorporation of smooth muscle cells [Vrancken *et al*,

1997]. Furthermore, remodeling of collateral vessels after arterial occlusion also depends on flow [Schaper *et al*, 1996]. Recent, gene inactivation studies have shown that shear-stress-induced vascular remodeling is affected by NO (Fig.3) and P-selectin [Zhou *et al*, 1998]. It is believed that mechanical forces alter vascular function through shear-stress-responsive gene transcription [Carmeliet, 2000].

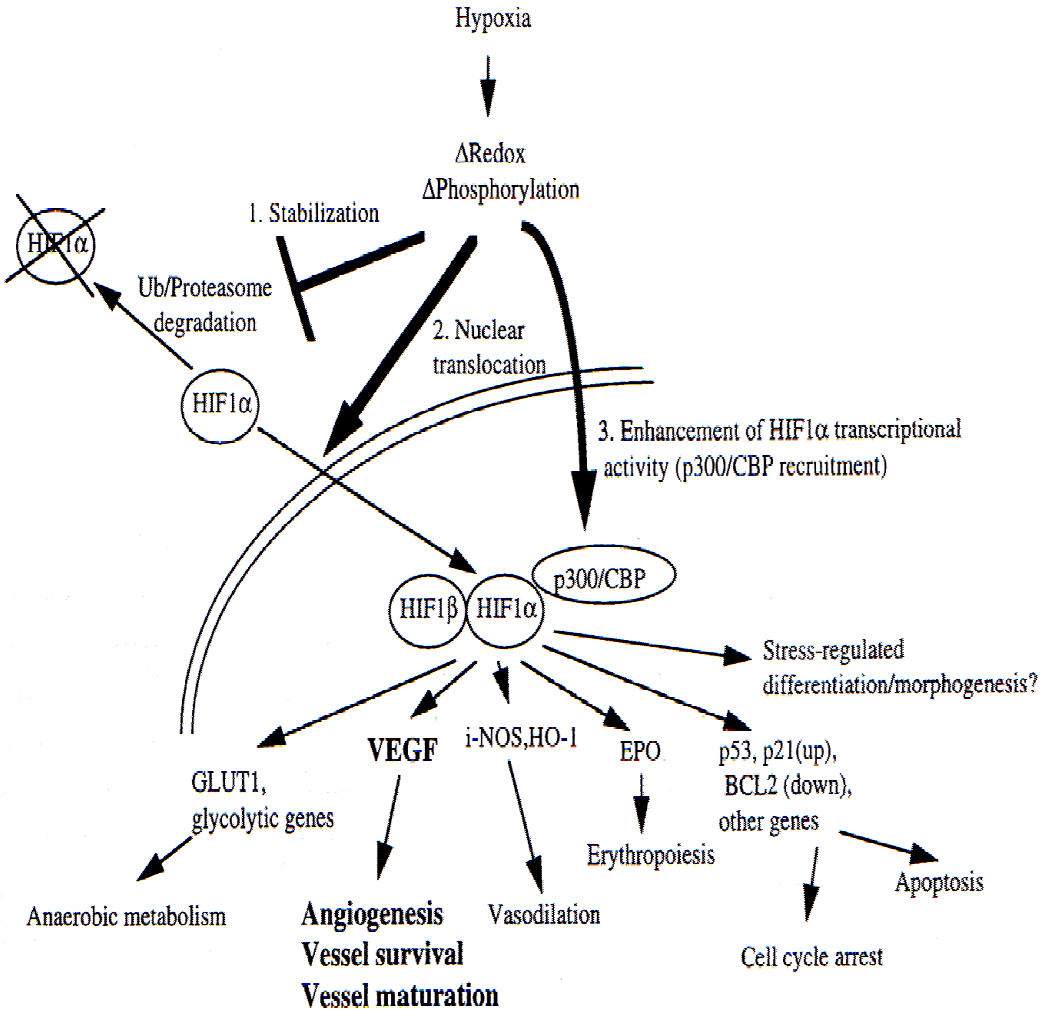

Figure 6. Current understanding of hypoxia signal transduction. Hypoxia is thought to activate, by still unknown mechanisms, HIF1 alpha via three independent mechanisms: stabilization of the protein, translocation to the nucleus, and promoting its transcriptional activity by recruitment of p300/CBP. The activated complex then up regulates its target genes that contain HRE [Rubanyi, 2000].

Physiologic vs. pathologic angiogenesis

Adult *de novo* blood vessel formation occurs by a combination of vasculogenic, angiogenic, and arteriogenic processes [Shi *et al*, 1998; Asahara *et al*, 1999] (Fig.4). While several mechanisms associated with pathologic angiogenesis now appear to correlate with normal fetal development, evidence is surfacing that supports distinct mechanisms between pathologic and physiologic blood vessel formation. Molecules solely involved with disease-induced vascular development include: Cox2, PLGF, $\alpha_v\beta_3$, proteinases, plasminogen activator inhibitor 1, NO, and TSP-2 [Heymans *et al*, 1999; Bajou *et al*, 1998; Varner *et al*, 1995; Murohara *et al*, 1998].

Furthermore, pathologic angiogenesis is often associated with some degree of inflammation. According to Carmeliet [2000], Monocytes/macrophages, platelets, mast cells and other leukocytes are "chemoattracted" to sites of inflammation or wound healing, in part by angiogenic factors such as VEGF. These blood-borne cells produce angiogenic and arteriogenic factors that, in turn, attract endothelial and smooth muscle cells, fibroblasts, leukocytes or platelets (Fig.7). These processes are highly correlated with and often induce ischemic driven tumor angiogenesis [Coussens *et al*, 1999; Schaper *et al*, 1996; Sunderkotter *et al*, 1994].

OSA and Hypertension

Sleep-disordered breathing (SDB) has been hypothesized to have a close relationship with hypertension but previous studies have produced mixed results. Garcia-Rio *et al*. [2000] assessed the relationship between hypertension and three forms of SDB (chronic snoring, breathing pauses and obstructive sleep apnea syndrome (OSAS) using representative samples of the non-institutionalized population of the UK,

Germany and Italy. OSAS was found in 1.9% (95% CI: 1.2% to 2.3%) of the UK sample, 1.8% (95% CI: 1.4% to 2.2%) of the German sample and 1.1% (95% CI: 0.8% to 1.4%) of the Italian sample.

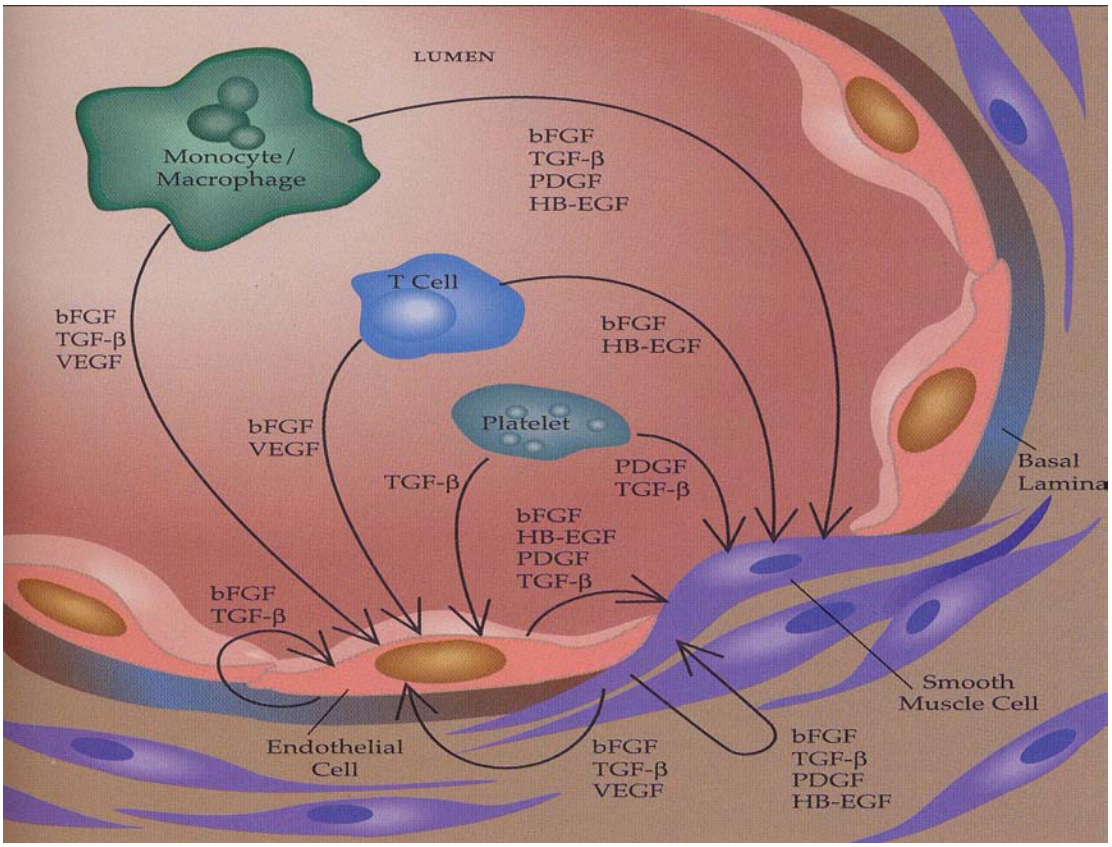

Figure 7. Inflammatory processes involved with pathologic angiogenesis. Arrows indicate growth factor targeting [Haber, 1995].

OSAS was found to be an independent risk factor for hypertension after controlling for possible confounding effects of age, gender, obesity, smoking, alcohol consumption, life stress, and, heart and renal disease. Others have found similar results. Grote *et al*. [2000] reported that SDB was independently associated with hypertension when potential confounders such as age, body mass index, sex, menopause and use of hormone replacement therapy, race, alcohol use, and smoking were controlled in the logistic regression analysis. The strength of the association decreased with age and was proportional to the severity of SDB. These findings appear to support the findings of Lavie *et al* [2000]. These researchers investigated the mortality rates of 1,442 male OSA patients from 1976 to 1988. The subjects' age at time of diagnosis ranged from 21 to 79 years. Observed /expected mortality rates were calculated by comparing the expected death rates derived from Israeli national mortality data to actual subject death rates in each decade of life. This study revealed significant excess mortality in the fourth and fifth decades and that myocardial infarction was the major cause of death. Surprisingly, this mortality ratio decreased from 1.34 in the sixth decade to 0.34 in the seventh decade. While Lavie provided no definitive explanation for their findings, this age-related phenomenon may be related to enhanced endothelial function and/or neovascularization with a subsequent decrease in peripheral resistance in response to chronic disease-induced hypoxia.

It is has been postulated that intermittent nocturnal decreases in arterial oxygen saturation may cause hypertension and endothelial dysfunction in Sleep Apnea Syndrome patients. To this end, Bixler and associates [2000] evaluated the central inspiratory drive response to hypoxia in patients with OSA and in healthy control subjects. They also examined the relationships among sleep architecture, hypoxic sensitivity, urinary catecholamine excretion, and BP. OSA patients were categorized as being normotensive (type 1), having BP elevation only during sleep (type 2), and as being hypertensive with

elevated BP at all times (type 3). They found a significant difference in the response to hypoxia among control subjects and type 1, type 2, and type 3 OSA patients. In the OSA patients, chemosensitivity was related to the apnea-hypopnea index and to the nocturnal excretion of epinephrine. Significant relationships between the nocturnal excretion of epinephrine and BP were also noted. On multiple linear regression analysis, the response to hypoxia was the only variable significantly related to diurnal and nocturnal mean BP. Their findings suggest a possible mediating role of peripheral chemosensitivity in the association between sleep apnea and hypertension. Furthermore, Kraicizi *et al* [2000] studied vasoconstrictor sensitivity and cholinergic responsiveness of the forearm vasculature in 10 male patients with OSA and 10 healthy controls. With the use of three dosage steps each, angiotensin II and acetylcholine were infused into the brachial artery. During infusion of angiotensin II, mean conductance was 39.6% lower in the OSA patients compared with that in the control subjects. Vascular responsiveness to increasing dosages of acetylcholine was not significantly altered in the OSA group. Their results suggest enhanced vasoconstrictor sensitivity in the forearm vasculature in OSA.

In hypertensive OSA that is associated with a damaged endothelium, vasoconstrictor influences predominate. Phillips *et al*. [1999] evaluated 22 patients with severe OSA and associated hypertension before and after continuous positive airway pressure (CPAP) therapy. They concluded that sleep apnea elicits increases in both blood pressure and endothelin-1 (ET-1); a potent vasoconstrictive and mitogenic peptide produced by endothelial cells and degraded predominantly in pulmonary vasculature, and a reduction in plasma renin activity. Although CPAP treatment had no effect on renin activity, it did decrease mean arterial pressure and plasma endothelin-1 levels. Their study design did not include the evaluation of NO production and iNOS expression, thus a suppression of the renin-angiotensin-aldosterone system after CPAP therapy could not be determined. Although the causes of primary

hypertension are presently unknown, secondary hypertension, as commonly seen in OSA patients with vascular dysfunction, may be endocrine and/or structurally related. Chronic hypertension abnormally affects vessel wall thickness, lumen diameter, and endothelium function, all of which can increase peripheral resistance.

A substantial body of evidence now exists to support the notion that increased cellular production and circulating plasma levels of ET-1 may be intricately involved in the altered vascular function associated with hypertensive OSA. Moreover, Increased circulating plasma ET-1 levels now appear to be significantly correlated to SAS. Saarelainen *et al*. [1997] found ET-1 levels were higher in both normotensive and hypertensive OSA groups than in 66 healthy controls.

Aging is an independent risk factor for cardiovascular and renal disease and may also be associated with increased expression of ET-1. Recently, Barton *et al.* [2000] investigated whether aging affects ET-1 and tissue levels of the NO metabolites, nitrite/nitrate, in the kidneys of rodents. They compared renal ET-1 protein levels in whole kidneys of young (3 month) and old (24 month) male Wistar Kyoto rats. Their analysis revealed a 3.6-fold increase in the older rats. Similarly, renal ET-1 protein increased 1.7-fold in 18-month-old C57BL/6J mice as compared to 8-month-old adult animals and in female RoRo-Wistar rats, tissue nitrite/nitrate levels in whole kidneys decreased with increasing age. Thus, aging in healthy rodents appears to be associated with marked upregulation of renal ET-1 protein content and a decrease in tissue nitrite/nitrate levels in whole kidneys, independent of blood pressure. These researchers concluded that activation of the ET pathway with aging might promote the development of age-dependent diseases such as glomerulosclerosis, hypertension and atherosclerosis.

There also appears to be a gender difference in the vasoconstrictor, but not the vasodilator effects of ET-1. Tatchum-talom *et al* [2000] investigated whether gender affects the pressor and constrictor effects of ET-1 in anesthetized rats. They found that ET-1 produced rapid and transient falls in arterial blood pressure and hindquarter resistance (HQR), followed by increases in blood pressure and HQR. The initial ET-1-induced hypotension and vasodilation were similar in both groups. However, the pressor and hindquarter vasoconstrictor effects were significantly higher in male than female rats. Thus, the increases in blood pressure and HQR to ET-1 were attenuated in female compared to male rats. These results indicate that there is an observed gender difference in female rats and that this difference may be responsible for the lower risk of developing coronary heart disease in premenopausal women.

Vascular compliance also appears to be highly correlated with an increased incidence of essential hypertension. McVeigh *et al*. [1991] evaluated proximal and distal arterial compliance in 38 patients with established essential hypertension and 32 age-matched normotensive control subjects. In the hypertensive subjects compared to the normotensive subjects mean arterial pressure was 25% higher, systemic vascular resistance 23% higher, proximal compliance 19% lower, and distal compliance 72% lower. The reduction in distal compliance was highly age-dependent. In the youngest age range (45 to 54 years) little overlap appeared between hypertensive and normotensive groups, whereas in the oldest subjects studied (65 to 75 years) distal compliance was comparably low in the two groups. Thus, these researchers concluded that distal vascular compliance provides a sensitive and specific marker for the abnormal vasculature associated with hypertension and may be particularly useful in identifying the disease in young individuals with borderline blood pressure.

Cardiopulmonary stress testing

Cardiopulmonary stress testing has been shown to be an important diagnostic and prognostic tool in the evaluation of patients with hypertension. An exacerbated blood pressure response to exercise in normotensive subjects is one of the best predictors for future hypertension. However, assessment of this disease state could be enhanced by a standardized test procedure, which would allow for comparable and reproducible blood pressure determinations. Furthermore, results from such a protocol might enable physicians and healthcare professionals to devise more effective treatment regimes for their patients. It would also be useful to have a standardized method for monitoring sympathetic activity, so that the occurrence and magnitude of abnormal stress responses could be assessed more accurately. Franz [1996] proposed a standardized bicycle ergometry test that begins at workloads of 50 to 100 watts and increases by increments of 10 watts/ min or 25-watts/2 min. This researcher evaluated blood pressure responses in patients between the ages of 20 and 50 years. The criterion for a hypertensive response during exercise was a blood pressure value of more than 200/100 mg Hg at a workload of 100 W. In the recovery phase, BP was considered hypertensive if a value of 140/90 mm Hg was exceeded in the fifth minute. Others have also shown that an abnormal recovery blood pressure response to exercise is a valid measure of determining CAD [Taylor *et al*, 1998]. This may prove to be a useful tool in assessing co-morbidity in SAS patients. In addition, Franz's study showed that patients suffering from mild hypertension had significantly higher blood pressures at 100 W and after exercise than age-matched normotensives but significantly lower values than hypertensives with stable hypertension. Exercise ergometry revealed that 50% of the patients with borderline hypertension at rest could be classified as hypertensives. Follow-up examinations ~ 4 years later revealed that 97% of the ergometric-positive borderline hypertensives developed established hypertension [Franz, 1996].

Lim *et al.* [1996] preformed a meta-analysis over a 10-year period from 1985 to 1995 that evaluated information on exercise testing in hypertensive patients and persons at risk for developing hypertension. Hemodynamic responses in hypertensive patients and persons at risk for developing hypertension and correlations to end-organ damage, mortality, and exercise tolerance were assessed. Their analysis was aimed at determining whether this type of methodology is a valuable screening procedure for the diagnosis, prognosis, or assessment of the effect of therapy. They found that the exercise capacity of hypertensive patients was reduced by as much as 30% compared with age-matched controls. In addition, this exercise impairment increased with age and end-organ damage, and its origin could be traced back to adolescence. Total peripheral resistance also progressively increased. These researchers believed that the associated changes were caused by functional and structural involvement of the cardiovascular system and abnormal blood pressure responses to exercise have prognostic value for the future development of hypertension, end-organ damage, and death. They went on to state that the adequacy of antihypertensive treatment should therefore be evaluated in terms of normalizing these stress-related blood pressure responses.

In 1992, Goodman *et al*. assessed the limiting factors associated with the exercise impairment in hypertensive patients associated with left ventricular hypertrophy. They also evaluated the relationship between peripheral function and exercise capacity. Cardiopulmonary exercise testing was conducted using two protocols: a graded exercise test to maximal effort established maximal exercise capacity, followed by a step-incremental test combining gas-exchange measures and radionuclide angiography. 12 patients with established hypertension and left ventricular hypertrophy were studied. Subjects were screened for co-morbidity and were un-medicated at time of testing. A volunteer sample of normal, healthy subjects acted as a control. Measures included maximal oxygen intake, ventilatory anaerobic

threshold, total peripheral resistance and plasma blood lactate levels. Cardiac function measures included ejection fraction and ventricular volumes. These researchers found that the hypertensive subjects demonstrated an increased pressure to volume ratio and a blunted ejection fraction at peak exercise. Although end-diastolic volume increased during exercise, values were lower during both levels of exercise compared with normal subjects. Mean end-systolic volume increased from 39 +/- 22 at rest to 42 +/- 23 mL during peak exercise. The hypertensive subjects had a lower VO_2 max compared with normals and a lower ventilatory anaerobic threshold. In addition, hypertensive patients had a significantly elevated total peripheral resistance at rest and at peak exercise than the normal subjects. Furthermore, total peripheral resistance and VO_2 max were linearly related ($r = 0.92$) in the hypertensive subjects. They concluded that peripheral factors, specifically a failure to reduce total peripheral resistance, limited exercise performance despite maintenance of left ventricular function during exercise in patients with moderate hypertension. Additionally, the use of cardiopulmonary exercise testing could help identify the underlying causes of exercise intolerance in this population.

Exercise testing is a simple procedure that appears to have the potential for assessing hypertensive patients. Additionally, the assessment of the pressor response to other laboratory stressors has been extensively evaluated in an attempt to develop a reliable means of determining the role hypertension in various disease states. Researchers in Japan [Murakami *et al*, 1996] investigated the relationship between pressor responses to laboratory stressors and 24-hour blood pressure (BP) variability or left ventricular mass. Responses to three stressors, mental arithmetic tests (MAT), isometric hand grip exercise (IHP), and bicycle ergometer exercise (BEE), were assessed in middle-aged normotensive subjects and in age-matched stage I and stage II patients with essential hypertension. MAT was associated with a greater rate of increase in plasma epinephrine than in norepinephrine, and the (IHP)

group was associated with a greater rate of increase in plasma norepinephrine than in epinephrine in all three groups. BEE caused a dramatic increase in plasma norepinephrine but only a mild increase in plasma epinephrine in all three groups. In MAT, pressor responses of hypertensive patients were significantly greater than those of normotensives and were highly correlated with 24-hour BP variability in all subjects. The pressor response to IHP increased with the stage of hypertension and was significantly correlated left ventricular mass. However, there was no difference in the pressor response to ergometer exercise between any of the groups. Their findings suggest that MAT is associated with 24 hr BP variability and that IHP pressor responses are correlated with target-organ disease associated with hypertension.

Numerous researchers have shown that the use of anti-hypertensives, such as beta-blockers, can positively affect exercise capacity in hypertensive patients. Vyssoulis *et al*. [1995] assessed the effect of antihypertensive therapy on the exercise capacity of 40 patients randomized to either celiprolol 200 mg or metoprolol 100 mg daily in a double-blind fashion. Patients were studied after a month of placebo and a year of active treatment. Both drugs normalized resting blood pressure and produced echocardiographic and electrocardiographic left ventricular hypertrophy regression. In symptom-limited peak exercise stress before and after treatment, exercise duration increased with celiprolol and metoprolol. Resting and peak heart rates were reduced with both medications. Furthermore, blood pressure at peak exercise was reduced with both celiprolol and metoprolol to a similar degree. These researchers concluded that exercise parameters were not related to patient age or the degree of left ventricular hypertrophy regression and that beta-blocker antihypertensive therapy improves exercise capacity, by decreasing heart rate and blood pressure responses to stress, irrespective of left ventricular

structural changes. However, it is still undetermined whether reducing the severity of a hypertensive state will have any impact on the degree of co-morbid states such as OSA.

Also associated with OSA is the possibility for myocardial infarction and stroke. The prevalence of OSA in patients with coronary artery disease (CAD) is estimated to be 20-35% [Chittenden *et al,* 2002]. However, the available evidence suggesting a link between OSA and myocardial infarction is equivocal [Weiss *et al*, 1999], likely because many studies failed to control for confounding variables. Obesity is a risk factor for the development of both CAD and OSA and without controlling for this factor; it is difficult to conclude a causal relationship. It has also been suggested that myocardial infarction may increase the risk for the development of OSA [Weiss *et al,* 1999]. Therefore, a link establishing OSA and coronary artery disease needs to be further evaluated before more definitive conclusions can be drawn [Chittenden *et al*, 2002]. Studies showing a link between OSA and stroke have come under similar scrutiny. As with myocardial infarction, it has been argued that stroke may contribute to the development of sleep apnea [Weiss *et al*, 1999]. While some studies have shown a diminished cerebral artery flow at the termination of an apneic event [Hajak *et al,* 1996; Balfors *et al,* 1994], no definitive evidence exists establishing a link between OSA and cerebrovascular events [Chittenden *et al*, 2002].

As a result of increased sympathetic activity secondary to apneic episodes, oscillations in both heart rate and blood pressure occur as well as an increase in the frequency of atrial and ventricular arrhythmias [Weiss *et al,* 1999]. In one large study of 400 patients, cardiac arrhythmias were noted in almost half the subjects [Guilleminault *et al*, 1983]. The majority of these cardiac disturbances noted were bradyarrhythmias with 43 of the 400 patients experiencing sinus arrest of 2.5 to 13 seconds. In subsequent studies, ventricular arrhythmias were relatively uncommon [Shepard *et al*, 1985]. Some investigators dispute the high prevalence of arrhythmias associated with OSA, claiming that the patients

in the aforementioned studies had severe sleep apnea and were not representative of a greater spectrum of disease severity [Weiss *et al*, 1999].

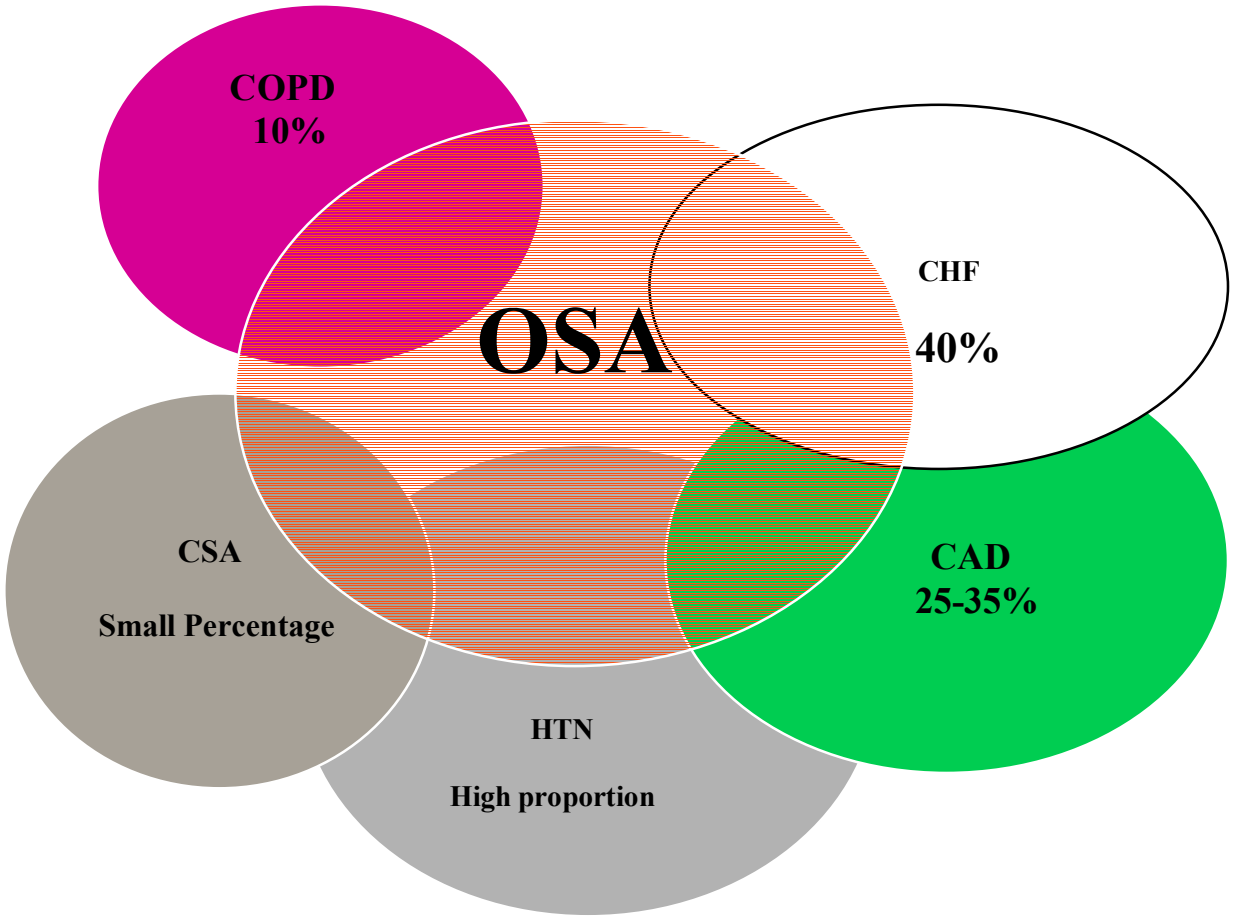

Figure 8. Representation of the chronic co-morbid conditions that may often accompany OSA and how prevalence may overlap.

While the available literature is equivocal in establishing a causal relationship between sleep apnea and cardiovascular morbidity, it seems likely that the hemodynamic changes associated with apneic events may contribute to both acute and chronic cardiac disorders in certain individuals [Chittenden *et al*, 2002].

Conclusions

Obstructive sleep apnea is a serious disorder that has gained attention over recent years. This condition predisposes individuals to increased cardiovascular morbidity and mortality [Hall *et al*, 1995]. The importance of early detection and treatment cannot be understated. Adequate intervention has been shown to improve daytime alertness and quality of life, daytime hypertension, neuropsychiatric performance, and reduce nocturnal oxyhemoglobin desaturation and respiratory arousals [Henderson *et al*, 1999;Strollo *et al*, 1998]. Therefore, in an attempt to develop improved early detection and treatment protocols, researchers have recently focused their attention on determining the complex pathogenic mechanisms of sleep-disordered breathing. These efforts have lead to the realization that cardiovascular dysfunction plays a major role in patient morbidity and mortality. Researchers now believe that disease-related HTN may be associated with abnormal functional and structural changes within the cardiovascular system. It appears that these changes may be directly attributed to pathologic suppression of NO-dependent vascular relaxation and subsequent hypoxic-release of inhibitory neovascular control mechanisms. These perturbations within the endothelium may constitute an adaptive angiogenic mechanism to counterbalance the onset of cardiovascular co-morbidity. Hypothetically, this disease related phenomenon might provide a feasible explanation for the reported decrease in morbidity and mortality associated with chronic sleep-disordered breathing. Moreover, while current medical assessment procedures provide an adequate means of diagnosing persons with OSA, cardiopulmonary exercise testing, in combination with polysomnography and various molecular and biochemical assessment techniques, may prove to be viable means of not only determining disease severity but also assessing the degree of cardiovascular dysfunction in these patients. These measures may eventually provide insight into the underlying pathology of this condition, which could ultimately lead to superior treatment regimes and improved quality of life for many OSA patients.

Chapter III

The results of this chapter are presented as a journal manuscript. A detailed description of the methodology is presented in Appendix F and detailed statistical analyses are presented in Appendix B.

Journal Manuscript

to

Sleep

Chronic Hypoxia and Cardiovascular Dysfunction

in Sleep Apnea Syndrome

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Abstract

The purpose of the current study was to test the hypothesis that chronic hypoxia associated with sleep-disordered breathing relates to abnormal Nitric Oxide (NO) production and vascular endothelial growth factor (VEGF) expression patterns that contribute to aberrancy of specific determinates of cardiovascular and cardiopulmonary function before, during, and after graded exercise. These patterns may further reflect pathologic alteration of signaling within the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt-1) transduction network. To this end, 7 medically diagnosed OSA patients (3 male, 4 female), mean age 48 years and 7 apparently healthy control subjects (3 male, 4 female), mean age 42 years, underwent baseline venous blood draws and maximal bicycle ergometry. Mononuclear cells isolated from peripheral blood were utilized as reporter cells for measurement of VEGF, Akt-1, hypoxia inducible factor-1 alpha (HIF-1 alpha), and vascular endothelial growth factor receptor-2 (VEGFR2) gene expression by redundant oligonucleotide DNA microarray and real-time

PCR technologies. Circulating angiogenic progenitor cells expressing VEGFR2 were profiled by flow cytometry. Plasma and serum concentrations of VEGF, nitrates/nitrites, catecholamines, and dopamine were measured by enzyme-linked immunosorbent assay (ELISA) and high performance liquid chromatography (HPLC). Arterial blood pressure, cardiac output, oxygen consumption and total peripheral resistance were determined at Baseline, 100W, and peak ergometric stress by standard techniques. There were no apparent differences ($p \leq .05$) observed in biochemical markers relating to vascular function and adaptation including, serum nitrates/nitrites, norepinephrine, dopamine, and plasma VEGF. No differences were found relative to cardiac output, stroke volume, cardiopulmonary or myocardial oxygen consumption, expired ventilation, heart rate, arteriovenous oxygen difference, total peripheral resistance, and mean arterial pressure. Due to methodological issues related to the redundant oligonucleotide DNA microarray and real-time PCR gene expression analyses, results of these experiments were uninterpretable. Thus, the research hypothesis was rejected. Conversely, significant ($p \leq .05$) differences were observed in waist: hip ratios, recovery: peak systolic blood pressure ratio at 1 minute post-exercise and %VEGFR2 expression. OSA was associated with elevations in both waist: hip ratios and recovery: peak systolic blood pressure ratio at 1 minute post-exercise as well as significant depression of %VEGFR2 profiles. Moreover, significant negative correlations were found regarding waist: hip ratios and %VEGFR2 expression ($r = -.69; p = .005$) and recovery: peak systolic blood pressure ratio at 1 minute post-exercise and %VEGFR2 expression ($r = -.65; p = .01$). These findings did not provide evidence that NO-dependent vasoactive mechanisms are suppressed nor did they support the supposition that angiogenic mechanisms are pathologically activated in sleep-disordered breathing.

Introduction

Systemic hypertension (HTN) is frequently associated with OSA and can be directly ascribed to the repeated and partial apneic events that disrupt autonomic cardiovascular reflexes during sleep [Young *et al*, 1997; Lies *et al*, 1996; Dempsey *et al*, 1997; Brooks *et al*, 1997]. The prevalence of HTN in OSA patients is now being reported at approximately 50% [Hall *et al*, 1995]. Diminished airflow during sleep results in decreased blood oxygen levels and increased carbon dioxide retention, both of which cause increased sympathetic nerve activity resulting in elevations in blood pressure [Somers *et al*, 1995]. Studies have also shown that patients with OSA exhibit increased sympathetic activity during the day [Hedner *et al*, 1988; Carlson *et al*, 1993]. These fluctuations in sympathetic nervous system activity may be involved in the pathogenesis of HTN. Furthermore, studies supporting this link between OSA and HTN have reported a decrease in resting sympathetic nervous system activity [Waradekar et al, 1996] and blood pressure [Wilcox *et al*, 1993; Suzuki *et al*, 1993] following nasal continuous positive airway pressure (nCPAP) treatment [Chittenden *et al*, 2002].

In addition, OSA is associated with both acute and chronic effects of sympathoexcitation, resulting in decreased vascular compliance [Chittenden *et al*, 2002]. It has long been postulated that the increased vascular tone in OSA leads to abnormal diurnal blood pressure control and increases in the incidence of myocardial infarction and stroke. Therefore, it is likely that OSA adversely affects several different cardiovascular homeostatic mechanisms. Although these cardiovascular regulatory mechanisms have not been thoroughly investigated in OSA patients, disease related hypoxia might abate vascular function by limiting vessel-derived vasoactive substances such as adenosine, prostanoids, endothelin, and/or nitric oxide (NO) [Ni *et al*, 1998].

In addition to its role in blood pressure regulation, NO stimulates of blood vessel growth and development. This process, referred to as neovascularization, is controlled by two general mechanisms: 1) vascular shear stress and 2) hypoxia. In fact, it is now believed that the decrease in blood oxygen tension (hypoxia) is one of the major contributing pathologic factors related to sleep-disordered breathing. Hypoxia has also been shown to stimulate the expression of many angiogenic peptides under both normal and pathologic conditions. Clearly the most studied protein related to neovascularization, vascular endothelial growth factor (VEGF), is an important mediator of blood vessel growth in a wide variety of tissues [Ware and Simons, 1999]. VEGF stimulates angiogenesis by activating receptors on endothelial cells of existing vessels. In concert with other growth/differentiation factors, VEGF stimulation results in basement membrane breakdown, migration and proliferation of endothelial cells, and formation of functional blood-carrying structures. Acutely, VEGF has vasodilatory activity mediated by NO [Ware and Simons, 1999].

While endothelium-derived vascular relaxation and NO-dependent mechanisms appear to be attenuated in OSA, studies have recently indicated that VEGF levels are elevated in sleep-disordered breathing [Gozal *et al*, 2001]. These perturbations within the endothelium may constitute an adaptive angiogenic mechanism to counterbalance the onset of cardiovascular co-morbidity. Although the exact mechanism for this phenomenon has not been delineated, chronic hypoxia may serve to explain at least in part the pathologic VEGF expression associated with this disease state. Furthermore, the Sleep Heart Health Study recently indicated that patients with mild OSA have a greater risk for developing occlusive vascular diseases [Shahar *et al*, 2001]. Conversely, these investigators found that this risk did not increase in patients with more severe OSA. In addition, Lavie *et al*. [2000] reported decreased

cardiovascular morbidity and mortality in ~1500 Israeli men after the eighth decade of life. This outcome is supported by the clinical observations that, even in the presence of severe nocturnal oxygen desaturations, not all OSA patients suffer from cardiovascular co-morbidity [Schulz *et al*, 2002]. Consequently, it may be considered that certain as yet unrevealed mechanism(s) shield some OSA patients from developing cardiovascular complications.

Neovascularization and the resultant decrease in vascular compliance may explain the decline in morbidity and mortality associated with long-standing sleep apnea pathology. Therefore, this study aimed to evaluate the relationship between chronic hypoxia and the intricate disease related pathogenic mechanism(s) of aberrant NO production and VEGF expression on cardiovascular and cardiopulmonary performance before, during, and after graded exercise in both OSA patients and non-experimental controls.

Methods and Procedures

Subjects

Seven medically diagnosed volunteer OSA patients (4 females and 3 males) referred to the Southwest Virginia Sleep Disorders Center (SVSDC) in Christiansburg were invited to participate. saO2<90%, measured by pulse oximetry, and RDI scores were determined during an overnight sleep study. Each subject gave informed consent; approval for the protocol and the informed consent process and form was obtained prior to the start of the study from the Virginia Tech Institutional Review Board for Human Subjects' Research. Exclusion criteria included: recent complicated myocardial infarction; recent episode of uncontrolled or increasing angina pectoris; recent revascularization; chronic

obstructive pulmonary disease; congestive heart failure; uncontrolled hypertension; uncontrolled diabetes mellitus; orthopedic, musculoskeletal, and neuromuscular disabilities; history of moderately vigorous physical activity ≥3 day/wk, ≥ 30 min/session, over the last 6 months; and prescription cardiovascular medications.

In addition, seven apparently healthy subjects (4 females and 3 males) matched for age and body mass index (BMI) were recruited to serve as controls. Absence of sleep-disordered breathing was confirmed in control subjects via a health history questionnaire, standardized sleep questionnaire (Epworth Sleepiness Scale, ESS), sleep-related symptom review, and clearance to participate from their primary care physician. Subjects answered questions related to snoring and apnea history, witnessed snoring, sleep quality, and daytime sleepiness. None of the subjects reported a history of nocturnal gasping, apnea or witness apnea (by bed partner) nor did any of the subjects report symptoms of excessive daytime sleepiness.

Blood Collection Procedures

Venous blood samples were drawn before exercise testing from the antecubital vein, with the subject in a seated position and using the limb opposite the one used for measurement of blood pressure. Blood (< 20mL) was collected in glass tubes containing either EDTA or no anticoagulant. Whole blood was separated for the determinations of VEGFR2 expression on angiogenic progenitors and isolation of mononuclear cells for mRNA collection. Collection tubes were centrifuged at room temperature for 15 min at 25, 000xg. Both serum and plasma samples were stored at -70°C. Centrifugation was performed no later than 2 hours post-blood draw.

Exercise Stress testing

Resting measures were taken in a seated position on electronically brake cycle ergometer (CardioO2, MedGraphics, St. Paul, MN). A Vmax 229® (SensorMedics, Yorba Linda, Ca.) metabolic cart was used to collect both expired respiratory gases and cardiac output (Qc) determinations as previously described [Warburton *et al*, 1998; Zenger *et al*, 1993]. Subjects were fitted to the breathing apparatus and two Qc measurements were taken before exercise testing. Blood pressure was measured with a sphygmomanometer and stethoscope. Heart rate was assessed using the Schiller AT-12 lead ECG recorder (Switzerland). Both assessments were taken at rest as well as immediately before each Qc measurement during exercise.

Graded exercise testing was performed on the electronically braked cycle ergometer. Subjects began at an initial work rate of 25 Watts and were continuously ramped at a rate of 5 Watts every 20 seconds until peak effort was achieved. The Vmax 229® (SensorMedics, Yorba Linda, Ca.) metabolic cart was used to collect expired respiratory gases. The highest VO_2 obtained during the last min of exercise was used as peak oxygen consumption (VO_2 pk). Blood pressure, Qc, and rating of perceived exertion (RPE) were taken every two minutes during exercise and heart rate (HR) was taken every 60 seconds. Blood pressure and HR were taken each minute during the 8-minute recovery period.

Cardiovascular and cardiopulmonary assessment determinations

The Qc procedure was explained and demonstrated in detail before each test. Each subject then practiced the breathing technique under resting conditions. The Qc breathing maneuver was performed in accordance with the manufacturer's protocol, using proprietary computer software. Cardiac index

(CI), total peripheral resistance (TPR), stroke volume (SV), SV index (SVI) and arteriovenous oxygen difference values were calculated from Qc determinations.

Determination of Circulating Mononuclear cell VEGFR2 Expression

Total Leukocyte populations expressing the VEGFR2 receptor were assessed using the fluorescently labeled monoclonal Flk-1 (A-3): sc-6251 antibody (mAb) (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Final antibody dilution was determined at 1:300 (see detailed methodology). One hundred microliters of anti-coagulated (EDTA) whole blood from each subject was mixed with 2ml of 1 X FACS lysing solution in 15ml glass tubes. Tubes were the centrifuged at 300xg (1291 rpm) for 5 min at 3°C. Supernatants were aspirated and cells were washed with 2ml of phosphate buffered saline (PBS) plus 0.1% sodium azide (NaN_3). This procedure was repeated at 200xg (1054 rpm) for 5 min at 3°C. Supernatants were again removed, and cell pellets were resuspended in 500ul of PBS/NaN_3. Cell pellets were incubated with 50ul of pre-diluted stock mAb Flk-1 solution for 30 minutes on ice. Tubes were then centrifuge at 200xg for 5 min at 3°C. Supernates were removed, and cell pellets were resuspended in 500ul of PBS/NaN_3. One microliter of fluorescently labeled secondary antibody (Fitc) was added to each tube and allowed to incubate in the dark for 30 min on ice. Samples were then centrifuged at 200xg for 5 min at 3°C. Supernates were removed, and cell pellets were resuspended in 500uL of paraformaldehyde fix and stored at -20°C until time of assay. Blood samples were analyzed using an Epics XL Flow Cytometer (Coulter Electronics, Hialeah, FL) at the Virginia-Maryland Regional College of Veterinary Medicine Flow Cytometry Laboratory. Total mononuclear cell gate was determined as previously described [Fichtlscherer *et al*, 2001].

VEGF ELISA Assay

All subject VEGF plasma concentrations were obtained by enzyme linked immunosorbent assay (ELISA) using a commercially available kit (R&D Systems, Inc., Minneapolis, MN). The lower sensitivity of the assay is 3 pg/mL and linear results are obtained at a range of 7.0 to 1,000 pg/ml. Calibration curves revealed linear sensitivity within the manufacturers' recommended range. Reproducibility agreement was r = 0.99. One hundred microliters of assay diluent RD1W, standard and/or plasma sample were added to each well and allowed to incubate for 2 hours at room temperature. Each well was aspirated and washed three times with 400ul of wash buffer. Two hundred microliters of VEGF conjugate was then added to each well and allowed to incubate for another 2 hours at room temperature. The aspiration/wash steps were repeated. Two hundred microliters of substrate solution was added to each well and allowed to incubate for 25 min in the dark at room temperature. Fifty microliters of stop solution was added and the plate was read at 450 nm.

Reporter cell mRNA isolation

Three milliliters of HISTOPAQUE-1077 (Sigma-Aldrich, St. Louis, MO) was added to 15ml conical centrifuge tubes and allowed to come to room temperature. Three milliliters of whole blood was then layered onto the HISTOPAQUE-1077. Samples were then centrifuged at 400x g for 30 minutes at room temperature. Plasma was aspirated to within 0.5 cm of the opaque interface containing mononuclear cells. The opaque interface was transferred to a clean conical centrifuge tube. Cells were washed with 10 mL PBS and centrifuged at 250x g for 10 minutes. Supernatant was removed and the cell pellet was resuspended in 5.0 ml of PBS. Samples were again centrifuged at 250x g for 10 minutes. This step was repeated and 600 ul of lysis buffer RLT (Qiagen, Bothell, WA) and 6 ul (1%) of Beta Mercaptoethanol (B-ME) were added for storage at -80°C until time of assay.

Affymetrix Gene Chip Protocol (Santa Clara, CA)

Frozen cell pellets were defrosted and then lysed with a 21-gauge needle in 100 ul of RNase-Free Water, 350 ul Buffer RLT, and 1% B-ME. Samples were vortexed and then transferred to 1.5 ml RNase-Free microtubules and mixed with 70% EtoH. Samples were then applied to RNeasy mini columns that were placed in 2 ml collection tubules. Samples were centrifuged at 10,000xg for 15 seconds. This step was repeated at 12,000xg for 15 seconds. Columns were transferred to new 2 ml collection tubules. Buffer RPE (500 ul) was added onto each column and centrifuged for 15 seconds. Another 500 ul Buffer RPE was added onto the columns and spun for 2 minutes. RNeasy columns were transferred to 1.5 ml collection microtubules and 30 ul RNase-Free Water was added. Samples were incubated at room temperature for 20 minutes and spun at 10, 000xg for 1 minute. Another 20 ul RNase-free water was added to each column and again incubated at room temperature for 20 minutes. Samples were spun at 10,000xg for 1 min and then read on spectrophotometer at 260 and 280 nm. For RNA precipitation, 1/10 volume 7.5 M NH_4Oac (pH 5.2), 2.5 volumes 100% cold EtoH, and 1 ul glycogen were mixed with 15ug of total pooled RNA from both controls and OSA patients, incubated at –20° C for 1 hour and centrifuged at 12,000xg for 20 min. Pellet was washed twice with 80% EtoH, spinning each time for 5 min at 4°C. Pellet was dried and resuspended in 14 ul DEPC water and again read on spectrophotometer at 260 and 280 nm.

Double stranded cDNA was synthesized by bringing total RNA samples to a volume of 9.1ul with DEPC water. One microliter of T7- (dT) 24 primer was added and allowed to incubate at 70°C for 5 min. Samples were then spun and put on ice. Ten point nine microliters of first strand master mix (Qiagen, Bothell, WA) were added to each sample and incubated at 42°C for1 hour. One hundred and thirty microliters of second strand master mix was then added to each sample and allowed to incubate at

16°C for 2 hours. Two microliters of (10U) T4 DNA Polymerase was added to each sample and incubated at 16°C for 5 min.

Double-Stranded cDNA cleanup was performed by microcentrifuging Pellet Phase Lock Gel (PLG) (Qiagen, Bothell, WA) at 12,000xg for 30 seconds. One hundred sixty two microliters (equal volume) of (25:24:1) Phenol: chloroform: isoamyl alcohol (saturated with 10mM Tris-HCL pH 8.0/1mM EDTA) were added with the final cDNA synthesis preparation (162 ul) for a final volume of 324 ul. The samples were vortexed and transferred to the PLG tube. cDNA was precipitated by adding 0.5 volume 7.5 M NH_4Oac (pH 5.2) and 2.5 volumes 100% cold EtoH and incubated at –20° C for at 20 min. Samples were centrifuged at 12,000xg at room temperature for 20 min and washed twice with 0.5 ml cold 80% EtoH. Cell pellets were allowed to air dry. Samples were resuspended in 12 ul RNase free water, and 1 ul of sample was evaluated for quality on a 1% agarose gel.

The in vitro transcription reaction (IVT) included the addition of 33 ul of IVT master mix (Qiagen, Bothell, WA) to 7 ul of sample in RNase-Free tubes (500 ul) for a total volume of 40 ul. Samples were spun and incubated at 37°C for 5 hours. The IVT cleanup was then performed. Samples were transferred to 1.5 ml RNase-Free microtubules and 100 ul of RNase-Free Water was added to 350 ul Buffer RLT and 1% B-ME and vortexed. Contents were then mixed with 250 ul 100% EtoH. Samples were then applied to RNeasy mini columns that were placed in a 2 ml collection tubule. Samples were centrifuged at 10,000xg for 15 seconds. This step was repeated at 12,000xg for 15 seconds. Columns were transferred to new 2 ml collection tubules. Buffer RPE (500 ul) was onto each column and centrifuged for 15 seconds. Another 500 ul Buffer RPE was added onto the columns and spun for 2 minutes. RNeasy columns were transferred to 1.5 ml collection microtubules and 30 ul RNase-Free

Water was added. Samples were incubated at room temperature for 20 minutes and spun at 10, 000xg for 1 minute. Another 20 ul RNase-free water was added to each column and again incubated at room temperature for 20 minutes. Samples were spun at 10,000xg for 1 min and then read on spectrophotometer at 260 and 280 nm.

Fragmentation of cRNA was performed by the addition of 20 ug of sample, RNase-free water, and 8 ul 5X Fragmentation Buffer (Total volume 40 ul). Samples were vortexed and incubated at 94°C for 35 min. Two microliters unfragmented and fragmented samples were run on a 1% agarose gel to provide quality assurance. Hybridization was performed by first heating the frozen stocks of 20X GeneChip Eukaryotic Hybridization Control Cocktails to 65^{o}C for 5 minutes**.** The redundant oligonucleotide probe arrays (HG-U133A) were equilibrated to room temperature immediately before use. The hybridization cocktail, which contained 11 ug of pooled fragmented cRNA, was then heated to 99^{o}C for 5 minutes. The array was pre-hybridized with 1X hybridization buffer for 10 minutes at 45^{o}C rotating. The hybridization cocktail was transferred to a 45^{o}C heat block for 5 minutes. The cocktail was maximally microfuged for 5 minutes. Pre-hybridization buffer was removed from the array and filled with 300 uL of clarified hybridization cocktail. The array was placed in a hybridization oven at 45^{o}C and 60 rpm for 16 hours. The hybridization cocktail was removed from the array and placed on ice. The array was washed with 300 ul of wash buffer A for 10 cycles of 2 mixes/cycle at 25°C. The array was again washed with 300 ul of wash buffer B for 4 cycles of 15 mixes/cycle at 50°C. The probe array was then stained for 10 minutes in 300u of R-Phycoerythrin Streptavidin (SAPE) solution at 25°C. A third wash was performed for 10 cycles of 4 mixes/cycle with wash buffer A at 25°C. A second stain was done with 300ul of antibody solution for 10 minutes at 25°C. The third stain was achieved by staining the array for 10 minutes in 300ul of SAPE solution at 25°C, after which a final wash was performed with

wash buffer A at 30°C for 15 cycles of 4 mixes/cycle. The Pixel value was set at 3um and the array was read at 570 nm. Please see detailed methodology in the appendices for a full description of reagents and materials.

Gel Electrophoresis Protocol

The gel box was wiped clean with RNase Away. The gel was allowed to solidify at room temperature and then refrigerated for 10 minutes. A 1% agarose gel was subjected to pre-electrophoresis at ~5 volts/ cm for 5 minutes in 1X MESA + 0.22M formaldehyde. Samples and markers were prepared as described in the detailed methodology section of the appendices. One percent agarose gels were run at ~100 volts for an hour and then photographed. These gels were used to determine the relative quality of RNA samples.

Quantitative PCR

Double stranded cDNA was synthesized from both control and patient total RNA as described in the microarray methods. Forward and reverse primers were designed with proprietary software (Primer Express, Applied Biosystems, Inc., Framingham, MA). The primer pairs (Ransom Hill Biosciences Inc, Ramona, CA) included:

HIF1 alpha, GCATCTTGATAAGGCCTCTGT/CACCAGCATCCAGAAGTTTCC;

HIF1 beta, CTGTAGTGCCCTGGCTCGAA/CGCAAGGACTTCATGTGAGAAAC;

KDR, GACTGGCTTTGGCCCAATAA/CCATCGCTGCACTCAGTCA;

VEGF, GCTGTCTTGGGTGCATTGG/GCAGCCTGGGACCACTTG;

vSrc homolog, ACCTCCCGCACCCAGTTC/AGGCCATCGGCGTGTTT;

PDK-1, TTGGTGGAAAAGGCAAAGGA/ATAACTTCAAGTACATTGCAGTTTGGA;

Akt-1, GTGACCATGAACGAGTTTGA/CAGGATCACCTTGCCGAAA;

iNOS, AGCTGGATGCAACCCCATT/CCCGCTGCCCCAGTTT;

eNOS, TCATCAACCAGTACTACAGCTCCATT/CTCGGCTTCCACCTCTTGAA.

GAPDH; CCACCCATGGCAAATTCC/ TGGGATTTCCATTGATGACAAG was used as a positive control. Reaction Mix contained: 2ul of template, 3ul of respective forward and reverse primers, 25ul of master mix (Qiagen Inc, Valencia, CA), 16.6ul [1:50,000] of SYBR green I, and 0.5ul [10nM; 1: 1000] of fluoriscene. The cycling protocol comprised: 3min at 95°C, 40x 30sec at 95, 50 and 72°C, and 10min at 72°C. A melt curve was performed to identify number of amplified products. Dilutions of 2 and 4ul of 32ng/ul cDNA template were used for each primer pair. GAPDH was used for normalization of groups. Relative expression for each gene was calculated by standard methods.

Serum Norepinephrine, Epinephrine, and Dopamine Determinations

Serum norepinephrine, epinephrine, and Dopamine concentrations were determined by high performance liquid chromatography (HPLC) using a Beckman "Gold" HPLC system (San Ramon, CA) and a Luna 5uC18 column (250 x 3 mm) (Phenomenex, Torrance, CA). On the day of determination, serum samples were thawed and aliquots were centrifuged for 30 minutes at 4000 rpm and filtered through Microcon YM-30 filters. A standard curve was determined prior to testing. Flow rate was at 1ml/min, and the mobile phase was 75mM $NaH_2PO_4*H_3O$, 1.7mM octane sulfuric acid, 10µM EDTA, 10% Acetonitrile, pH 3.1 with H_3PO_4. Retention times for norepinephrine, epinephrine, dopamine, and serotonin were 6.7 min, 8.5 min, 15.2 min, and 22.9 min, respectively.

Serum nitrate/nitrite Determination

Nitrate (NO_3^-) and nitrite (NO_2^-) were determined by high performance liquid chromatography (HPLC) using a Beckman Gold HPLC system (San Ramon, CA) and a Wescan Silica-based anion and cation

exchanger (column 10 x 4 mm: Alltech, Deerfield, IL). On the day of determination serum samples were thawed, centrifuged at 4000 rpm for 30 minutes, and filtered through YMT ultra-filtration membranes (Amicon, Inc., Beverly, MA). The mobile phase was 1.5 mM sulfuric acid. Nitrate and nitrite standards (LabChem, Inc., Pittsburgh, PA) were injected at a flow rate of 3 min. Retention times for NO_2^- and NO_3^- were 2.4 min and 7.1 min, respectively.

Statistical Analysis

Values are reported as mean $\pm$ SD. Pearson's product moment correlation coefficients were calculated to evaluate relationships between markers of cardiovascular biologic function and polysomnography (PSG) indicators of disease severity. The PSG variables included both the respiratory disturbance index (RDI) and total sleep time spent below 90 percent saO_2. Two-tailed independent samples t-tests were used to assess the differences between groups. The statistical significance level for the investigation was set at $p \leq 0.05$.

Results

The OSA and control groups were statistically similar with respect to demographic data including age, weight, body mass index (BMI), body surface area (BSA), neck circumference, and peak workload and oxygen consumption. However, significant differences were witnessed in scores related to both the Epworth Sleepiness Scale and waist: hip ratios (Table 1). Significant correlations were also observed among OSA patients. Both positive and negative relationships were noted between RDI and waist: hip ratio ($r = 0.85$; $p = 0.01$) and RDI and BMI ($r = -0.77$; $p = 0.04$). RDI did not significantly correlate with $saO_2<90\%$ ($r = 0.08$; $p = 0.87$) nor did $saO_2<90\%$ significantly correlate with any of the patient demographic data.

Table 1. Characteristics for both the OSA and control subjects. * Significance at $p \leq 0.05$ ** Significance at $p \leq 0.01$

Table 1. Patient Characteristics	OSA Group (N = 7)	Control Group (N = 7)
Age	48 ± 4.1	42 ± 2.5
Male	3	3
Female	4	4
RDI	33 ± 6.5	N/A
SaO_2 <90%	4.62 ± 1.7	N/A
Epworth Score	11.7 ± 1.5**	5.6 ± 1.2
Weight (kg)	91.8 ± 10.5	80.4 ± 14.8
BMI	31 ± 2.03	29 ± 2.17
Waist: Hip Ratio	.93 ± .03*	.81 ± .04
Neck Circ (cm)	39 ± .97	36 ± 1.34
BSA	2.10 ± .04	1.93 ± .07
Peak VO_2 (ml/kg/min)	20.40 ± 1.7	24 ± 2.2
Peak Watts	149.3 ± 12	157.8 ± 19

Table 2. Relationships between subject characteristics and indicators of disease severity.

Table 2. Correlation Coefficients	Neck circ	Waist: Hip	BMI	BSA
RDI	r=.59;p=.15	r=. 84;p=.01	r=-.76;p=. 04	r=-.05;p=.90
Sa02<90%	r=-.10;p=.82	r=.09;p=.84	r=.09;p=.83	r=.18;p=.69
NO3	r=-.41;p=.14	r=-.46;p=.09	r=-.12;p=.66	r=-.31;p=.27
VEGF	r=.11;p=.70	r=.19;p=.50	r=-.27;p=.34	r=-.23;p=.43
VEGFR2	r=-.72;p=. 003	r=-.69;p=. 005	r=-.10;p=.70	r=-.60;p=. 02

Interestingly however, VEGFR2 significantly correlated with neck circumference, waist: hip ratio, and BSA. Results from the redundant oligonucleotide DNA microarray experiment indicated that the signal log ratios for the housekeeping genes of the pooled OSA and non-experimental chips were equivalent at $p \leq 0.05$ (Figure 1). However, The peak 3': 5' ratio for the OSA and non-experimental chips were 9 and 4.9, respectively. There was also a 4.5% difference in hybridization efficiency noted between the 2 chips. Analysis revealed 12.6% total hybridization to the OSA chip and 17.1% binding to the non-experimental chip. These findings may represent pooled RNA contamination for the OSA chip. Thus, results of these experiments are equivocal.

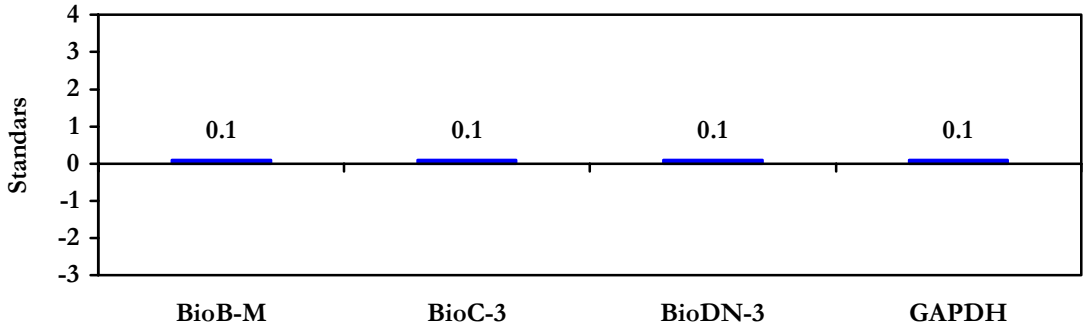

Figure 1. Signal log ratios between the pooled OSA and non-experimental redundant oligonucleotide DNA microarray chips for 4 of the standard housekeeping genes

With the exception of HIF-1 alpha, microarray analysis revealed absence of fluorescent signal at $p \leq 0.05$ for select transcripts within the PI3K/Akt-1 signal transduction network. However, figure 2 represents the signal log ratio values between OSA and non-experimental subjects for these transcripts at $p > 0.05$. These findings are more than likely associated with the decreased hybridization efficiency observed on the OSA chip.

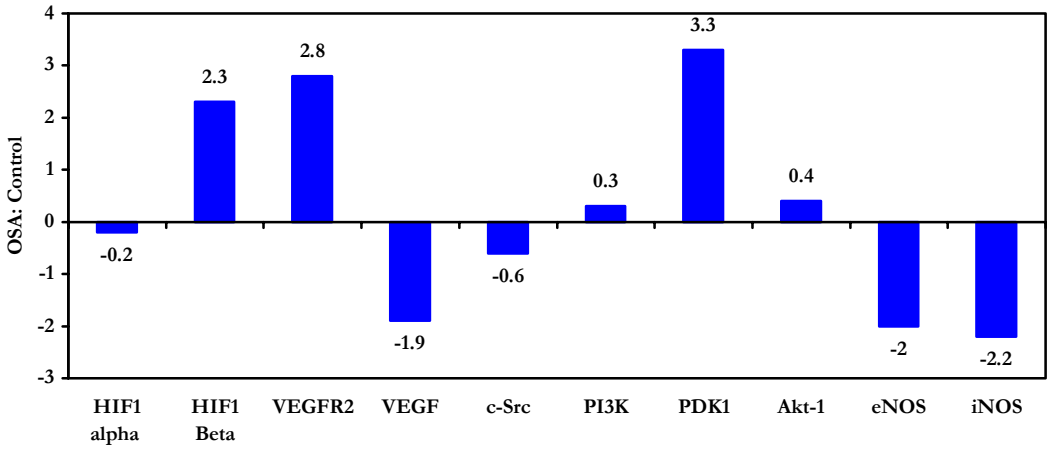

Figure 2. Signal log ratio values at $p > 0.05$ for select transcripts within the PI3K/Akt-1 signal transduction network. Values represent OSA: Non-experimental Control

In an attempt to rectify the concerns associated with the microarray experiment, real-time quantitative PCR was used to quantify relative expression of select transcripts within the PI3K/Akt-1 signal transduction pathway. However, due to limitations related to sample availability (cDNA) for both OSA and non-experimental control groups, standard curves were not generated. Additionally, because no apparent differences in relative expression of GAPDH were observed in the microarray analysis, this gene was used for normalization in the quantitative PCR experiments. It was subsequently discovered that hypoxia up-regulates GAPDH. Therefore, results of these experiments are also equivocal.

Nonetheless, Table 3 indicates the relative expression values without demonstration of linearity of Ct values verses target copy number for select transcripts within the PI3K/Akt-1 signal transduction pathway.

Table 3. Relative expression for VEGF, HIF1 alpha, and Akt-1. RE1: relative expression values incorporating all primer pairs with baseline parameters set at 2-10 cycles. RE2: relative expression values incorporating all primer pairs with baseline parameters set at 2-15 cycles. RE3: relative expression values incorporating individual primer pairs with baseline parameters set at 2-10 cycles. RE4: relative expression values incorporating individual primer pairs with baseline parameters set at 2-15 cycles.

Table 3 Transcript	RE 1	RE 2	RE 3	RE 4
VEGF	**477**	**12.3**	**181**	**8**
HIF1 Alpha	**119**	**2.2**	**39**	**1.51**
Akt-1	**0.230**	**0.314**	**0.08**	**0.189**

The OSA and Control groups were similar with respect to specific biochemical markers related to vascular function and adaptation including, serum nitrates/nitrites, norepinephrine, dopamine, serotonin, and plasma VEGF (Table 4). Conversely, %VEGFR2 expression was significantly suppressed in OSA subjects ($p = 0.04$). Though not evaluated in OSA, the degree of %VEGFR2 suppression in these patients is similar to the findings of Fichtlscherer *et al.* [2001]. These researchers observed similar results in hypertensive CAD patients. Although none of the biochemical markers adequately correlated with RDI, a marginal relationship between $sa0_2<90\%$ and VEGF (Figure 4) was observed. Additionally, serum nitrate concentrations did not significantly correlate with plasma VEGF levels. VEGFR2 analysis included percent quantification of circulating mononuclear cell populations. Figure 3 indicates flow cytometry gates for circulating lymphocyte, monocyte, and granulocyte populations.

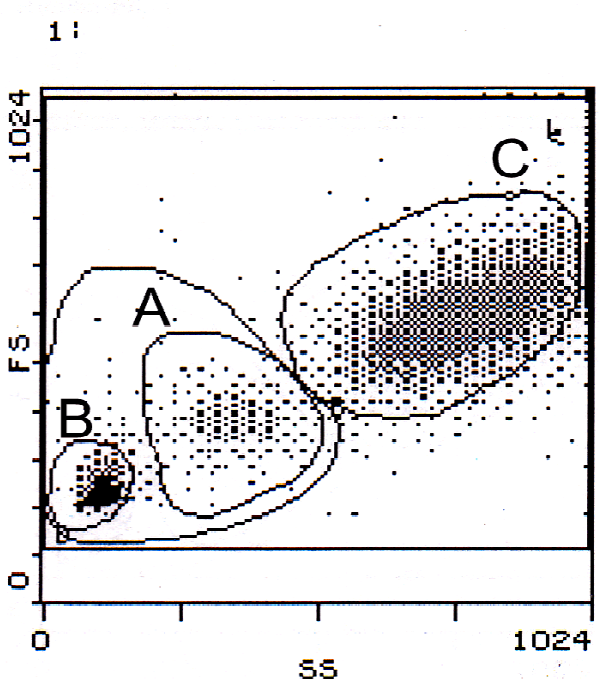

Figure 3. VEGFR2/KDR positive cells were detected by flow cytometry. Forward/sideward scatter with monocyte (A), lymphocyte (B), total mononuclear cell (A + B), and granulocyte (C) gates are indicated.

Table 4. Biochemical markers of vascular function and adaptation for both OSA and non-experimental control groups. *Statistical significance at $p \leq 0.05$

Table 4. Biochemical Markers of vascular function and adaptation	**OSA Group (N = 7)**	**Control Group (N = 7)**
Serum Nitrate (uM)	**7.32 ± 3.80**	**14.08 ± 15.90**
Serum Nitrite (uM)	**0.08 ± 0.21**	**0**
Plasma VEGF (pg/mL)	**74.60 ± 53.30**	**56.30 ± 24.60**
% VEGFR2 Expression	**53.30 ± 28.60**	**81.70 ± 13.80***
Norepinephrine (ug/mL)	**55.5 ± 12.14**	**44.81 ± 25.5**
Dopamine (ng/mL)	**27.20 ± 16.23**	**26.20 ± 26.34**
Serotonin (ng/mL)	**22.91 ± 23.80**	**25.90 ± 22.70**

Table 5. Relationships between Biochemical markers of vascular function and indicators of disease severity.

Table 5. Correlation Coefficients	NO3	VEGF	VEGFR2	NE
RDI	r=-.17;p=.71	r=.29;p=.52	r=-.43;p=.32	r=.38;p=.39
Sa02<90%	r=.66;p=.10	r=.7;p=.07	r=-.49;p=.25	r=-.47;p=.28
NO3	1.00	r=-.09;p=.74	r=.01;p=.96	r=.008;p=.97
VEGF	r=-.09;p=.74	1.00	r=-.13;p=.63	r=-.22;p=.44
VEGFR2	r=.01;p=.96	r=-.13;p=.63	1.00	r=-.33;p=.24

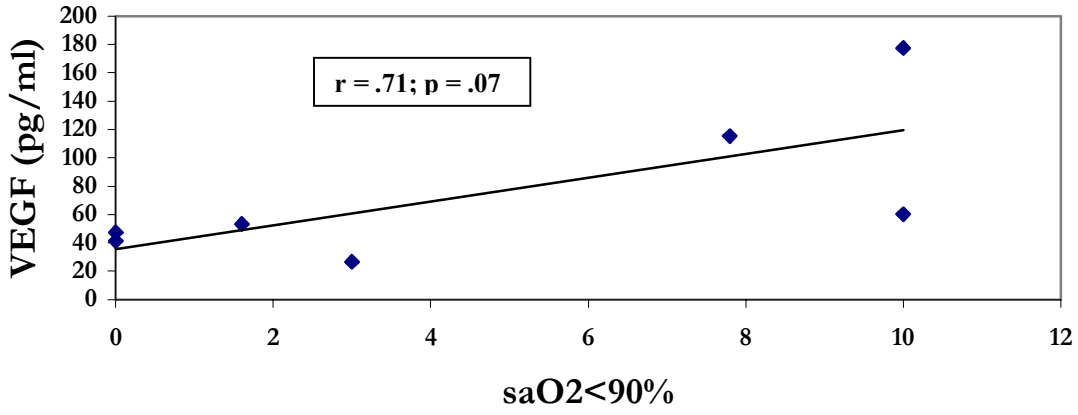

Figure 4. The correlation between hypoxia and circulating plasma VEGF concentrations for 7 OSA patients. r = .71; p = .07

Both cardiopulmonary and cardiovascular function profiles were also similar between the two groups. No differences were observed relative to cardiac output, stroke volume, cardiopulmonary or myocardial oxygen consumption, expired ventilation, true O_2, heart rate, arteriovenous oxygen difference, total peripheral resistance, and mean arterial pressure before, during and after graded exercise.

However, a significant difference (p = .0017) was observed in the recovery: peak systolic blood pressure ratio at 1 minute post-exercise (Figure 5).

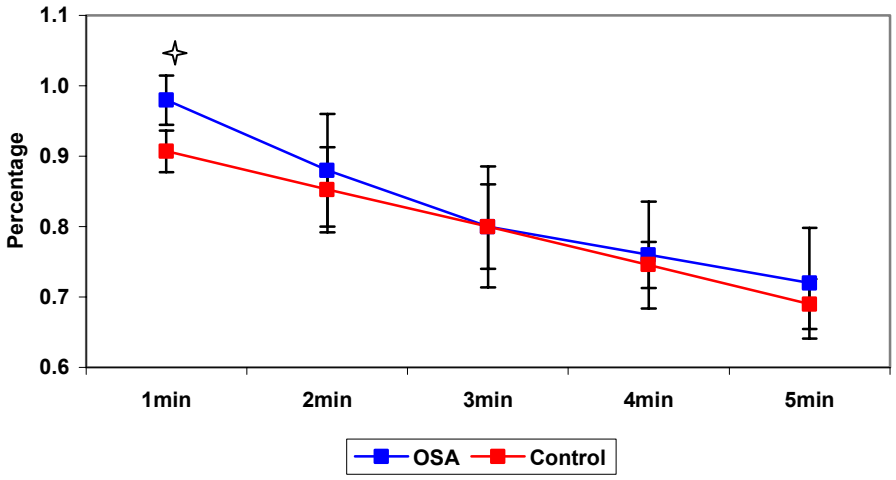

Figure 5. Recovery: peak systolic blood pressure ratios for both groups at each minute of recovery. Minute 1 revealed a significant statistical difference between the groups (p = 0.0017)

Heart rate responses did not significantly associate with saO_2<90%, however, heart rate did negatively correlate with RDI at 100 watts (r = -0.74; p = 0.05), peak exercise (r = -0.74; p = 0.05), minute 1 of recovery (r = -0.79; p = 0.03), and minute 3 of recovery (r = -0.76; p = 0.04). Interestingly, VEGFR2% strongly associated with the recovery: peak systolic blood pressure response at min 1(r = -0.65;p=. 01) and both recovery min 2 (r = -0.60;p=.02) and 5 (r = -0.54;p=. 04) mean arterial pressure. No significant associations were observed between RDI or saO_2<90% and arteriovenous oxygen difference before or during exercise.

Table 6. Associations between RDI and heart rate during and after graded exercise. HR100W: heart rate at 100 watts; HRpeak: heart at peak exercise; HR1: heart rate at recovery min 1; HR3: heart rate at recovery min 3.

Table 6. Correlation Coefficients	HR100W	HRpeak	HR1	HR3
RDI	r=-.74;p=.05	r=-.74;p=.05	r=-.79;p=.03	r=-.76;p=.04

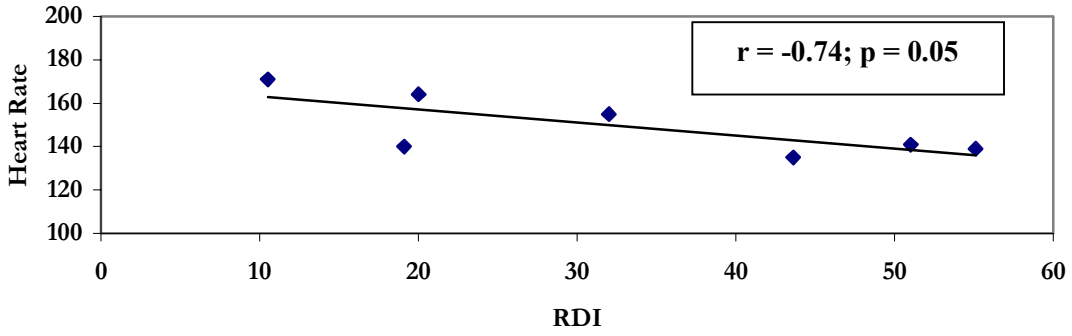

Figure 6. The relationship between RDI and peak exercise heart rate response during graded exercise in 7 OSA patients.

Table 7. Associations between VEGFR2 and blood pressure responses after graded exercise. RP1: recovery: peak systolic blood pressure min 1 of recovery; MAP2: mean arterial pressure min 2 of recovery: MAP5: mean arterial pressure min 5 of recovery.

Table 7. Correlation Coefficients	**RP1**	**MAP2**	**MAP5**
VEGFR2	**r=-.65;p=.01**	**r=-.60;p=.02**	**r=-.54;p=.04**

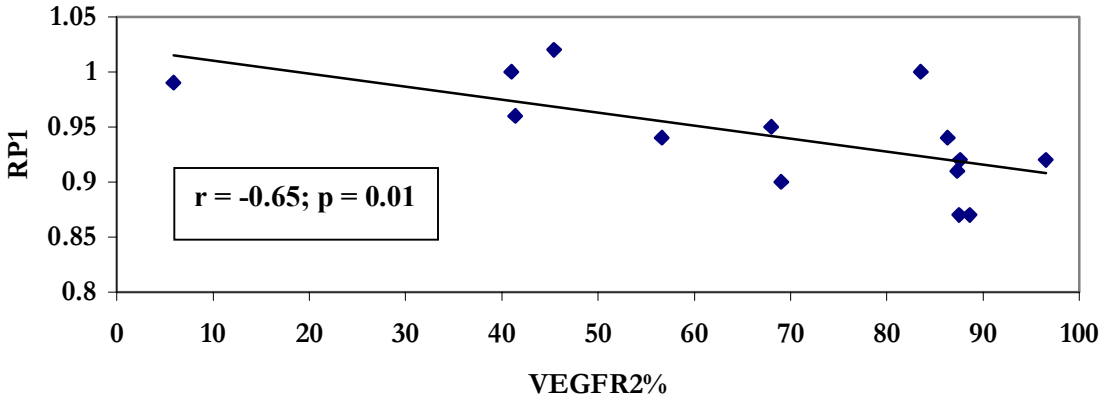

Figure 7. The relationship between VEGFR2 expression on circulating mononuclear cells and the recovery: peak systolic blood pressure response minute 1 in both OSA and non-experimental subjects.

Discussion

Sleep apnea syndrome is an independent risk factor for hypertension, myocardial infarction, and stroke [Hall *et al*, 1995; Grote *et al*, 2000; Ip *et al*, 2000]. This disease state is related to both acute and chronic hemodynamic changes that adversely affect arterial blood pressure, heart rate, stroke volume, and cardiac output [Ip *et al*, 2000]. In order to discern the causes of these cardiovascular co-morbidities, recent emphasis has been placed on the evaluation of possible vascular pathogenic mechanisms in OSA. Therefore, the current study aimed to systematically determine whether chronic hypoxia associated with sleep-disordered breathing relates to pathologic alteration of signaling within the PI3K/Akt-1 transduction network, and if so, does this disturbance pertain to abnormal NO production and VEGF expression patterns that might possibly contribute to the aberrancy of specific determinates of cardiovascular and cardiopulmonary function before, during, and after graded exercise.

In the present study, OSA and control groups were statistically similar with respect to demographic data including age, weight, body mass index (BMI), body surface area (BSA), and neck circumference. There were no apparent differences observed in biochemical markers relating to vascular function and adaptation including, serum nitrates/nitrites, norepinephrine, dopamine, serotonin, and plasma VEGF. Additionally, no differences were found relative to cardiac output, stroke volume, cardiopulmonary or myocardial oxygen consumption, expired ventilation, heart rate, arteriovenous oxygen difference, total peripheral resistance, and mean arterial pressure before, during and after graded exercise in these subjects. These findings may be attributed to the small sample size of the current experimental design. Furthermore, due to methodological issues related to the redundant oligonucleotide DNA microarray and real-time PCR gene expression analyses, results of these experiments were uninterpretable. While these concerns were addressed in the results section, equivocations associated with these findings were

quite possibly related to the quality of pooled RNA in the experimental group. Additively, these results did not support the research hypothesis.

Conversely, differences were observed in waist: hip ratios, recovery: peak systolic blood pressure ratio at 1 minute post-exercise and %VEGFR2 expression. OSA was associated with elevations in both waist: hip ratios and recovery: peak systolic blood pressure ratio at 1 minute post-exercise as well as significant depression of %VEGFR2 profiles. Moreover, significant negative correlations were found regarding waist: hip ratios and %VEGFR2 expression and recovery: peak systolic blood pressure ratio at 1 minute post-exercise and %VEGFR2 expression. This represents a unique finding in OSA research. Presently, circulating %VEGFR2 expression has not been evaluated in sleep-disordered breathing. However, Fichtlscherer *et al.* [2001] found by multivariate analysis that HTN was a major independent predictor for impaired endothelial progenitor cell (EPC; KDR-positive cells) migration in patients with coronary artery disease. While the cause of this hypertensive response is unknown, impaired EPC migration might be related to hypoxic perturbations of VEGF expression found in many OSA patients.

The findings of the present study did not support the research hypothesis nor did they provide evidence that NO-dependent vasoactive mechanisms are suppressed in sleep-disordered breathing. Additionally, these results did not support the supposition that angiogenic mechanisms are pathologically activated in this disease state. The finding that circulating VEGFR2 positive cell mobilization was impaired in these select OSA patients may indicate suppression of neovascular processes. However, limitations associated with inadequate sample size may explain the apparent discrepancies with current research literature. Additional basic and clinical investigations are needed in order to fully delineate the relationship between cardiovascular dysfunction and sleep-disordered breathing.

Chapter IV

Recommendations for Future Research

Based both on the findings of this study and on concurrent research literature germane to cardiovascular dysfunction in sleep disordered breathing, the following recommendations are reasonably justified:

1) Sample size has been problematic in terms of delineating true biologic response variable differences. Another concern in this regard is the lack of polysomnographic measures for controls subjects. In an attempt to control for disease presence in non-experimental subjects, controls were evaluated by means of sleep related questionnaires. Consequently, observes were unable to determine hypoxia and RDI scores for these subjects. Both these concerns may be alleviated with the allocation of research funding for both direct compensation of subject participation and for control subject polysomnographic studies. Presently, an ample amount of both basic and clinical preliminary research data indicating biologic differences in cardiovascular and cardiopulmonary function has now been generated. These findings now warrant grant application to the NIH and/or AHA.

2) Delineations in the mechanisms related to regulatory control of integrative cardiovascular biology in sleep-disordered breathing might be enhanced by the implementation of animal and cell culture models. Presently, *ex vivo* data signifying both positive and negative associations between hypoxia, molecular and biochemical markers of vascular function, and physiologic parameters related functional capacity have now been determined. The addition of various *in vivo*, *in situ*, and *in vitro* protocols may afford for the establishment of causal relationships among these discriminate biologic variables.

3) In the present study, associations were determined between established and proposed indicators of disease severity, molecular and biochemical markers of endothelial function, and blood pressure responses before, during, and immediately after ergometric stress. In an attempt to indirectly determine vascular function in these patients, estimates of total peripheral resistance were calculated from cardiac output and blood pressure response values. The addition of plethysmography to the current experimental design would allow for noninvasive assessment of vascular function and perfusion. Moreover, this technology may reveal the degree to which peripheral factors contribute to aberrant blood pressure regulation in OSA.

4) In an attempt to explain the decrease in cardiovascular co-morbidity associated with long-standing OSA, a connection between disease severity and elevated serum and plasma concentrations of vascular endothelial growth factor have recently been reported in the OSA literature. However, researchers have currently not determined whether this phenomenon is linked to the induction of neovascularization in these patients. Therefore, the implementation of an animal model into the present experimental design may divulge the existence of angiogenic processes and the determination of its impact on sleep disordered breathing.

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