The Relationship between Circulating Biomarkers of Nitric Oxide and Endothelin-1 and Hemodynamic Function in Obstructive Sleep Apnea

by

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(ABSTRACT)

Obstructive sleep apnea (OSA) is a disorder that affects a significant portion of middle-aged adult population. Patients exhibit recurring episodes of upper airway obstruction during sleep that decrease blood oxygen concentration (hypoxia) and are terminated by brief arousals. Epidemiologically, OSA has been extensively linked to cardiovascular dysfunction and is an independent risk factor for the development of hypertension. The proposed mechanism of cardiovascular dysfunction in patients is chronic sympathoexcitation and altered vascular tone, with a predominance of the vasoconstrictor endothelin-1 (ET-1) and removal of the vasodilator nitric oxide (NO). Means to reduce the effects of ET-1 and increase synthesis of NO may have beneficial effects on the cardiovascular co-morbidity commonly associated with OSA. **OBJECTIVES:** The major aim of this study was to assess the relative importance of circulating biomarkers of ET-1 and NO in hemodynamic function in OSA patients. Potential production of ET-1 by circulating mononuclear cells was also measured to assess their contribution to plasma ET-1 levels. Biomarker levels before and after 12 wk of continuous partial airway pressure (CPAP) therapy were used to assess standard treatment. Mild/moderate exercise training was initiated with CPAP therapy in a subgroup of OSA patients to evaluate the potential benefits of physical activity on hemodynamic function and NO and ET-1 levels. **METHODS:** Overall, 16 newly diagnosed OSA patients (5 female, 11 male; age 45.4 ± 2.7 yr; RDI 24.6 ± 4.0 events/hr) were selected for study. Seven apparently healthy control volunteers (5 female, 2 male; age 39.43 ± 2.6 yr) screened for OSA served as control subjects. Blood pressure was recorded over one complete day and prior to, during, and following maximal exercise testing on a cycle ergometer. Blood samples were taken prior to exercise testing and assessed for nitrate and nitrite by HPLC and for big endothelin-1 and ET-1 by ELISA. Relative gene expression of preproendothelin-1 was measured by real-time RT-PCR. Following initial testing, patients were stratified into either a standard therapy group (nCPAP) or a standard therapy group with a mild/moderate intensity aerobic training regimen (nCPAP+Ex). Baseline testing was repeated following 12 wk of treatment. Statistical significance was set at $p<0.05$ *a priori*. **RESULTS:** 24 hr ambulatory systolic and diastolic blood pressure were elevated in OSA patients vs. control subjects (systolic: 128.9 ± 3.8 mmHg vs. 108.8 ± 1.3 mmHg, respectively; diastolic: 97.5 ± 2.0 mmHg vs. 82.1 ± 1.9 mmHg, respectively). OSA patients experienced significant elevations in systolic (OSA 209.7 $\underline{+}$ 5.7 mmHg; Control 174.5 $\underline{+}$ 6.2 mmHg) and mean arterial pressures (OSA 125.8 $\underline{+}$ 3.2 mmHg; Control

109.05 ± 4.5 mmHg) at peak exercise. No differences in nitrate, nitrite, or big endothelin-1 were noted. Plasma endothelin-1 concentrations were below assay detection limit. Big endothelin-1 levels were significantly correlated with BMI in both OSA patients (r=0.955; p=0.001) and control subjects (r=0.799; p=0.045). Relative gene expression of preproendothelin-1 was not elevated in OSA patients (0.40±0.20 fold increase over control subjects). Group nCPAP usage was above minimum therapeutic threshold, but was non-uniform in both groups, with an overall range of 182 to 495 min mean usage per night. A mild/moderate exercise training program failed to elicit a training response through standard hemodynamic or cardiopulmonary indices. Plasma nitrite levels rose from 55.3±4.7 μg/ml to 71.0±7.6 μg/ml in the nCPAP group.
CONCLUSIONS: Moderate OSA is associated with elevated blood pressure at rest and during exercise stress that bears no relationship to circulating biomarkers of NO and ET-1 or immune preproendothelin production in patients without diagnosed hypertension. nCPAP therapy failed to elicit significant improvements in hemodynamic function, with or without moderate exercise. Plasma nitrite levels rose following nCPAP therapy, indicating a possible increase in basal nitric oxide formation. Higher intensity exercise regimens may be needed to elicit the positive benefits of exercise training in OSA patients without significant cardiovascular dysfunction.

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Ange, thank you for your stabilizing influence and affection. Finally, to my niece Katie and nephew Mitchell, thank you bringing out the child inside me. I look forward watching you grow and to the day when I can bring out the child in you. I feel truly blessed to have such a loving family and look forward to coming home soon.

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Chapter I

Introduction

Obstructive sleep apnea (OSA) is a disorder that affects approximately 2% to 4% of middle-aged adults. However, recent estimates have suggested that some degree of OSA may be present in approximately ~25% of the middle-aged population (Henderson and Strollo 1999). Patients exhibit recurring episodes of upper airway obstruction during sleep that decrease blood oxygen concentration (hypoxia) and are terminated by brief arousals (McNicholas 1997). This pattern fragments sleep and has numerous physiological consequences. Recently, OSA has even been recommended as an independent risk factor for the development of hypertension (Peppard, Young et al. 2000; Peker, Hedner et al. 2002). Unfortunately, most OSA sufferers go unrecognized and untreated by current medical practice. Therefore, the deleterious effects of OSA mount with age and culminate in increased medical costs and disability later in life (Kapur, Blough et al. 1999). The stresses placed on the cardiovascular (CV) system by OSA have lead to the discovery of a CV mortality rate over three and a half times that of the general population between the fourth and fifth decades of life (Lavie, Herer et al. 1995).

The proposed mechanism of cardiovascular dysfunction in OSA patients is chronic sympathoexcitation and altered vascular tone. A hypothetical mechanism for this impaired vascular tone can be seeen in Figure 1. OSA patients experience significant reductions in vascular reactivity in both arterial (Kraiczi, Caidahl et al. 2001) and venous (Duchna, Guilleminault et al. 2000) vessels compared to non-disordered individuals. Correction of sleep disordered breathing with nasal continuous positive airway pressure

(nCPAP) partially restores vascular function in patients (Imadojemu, Gleeson et al. 2002), indicating a causal relationship between OSA and vascular impairment. Evidence also exists for decreased vascular production of vasodilators such as nitric oxide (NO) (Kato, Roberts-Thomson et al. 2000) and enhanced sensitivity to vasoconstrictors such as endothelin (ET-1), epinephrine (EPI), norepinephrine (NE), and angiotensin II (AngII) (Kraiczi, Hedner et al. 2000). Unfortunately, the exact mechanism by which OSA leads to vascular dysfunction is incompletely understood. Further, impaired cardiovascular regulation in patients is believed to occur following years of intermittent hypoxia during sleep, making prospective study in humans difficult.

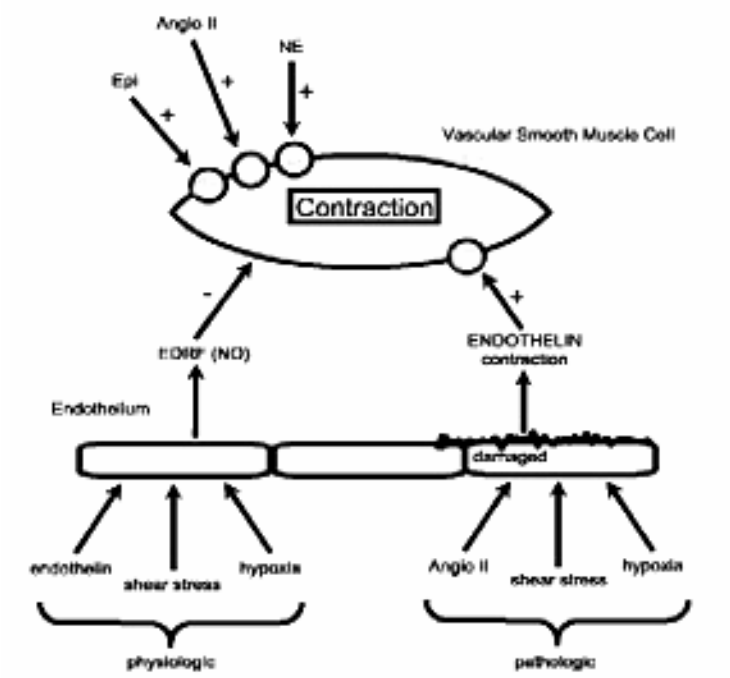

Figure 1. Hypothetical Mechanism for Vascular Dysfunction in Obstructive Sleep Apnea

Under physiologic conditions seen above left, hypoxia, shear stress, and endothelin-1 stimulate the release of endothelial derived relaxing factor (EDRF), or nitric oxide, to facilitate relaxation of the vascular smooth muscle cells. However, under pathologic conditions as seen above right, angiotensin II (Angio II), shear stress, and hypoxia stimulate the release of endothelin-1, causing vascular smooth muscle cell contraction and increased vascular resistance (modified from Opie, 1991).

Under physiologic conditions, the vascular endothelium plays a key role in modulating blood pressure, vascular smooth muscle proliferation, platelet adhesion and aggregation, coagulation, and monocyte adhesion (Luscher and Noll 1995). In the systemic vasculature, resting tone is maintained by basal release of NO (Li and Forstermann 2000). NO is a relatively stable reactive species produced in response to the substances bradykinin, ET-1, circulating catecholamines, and acetylcholine (Luscher and Noll 1995) and by vascular shear stress (Fisher, Al-Mehdi et al. 2002). In endothelial cells, NO is produced constitutively to maintain vascular tone by endothelial NOS (eNOS). Animals without functional eNOS subsequently develop hypertension and exhibit an impaired vasodilatory capacity (Huang, Huang et al. 1995). In vascular smooth muscle cells, endothelial-derived NO facilitates vasodilation through decreases in intracellular calcium (Gewaltig and Kojda 2002). Unfortunately, NO has a short half-life of approximately 3 seconds (Palmer, Ferrige et al. 1987), making in vivo measurement difficult. In an aqueous environment or in the presence of hemoglobin, NO is rapidly converted to nitrite and nitrate, respectively (Kelm, Preik-Steinhoff et al. 1999). Measurement of both nitrates and nitrites in serum consequently provide a means to assess overall NO production *in vivo*. Previously, depressed nitrate and nitrite levels have been observed in OSA patients (Ip, Lam et al. 2000; Schulz, Schmidt et al. 2000). Successful nCPAP therapy only partially restores nitrate and nitrite levels to that of control patients (Ip, Lam et al. 2000).

To counterbalance the vasodilatory properties of NO, endothelial cells have the ability to synthesize powerful vasoconstrictors when necessary. A prominent vasoconstrictor

substance implicated in the cardiovascular impairment of OSA is ET-1 (Lavallee, Takamura et al. 2001). ET-1 is a 21 amino acid peptide primarily produced in response to elevated blood pressure, Ang II, vasopressin, and low density lipoprotein cholesterol (Hunley and Kon 2001). ET-1 regulation occurs primarily at the transcription level as preproendothelin (Wagner, Christ et al. 1992). Preproendothelin is then converted into the precursor big endothelin (BE) (Hunley and Kon 2001). Functional ET-1 is formed by the catalytic cleavage of BE by endothelin converting enzyme (ECE) both intra and extracellularly (Hunley and Kon 2001). Although BE itself has mild vasoconstrictory properties, the primary action of the endothelin system occurs through the active ET-1 form (Pacher, Bergler-Klein et al. 1993).

Under physiologic conditions, intimal release of ET-1 promotes initial vasodilation via stimulation of the NO pathway (Bassenge 1995). Sustained release subsequently facilitates vasoconstriction and appears paramount in blood flow redistribution during bouts of increased metabolic activity (Tanabe, Yamamoto et al. 2000; Maeda, Miyauchi et al. 2002). In fact, impaired local release of ET-1 may directly affect functional ability (Ishikawa, Miyauchi et al. 1998). Under pathophysiologic conditions such as essential hypertension, ET-1 induces marked vasoconstriction and increased blood pressure (Cardillo, Kilcoyne et al. 1999). Pathologic conditions are further marked by increased levels of ET-1 in the plasma, which may be indicative of altered synthesis or clearance (Schmitz-Spanke and Schipke 2000).

While the implications of plasma ET-1 are poorly understood, observed elevations in resting plasma levels are seen in congestive heart failure patients which positively correlate with disease severity (Margulies, Hildebrand et al. 1990). Elevated levels of the ET-1 precursor BE have also been observed in heart failure patients (Kiowski, Suetsch et al. 2001), indicating increased synthesis in this disease state. Plasma ET-1 levels in heart failure patients are partially attributed to chronic immunologic activation of circulating mononuclear cells (Krum and Itescu 1994), possibly as a means to deliver ET-1 to needed areas or as a means of stimulating NO release. Theoretically, systemic elevations in plasma ET-1 may enhance vasoconstriction and blood distribution during stress, thereby acting as an early compensatory mechanism in the development of congestive heart failure (Best and Lerman 2000).

Documented increases in plasma ET-1 occur are seen in OSA patients regardless of hypertensive status (Saarelainen, Seppala et al. 1997). During untreated apnea, these levels positively correlate with both changes in mean arterial pressure and oxygen saturation and improve following acute nCPAP treatment (Phillips, Narkiewicz et al. 1999). In opposition, recent study has found no differences in plasma ET-1 between OSA patients and controls both before and after nCPAP therapy (Grimpen, Kanne et al. 2000).

Statement of the Problem

Normal vascular function involves an intricate interplay between vasodilator and vasoconstrictor substances to redirect blood flow where needed. The primary vasodilator

associated with systemic vascular tone is NO, and prior investigations have shown depressed levels of the NO derivatives in the plasma of OSA patients (Ip, Lam et al. 2000; Schulz, Schmidt et al. 2000). Reduced NO metabolites indicate a potential loss of vasodilatory capacity in the vasculature. In concert with the withdrawal of NO-induced vasodilation, it appears OSA patients experience an elevated vasoconstrictor influence by the peptide ET-1. However, conflicting results exist as to the importance of plasma ET-1 in OSA patients and additional study is needed. Further, it remains to be seen whether plasma ET-1 levels have a potential role in the cardiovascular control of OSA patients, manifested by systemic blood pressure both at rest and during physiologic stress.

In plasma, ET-1 has a relatively short half-life owing to its rapid clearance by endothelial receptors (Levin 1995). Due to its short existence, circulating levels of BE are important for the rapid formation of ET-1 when needed. Increased levels of circulating BE may therefore be indicative of increased ET-1 synthesis in OSA. To date, no studies investigated the relationship between plasma levels of BE and ET-1 in OSA patients either prior to or following nCPAP therapy have been found in the published scientific literature.

The exact source of plasma ET-1 in OSA patients remains unclear, and may be attributed to either an increased synthesis or decreased clearance. Circulating immune cells have previously been shown as a possible contributor to increased levels of plasma ET-1 in heart failure patients (Krum and Itescu 1994). To date, it remains to be seen if circulating immune cells contribute to elevated ET-1 levels in OSA patients. The

contribution of immune cells to ET-1 production may further implicate OSA in the development of cardiovascular disease, especially considering OSA patients in general experience a greater than three-fold risk for heart failure (Smith, Ronald et al. 2002). Given the vital role of ET-1 in stress-induced circulatory redistribution, no studies have yet evaluated ET-1 and exercise in OSA patients. Vascular regulation involves the integration of both vasoconstrictors and vasodilators to properly control blood flow both at rest and during exercise. However comprehensive evaluation of the biomarkers of vascular function, especially ET-1, has not been attempted, especially with regard to clinical measures such as resting and exercise blood pressure and as a possible marker of treatment.

ET-1 has demonstrated an important role in blood flow redistribution during exercise (Maeda, Miyauchi et al. 1998; Tanabe, Yamamoto et al. 2000; Maeda, Miyauchi et al. 2002). In healthy older humans, chronic exercise training decreases resting plasma ET-1 levels and blood pressure (Maeda, Tanabe et al. 2003). However, little information is known with respect to the effect of chronic exercise on abnormal ET-1 production observed in cardiovascular disease. Exercise training has long been advocated as an effective means of treatment in cardiovascular disease. Recent study has demonstrated dramatic improvements in endothelial function following exercise training in heart failure patients (Hambrecht, Wolf et al. 2000). To date, three published studies demonstrate a decrease in OSA severity without improvements in cardiovascular fitness, body parameters, or blood pressure following exercise training (Netzer, Lormes et al. 1997; Giebelhaus, Strohl et al. 2000; Norman, Von Essen et al. 2000). However, varied

experimental methods and a detailed evaluation of vasoactive biomarkers do not allow for adequate explanation of the positive effect of exercise in this population.

Significance of the Study

This experiment is the first to comprehensively assess ET-1 dysfunction and blood pressure regulation both before and after nCPAP therapy. Quantitative measurement of both ET-1 and its precursor, BE, in OSA patients devoid of co-existing cardiovascular disease lends evidence to whether increased plasma levels are a consequence of, and not simply associated with, sleep disordered breathing. Assessment of preproendothelin gene expression by circulating mononuclear cells provides evidence of a potential immune contribution to plasma ET-1 and BE levels. Finally, evaluation of NO metabolites helps elucidate the interplay between NO and ET-1 in this disease state.

Research Hypothesis

OSA promotes enhanced ET-1 synthesis in untreated patients and is associated with blunted NO production. Elevated plasma levels of ET-1 and its precursor, BE, coincide with increased transcription of the preproendothelin gene in circulating mononuclear cells and demonstrate a relationship with markers of NO production (serum nitrate and nitrite). Further, aberrant ET-1 levels are associated with cardiovascular function before, during, and after graded exercise. nCPAP therapy reduces circulating levels of ET-1 and BE, increases serum levels of nitrate and nitrite, and improves cardiovascular function. Concurrent physical training results in further improvements in circulating biomarker levels and cardiovascular function than nCPAP alone.

Research Aims

The major aim of this study was to assess the relative importance of circulating ET-1 on cardiovascular function in obstructive sleep apnea patients. Alterations in ET-1 production were assessed following treatment to assess standard therapy and the additive effects of exercise as a possible adjunct treatment.

1) Specific Aim 1: To evaluate the hypothesis that ET-1 production was enhanced in OSA, serum levels of ET-1 and its precursor, BE, were assessed by ELISA in patients prior to exercise testing and the initiation of nCPAP therapy. Mononuclear cells isolated from peripheral blood were used as reporter cells for measurement of preproendothelin gene expression by real time PCR technology. Circulating levels of serum nitrate and nitrite were determined by high performance liquid chromatography (HPLC) to evaluate the relationship between NO and ET-1 production.

2) Specific Aim 2: To assess the hypothesis that aberrant ET-1 levels affect cardiovascular function, the relationship between resting plasma levels of ET-1, BE, and serum nitrates and nitrites and blood pressure regulation were evaluated in response to maximal cycle ergometer exercise stress. Blood pressure before, during, and following exercise was measured manually by trained personnel. Ambulatory blood pressure was recorded at four specific time points during wakefulness.

3) Specific Aim 3: To evaluate the hypothesis that OSA treatment reduces ET-1 production, enhances NO production, and increases cardiovascular and vascular

function, study participants were evaluated following 12 weeks of nCPAP therapy by identical means as baseline testing. The additive effects of exercise training to nCPAP therapy were assessed in a randomly selected sample subset at 12 weeks post nCPAP initiation by identical means as baseline.

Assumptions

1. Subjects accurately answered the medical/health history questionnaire and did not exhibit co-morbid exclusion criteria.
2. Subjects correctly reported their physical activity levels.
3. Subjects exerted maximal effort during cycle ergometry.
4. All exercise testing equipment was accurately calibrated and maintained.
5. All blood pressure recording devices were accurately calibrated and maintained.
6. Home screening device for sleep apnea was accurately calibrated and maintained.
7. Metabolic cart accurately measured all cardiopulmonary variables.

Delimitations

1. Experimental subjects were volunteers consecutively referred to the Allergy and Sleep Disorder Center in Christiansburg, VA for evaluation of a suspected sleeping disorder.
2. Control subjects were volunteers from local communities.
3. Both experimental and control subjects did not engage in regular physical activity (> 2 day/wk) for at least 6 months prior to study participation.

4. Both experimental and control subjects did not have preexisting cardiovascular, pulmonary or metabolic conditions as well as significant musculoskeletal impairment that would prohibit repetitive physical training.
5. Both experimental and control subjects were not under treatment of either cardiovascular medication or medications that significantly influence vascular function.

Limitations

1. Due to logistical limitations, exercise testing and blood draw procedures were not restricted to a specific time of day.
2. Testing staff availability did not allow for immediate processing and storage of biological samples.
3. Blood sampling was not performed during a fasting state, as patients were expected to complete a maximal exercise effort immediately afterward.
4. Home screening for underlying OSA in control subjects was not performed in concert with other data collection procedures.
5. The experimental design included OSA patients and control subjects with wide variation in disease severity and demographic features.
6. Exercise training intensity may have varied between subjects due to the inherent independency of the training protocol.
7. Normal plasma levels of ET-1 are in the low pg/ml range, and may subsequently reside below assay detection limits in some patients.

8. Circulating cholesterol levels were not measured to account for the effect of oxidized LDL as a known stimulant of ET-1 release.

Definitions of Terms

Apnea: cessation of airflow for at least 10 sec (Malhotra and White 2002).

Apnea/Hypopnea index (AHI): the number of apneic/hypopneic episodes recorded per hour of sleep (Bradley and Floras 2000).

Arousal: abrupt change in sleep state (Bradley and Floras 2000).

Big Endothelin: 41 amino acid peptide created from the cleavage of ~200-residue preproendothelin polypeptide by furin-like protease that serves as the precursor for endothelin-1 (Kedzierski and Yanagisawa 2001).

Binding Site: a region on the surface of one molecule (usually a protein or nucleic acid) that can interact with another molecule through noncovalent bonding (Alberts 1994).

Complementary DNA (cDNA): DNA molecule made as a copy of messenger RNA and therefore lacking the introns that are present in genomic DNA (Alberts 1994).

Constitutive: produced in a constant amount: opposite of regulated (Alberts 1994).

Denaturation: dramatic change in conformation of a protein or nucleic acid caused by heating or by exposure to chemicals and usually resulting in the loss of biological function (Alberts 1994).

Desaturation: the unbinding of oxygen from hemoglobin (Myers 1996).

Deoxyribonucleic acid (DNA): polynucleotide formed from covalently linked deoxyribonucleotide units. It serves as the store of hereditary information within a cell and the carrier of this information from generation to generation (Alberts 1994).

DNA polymerase: enzyme that synthesizes DNA by joining nucleotides together using a DNA template as a guide (Alberts 1994).

Endothelin-1: 21 amino acid vasoconstricting factor formed primarily by endothelial cells to act as a paracrine or autocrine regulator of vascular tone (Kedzierski and Yanagisawa 2001).

Endothelin Converting Enzyme: member of a family of membrane-bound zinc metalloproteases from the neprilysin superfamily that cleaves big endothelin into the active peptide endothelin-1 (Kedzierski and Yanagisawa 2001).

Enzyme: protein that catalyzes a specific chemical reaction (Alberts 1994).

Exon: segment of a eukaryotic gene that consists of a sequence of nucleotides that will be represented in messenger RNA or the final transfer RNA or ribosomal RNA. In protein-coding genes, exons encode amino acids in the protein. An exon is usually adjacent to a noncoding DNA segment called an intron (Alberts 1994).

Electroencephalogram: recording of the electrical activity of the brain (Bradley and Floras 2000).

Electromyogram: recording of electrical activity of the muscles (Bradley and Floras 2000).

Electrooculogram: recording of eye position shifts (Bradley and Floras 2000).

Heart Rate Variability: beat-to-beat alterations in heart rate (Bradley and Floras 2000).

Hypopnea: a substantial reduction in airflow >50%, a moderate reduction in airflow (<50%) with desaturations (>3%), or moderate reduction in airflow (<50%) with electroencephalographic evidence of arousal (Malhotra and White 2002).

Gene: region of DNA that controls a discrete hereditary characteristic, usually corresponding to a single protein or RNA. This definition includes the entire functional

unit, encompassing coding DNA sequences, noncoding regulatory DNA sequences, and introns (Alberts 1994).

Genome: the totality of genetic information belonging to a cell or an organism; in particular, the DNA that carries this information (Alberts 1994).

Hormone: signal molecule secreted by an endocrine cell into the blood-stream, which can then carry it to distant target cells (Alberts 1994).

***in situ* Hybridization:** technique in which a single-stranded RNA or DNA probe is used to locate a gene or a messenger RNA molecule in a cell or tissue by hybridization (Alberts 1994).

***in vitro*:** term used by biochemists to describe a process taking place in an isolated cell-free extract. Also used by cell biologists to refer to cells growing in culture (*in vitro*), as opposed to in an organism (*in vivo*) (Alberts 1994).

***in vivo*:** in an intact cell or organism (Alberts 1994).

Messenger Ribonucleic Acid: RNA molecule that specifies the amino acid sequence of a protein. Produced by RNA splicing (in eukaryotes) from a larger RNA molecule made by RNA polymerase as a complementary copy of DNA. It is translated into protein in a process catalyzed by ribosomes (Alberts 1994).

Nitric oxide: gaseous signal molecule in both animals and plants. In animals it regulates smooth muscle contraction, for example; in plants it is involved in responses to injury or infection (Alberts 1994).

Nucleic Acid: RNA or DNA, a macromolecule consisting of a chain of nucleotides joined together by phosphodiester bonds (Alberts 1994).

Oxygen consumption: the rate at which oxygen is consumed per minute (Myers 1996).

Peak oxygen uptake: the highest oxygen uptake attainable during graded exercise testing (Myers 1996).

Polysomnography: test used to diagnose sleep disorders (Bradley and Floras 2000).

Oxygen uptake: the common biological measure of total body work. It is defined by the rate at which oxygen is consumed by the body, expressed in L O_2/min or ml O_2/kg/min (Myers 1996).

Paracrine signaling: short-range cell-cell communication via secreted signal molecules that act on adjacent cells (Alberts 1994).

Pharynx: Air passage between nose and larynx (Bradley and Floras 2000).

Polymerase chain reaction: technique for amplifying specific regions of DNA by the use of sequence-specific primers and multiple cycles of DNA synthesis, each cycle being followed by a brief heat treatment to separate complementary strands (Alberts 1994).

Polypeptide: linear polymer composed of multiple amino acids. Proteins are large polypeptides, and the two terms can be used interchangeably (Alberts 1994).

Promoter: nucleotide sequence in DNA to which RNA polymerase binds to begin transcription (Alberts 1994).

Respiratory Disturbance Index: total number of apneas and hypopneas per hour of sleep (Bradley and Floras 2000).

Reverse transcriptase: enzyme first discovered in retroviruses that makes a double-stranded DNA copy from a single-stranded RNA template molecule (Alberts 1994).

Ribosomal RNA: any one of a number of specific RNA molecules that form part of the structure of a ribosome and participate in the synthesis of proteins. Often distinguished by their sedimentation coefficient, such as 28S rRNA or 5S rRNA (Alberts 1994).

Ribosome: particle composed of ribosomal RNAs and ribosomal proteins that associates with messenger RNA and catalyzes the synthesis of protein (Alberts 1994).

Ribonucleic Acid: polymer formed from covalently linked ribonucleotide monomers (Alberts 1994).

Total Peripheral Resistance: the resistance of the entire systemic circulation (Myers 1996).

Transcription: copying of one strand of DNA into a complementary RNA sequence by the enzyme RNA polymerase (Alberts 1994).

Transcription factor: term loosely applied to any protein required to initiate or regulate transcription in eukaryotes. Includes both gene regulatory proteins as well as the general transcription factors (Alberts 1994).

Translation: process by which the sequence of nucleotides in a messenger RNA molecule directs the incorporation of amino acids into protein. It occurs on a ribosome (Alberts 1994).

Abbreviations of Terms

AHI	Apnea/Hypopnea Index
Ang II	Angiotensin II
BE	Big Endothelin
cDNA	Complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
ECE	Endothelin Converting Enzyme
ECG	Electrocardiogram
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
EPI	Epinephrine
ET-1	Endothelin-1
GXT	Graded Exercise Test
HPLC	High Performance Liquid Chromatography
HRV	Heart Rate Variability
mRNA	Messenger Ribonucleic Acid
nCPAP	Nasal Continuous Positive Airway Pressure
NE	Norepinephrine
NO	Nitric Oxide
O_2	Oxygen
OSA	Obstructive Sleep Apnea
PCR	Polymerase Chain Reaction
PSG	Polysomnography

RDI Respiratory Disturbance Index

RNA Ribonucleic Acid

RPE Ratings of Perceived Exertion

RT Reverse Transcriptase

RXT Ramped Exercise Test

VO_2 Oxygen Consumption ($L \bullet min^{-1}$)

VO_{2pk} Peak Oxygen Consumption ($L \bullet min^{-1}$)

Chapter II

Review of Literature

Introduction

Obstructive Sleep Apnea (OSA) is a wide-ranging disorder that encompasses multiple behavioral and biological systems. This review is subsequently organized to introduce the pathophysiology, risk factors, and consequences of OSA from a general view. Following background material, the focus shifts to the cardiovascular consequences of OSA. In particular, the vital role of the vasodilator substance NO and the vasoconstrictor ET-1 are profiled in both the physiologic and pathologic state. Finally, the potential utility of exercise training is discussed as it relates to both OSA treatment and as a means of altering vascular function through the endothelin and nitric oxide systems.

Epidemiology and Scope of Health Problem

Obstructive sleep apnea is a condition characterized by repetitive respiratory cessations throughout sleep, resulting in varying degrees of systemic hypoxia and/or sleep fragmentation (McNicholas 1997). These nocturnal disturbances constitute the primary pathophysiologic event in patients. Epidemiological studies have indicated that OSA is present in approximately 2% to 4% of middle-aged adults (Young, Palta et al. 1993). However, most sufferers go unrecognized and untreated by current medical practice. As a result, potentially up to 25% of the middle aged population may have varying degrees of the disease (Henderson and Strollo 1999). Unfortunately, the societal impact of OSA, both diagnosed and undiagnosed, is difficult to assess. The direct burden on placed on healthcare services is overwhelming, as OSA patients require substantially more

treatment than normal individuals (Kryger, Roos et al. 1996). The cost associated with undiagnosed cases is even more striking, with patients experiencing over a two-fold increase in medical costs prior to diagnosis (Kapur, Blough et al. 1999).

Pathophysiology of Obstructive Sleep Apnea

Clinically, humans present with either central or OSA, or a mixture of the two. Central sleep apnea results when respiration ceases without airway obstruction due to a withdrawal of central nervous innervation (Bradley and Floras 2000). This differs fundamentally from OSA, in which airway obstruction is the primary pathophysiologic event. Increased attention to the impact of OSA has led to a numerous research avenues into various aspects of the disease. OSA is a disorder linked to considerable physiological consequences. Unfortunately, the elusive nature of the disease and lack of a definitive experimental model make detailed experimentation difficult. As a result, the exact etiology of OSA remains largely unknown.

Unlike most mammals, humans lack a rigid skeletal support for the upper airway (Suratt, Dee et al. 1983). The lack of rigid support is believed a result of the movement necessary for speech and humans therefore require additional airway support via soft tissue structures (Malhotra and White 2002). During normal respiration, negative inspiratory pressure is counterbalanced by increased tone in the muscles of the upper airway (Series 2002). This increased muscle tone reduces the tendency for pharyngeal collapse by negative pressure, or suction. In individuals without upper airway obstruction, neuromuscular activation of the upper airway is sufficient to maintain airway patency. In

OSA patients, increased genioglossus muscle activation is observed and most likely provides a compensatory mechanism to avoid airway collapse during wakefulness (Malhotra, Fogel et al. 2000; Fogel, Malhotra et al. 2001). However, sleep induces a general decrease in neural output to the respiratory muscles (Orem and Lydic 1978). Therefore, alterations in respiratory muscle tone would have dramatic effects during sleeping conditions. A general diagram of the process of obstructed breathing during sleep can be seen in Figure 2.

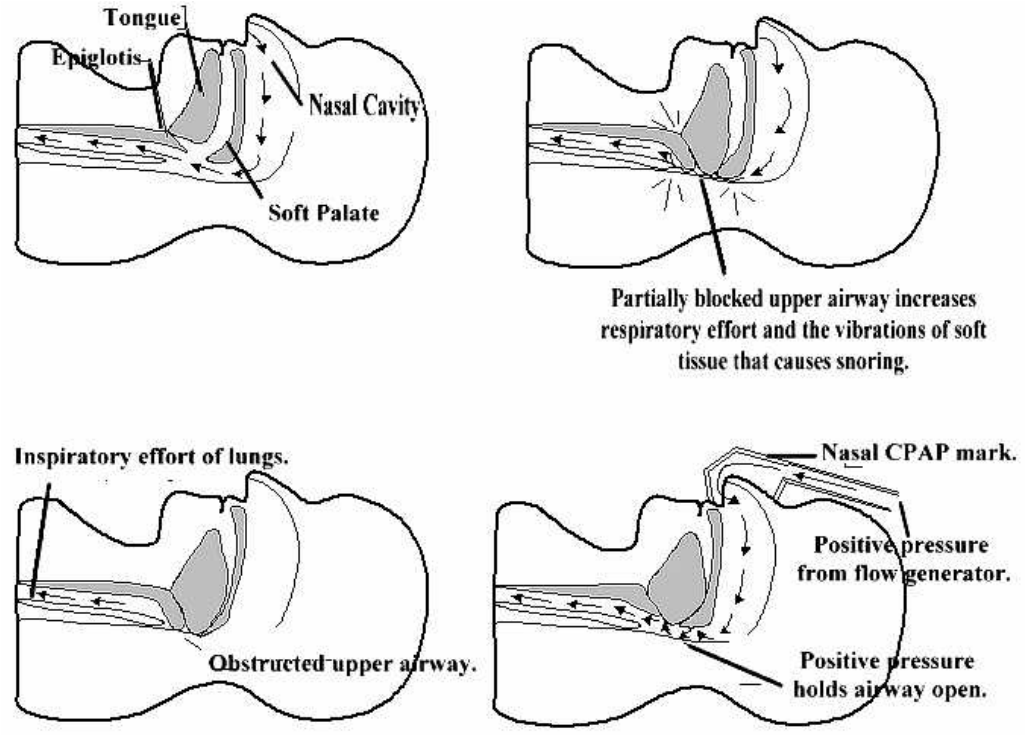

Figure 2. Airway Obstruction in Obstructive Sleep Apnea
During sleep, airway patency is maintained by the soft palate, tongue, and epiglottis. In obstructive sleep apnea patients, increased resistance and soft tissue vibrations cause the aforementioned structures to lose their support, and airway obstruction occurs. This obstruction can be avoided by application of nasal continuous positive airway pressure (CPAP) to force the airway open during periods of reduced airflow.

Airway collapse during sleep does not normally occur unless adequate insult is applied, such as in the case of alcohol consumption (>2 mg/kg) (Taasan, Block et al. 1981) or under certain types of anesthesia (Hansen-Flaschen, Brazinsky et al. 1991). In contrast, OSA patients in general experience depressed respiratory muscle activation during sleep. While the exact mechanism of this decreased respiratory muscle activity is unclear, altered pharyngeal dilator muscle structure and function (Oliven, Carmi et al. 2001; Series 2002) and both local and central neural factors (Pillar, Fogel et al. 2001) have been implicated. In OSA patients, it appears that the compensatory action of the genioglossus muscle is removed, causing a dramatic decrease in the pressure needed for airway collapse, also known as the critical closing pressure (Schneider, Boudewyns et al. 2002). This decrease in closing pressure allows the airway to easily collapse during the elevated negative pressures of inspiration. Once obstructed, hypoventilation leads to a progressive decline in blood oxygen saturation and systemic hypoxia (Findley, Wilhoit et al. 1985). Suction pressure concurrently increases in an attempt by the lungs to overcome obstruction. Both hypoxia (Remmers, deGroot et al. 1978) and suction pressure (Horner 2001) are strong stimuli for inspiration. In OSA patients these two stimuli are unfortunately insufficient to correct airway obstruction, and an arousal from sleep is needed to reset respiratory drive and restore patency (Remmers, deGroot et al. 1978). Following arousal, the patient again resumes sleep and the cycle begins anew.

Obstructive sleep apnea is officially defined by the quantity of nocturnal respiratory cessations during sleep, known as the respiratory distress index (RDI) or, more recently, the apnea/hypopnea index (AHI). Of this index, an apnea is simply defined by at least a

10 second cessation of airflow (Malhotra and White 2002). The definition of hypopnea, on the other hand, is more complicated. Recently, a consensus statement by the American Academy of Sleep Medicine states that hypopnea must include one of the following: a substantial reduction in airflow greater than 50%; a moderate reduction in airflow of (<50%) with blood oxygen desaturations of greater than 3%; or a moderate reduction in airflow (<50%) with evidence of arousal by electroencephalography (1999). Five apneic or hypopneic episodes per hour during sleep are considered the threshold for OSA and constitutes mild disease (Roux, D'Ambrosio et al. 2000). Subsequently, OSA severity rises with the quantity of events per hour. Patients experiencing between 5 and 15 events per hour are diagnosed with mild disease, a value between 15 and 30 events per hour is considered moderate, and a value greater than 30 events per hour has been designated as severe disease (Roux, D'Ambrosio et al. 2000). Due to the complicated nature of hypopneas and the need to assess respiratory effort and blood oxygen saturation, official diagnosis of OSA occurs only following overnight sleep study, known as polysomnography (PSG). PSG studies further record brain activity via electroencephalogram (EEG), eye movement via electrooculogram (EOG), muscle activity via electromyogram (EMG), and cardiac activity via electrocardiogram (ECG) to accurately diagnose and assess the presence or absence of OSA (Yamashiro and Kryger 1995; Yamashiro and Kryger 1995; Skomro and Kryger 1999).

Risk Factors for Obstructive Sleep Apnea

Nocturnal reductions in respiratory dilator muscle activity occur in all individuals, regardless of the presence of OSA. However, a large proportion of the population does

not experience airway collapse. Therefore, additional factors are necessary for OSA to develop in certain individuals. A common characteristic of all OSA patients is the presence of habitual snoring. Individuals who snore are at increased risk of the development of OSA (Ohayon, Guilleminault et al. 2000). Conservative estimates predict approximately 40% of the general population may snore, although differences are noted among groups. In a college-aged population, it has been reported that up to 25% of students may exhibit chronic snoring, while only 0.1% had a positive diagnosis of OSA (Hui, Chan et al. 1999). Among commercial bus drivers, snoring was noted in 37% of study participants and was considered an independent risk factor for the development of OSA (Hui, Chan et al. 2002). Community based PSG follow-up reveals between 1% and 5% of snorers experience OSA (Ohayon, Guilleminault et al. 2000). Unfortunately, the presence of snoring does not necessarily indicate OSA (Teculescu 1998) and may be dependent on body posture (Makofsky 1997). Consequently, it remains to be seen whether chronic snoring leads to or simply worsens sleep apnea (Teculescu 1998).

In concert with snoring, OSA patients commonly exhibit a narrowed upper airway (Haponik, Smith et al. 1983). A definitive example of the narrowed airway present in OSA patients deals with children. In a recent study (Arens, McDonough et al. 2001), the volume of the airway was 40% smaller in children with OSA than among those without OSA. Significant reductions in airway size are also seen in adults, although distinct differences in adults and children occur (Schwab, Gupta et al. 1995). In children, airway reduction results primarily from a greater adenoid and tonsil area (Arens, McDonough et al. 2001), whereas adults tend to demonstrate a general thickening of the

lateral pharyngeal walls secondary to fat deposition (Schwab, Gupta et al. 1995; Mortimore, Marshall et al. 1998). Nonetheless, OSA patients experience a dramatic decrease in overall airway size regardless of the prevailing pattern of tissue deposition (Schwab, Gupta et al. 1995).

As stated previously, fat deposition in the upper airway causes a general narrowing of the airway. In fact, the volume of fat in the lateral pharyngeal walls positively correlates with OSA severity (Shelton, Gay et al. 1993; Shelton, Woodson et al. 1993). OSA patients in general exhibit increased global fat deposition, with upwards of 70% classified as obese (Malhotra and White 2002). Further, the degree of obesity positively relates to the severity of OSA (Friedman, Tanyeri et al. 1999). Even more striking is the observation that recent weight gain occurs prior to diagnosis of OSA (Phillips, Hisel et al. 1999). Naturally, weight loss may theoretically alleviate the degree of respiratory cessations in OSA patients. Reductions in disease severity in fact do occur following weight loss in OSA patients, although complete restoration of airway control normally does not occur(Strobel and Rosen 1996; Goldberg 2000).

Specifically, fat accumulation in the neck region creates an increased workload on the respiratory dilator muscles to maintain airway patency (Benumof 2001). Direct evidence for the role of upper airway fat accumulation is easily observed by the application of external compression weight to an animal model of disease. In this instance, animals with the greatest amount of external fat weight experienced the greatest increase in airway resistance (Koenig and Thach 1988). These results are similar to those seen in

humans, with the incidence of OSA severity more directly correlated with neck circumference than with general obesity (Davies and Stradling 1990). Clinically, a neck circumference greater than 17 inches is a predictor of OSA (Gregg, Zedalis et al. 2000).

Other predictors for the presence of OSA include both inherent and modifiable factors. Of the inherent factors, disease diagnosis is six-fold greater in men than in women (Redline, Kump et al. 1994), although in the general population it appears that difference is only approximately two-fold. OSA is more prevalent with increasing age (Young, Peppard et al. 2002), although a survivor effect in older people may skew this finding (Lavie, Herer et al. 1995). Recently, it has also been shown that race is an independent predictor of OSA, with blacks experiencing an increased incidence compared to whites (Meetze, Gillespie et al. 2002). Alcohol consumption and tobacco use are modifiable risk factors in the development of OSA (Malhotra and White 2002). A table of risk factors and their relative magnitude in disease development is summarized in Table 1.

Table 1. Risk Factors for the Development of Obstructive Sleep Apnea

Risk Factor	Magnitude	Possible Mechanism
Male Sex	+ +	Anatomy, vent control
Age	+ +	Anatomy, neural reflex impairment
Obesity	+ + +	Anatomy, stability of airway
Menopause	+	Unknown
Black Race	+	Possibly anatomy
Alcohol	+ +	Impaired dilator muscle activity
Smoking	+	Airway inflammation, edema

Modified from Malhotra and White, 2002

Consequences of Obstructive Sleep Apnea

The most common clinical outcome of OSA is excessive daytime sleepiness (Resta, Foschino-Barbaro et al. 2001). In fact, OSA sufferers rate diurnal symptoms of sleepiness as the most significant disability (Lacasse, Godbout et al. 2002). Further, workers affected by OSA complain of excessive daytime sleepiness and physical problems that impact daily activities (Lavie 2002). These subjective values of daytime sleepiness in turn exhibit a strong relationship to workplace accidents (Lindberg, Carter et al. 2001). Unfortunately, the degree of daytime sleepiness does not always correspond to OSA severity (Sauter, Asenbaum et al. 2000). It is commonly believed that excessive daytime sleepiness is attributed to non-restorative sleep caused by the numerous nocturnal arousals observed in OSA patients. However, patients experience differing degrees of sleep fragmentation and varied changes in sleep architecture (Stepanski 2002). Therefore, sleep deprivation can not be considered the sole reason for the excessive daytime sleepiness in OSA patients.

Aside from direct medical costs and daytime functioning, OSA is associated with numerous societal and physiological consequences. One of the most important societal implications of nocturnal respiratory cessation is roadway safety. OSA patients are at increased risk of vehicular accidents than the general population (Shiomi, Arita et al. 2002; Vorona and Ware 2002). If fact, study has shown that patients are over six times more likely to be involved in a motor vehicle accident than non-apneic individuals regardless of alcohol consumption, visual impairments, body type, driving experience, age, traffic history, use of medications causing drowsiness, and sleep schedule (Teran-

Santos, Jimenez-Gomez et al. 1999). The most probable explanation for this increased risk may be excessive daytime sleepiness secondary to sleep fragmentation. Direct evidence for the role of OSA in vehicle accidents indicates that patients experience a gradual deterioration of driving capacity under simulated conditions lasting 60 minutes (Risser, Ware et al. 2000). Similar findings are observed in subjects under sleep deprived conditions (Hack, Choi et al. 2001). Subsequently, subjective measures of daytime sleepiness demonstrate a positive relationship with accident rate (Lloberes, Levy et al. 2000). Coincidently, proper OSA treatment has been shown to enhance driving ability (George 2001). Undiagnosed OSA may therefore contribute to the high incidence of unexplained vehicle accidents (Fuchs, McMaster et al. 2001). Ultimately, failure to properly diagnose and treat OSA may have dire consequences in overall public safety.

The impact of OSA on healthcare utilization and public safety has been well established. However, one cannot consider the full impact of OSA without noting its numerous physiological consequences. OSA patients exhibit noticeable morphological differences in brain structure (Macey, Henderson et al. 2002) as well as behavioral and cognitive impairment (Beebe and Gozal 2002) compared to normal individuals. Even in children, sleep disordered breathing and snoring result in quantifiable alterations in both behavior and learning ability (Owens, Opipari et al. 1998; O'Brien and Gozal 2002). Systemically, OSA is associated with an increased incidence of diabetes (Elmasry, Lindberg et al. 2001), and patients in general display increased glucose resistance over non-diseased individuals (Stoohs, Facchini et al. 1996; Punjabi, Sorkin et al. 2002). While its role in the development of diabetes and behavior and neurological function are a subject of

continued study, the most common consequence of sleep disordered breathing involves impaired vascular function and cardiovascular disease. To this end, OSA has been extensively linked to pulmonary hypertension (Weitzenblum, Krieger et al. 1988; Marrone and Bonsignore 2002; Yamakawa, Shiomi et al. 2002), cerebrovascular complications (Wessendorf, Teschler et al. 2000; Franklin 2002; Hermann and Bassetti 2003), and most notably, hypertension (Lavie, Herer et al. 1995; Peppard, Young et al. 2000; Bananian, Lehrman et al. 2002; Peker, Hedner et al. 2002).

Cardiovascular Impairment in Obstructive Sleep Apnea

Over half of OSA patients have systemic hypertension (Hla, Young et al. 1994) and OSA is widely recognized as an independent risk factor in the development of hypertension (Peppard, Young et al. 2000; Peker, Hedner et al. 2002). Conversely, approximately 25% of hypertensives were diagnosed with OSA by subsequent PSG (Fletcher, DeBehnke et al. 1985). In fact, the stresses placed on the cardiovascular (CV) system by OSA have lead to the discovery of a CV mortality rate over three and a half times that of the general population between the fourth and fifth decades of life (Lavie, Herer et al. 1995). Overall, OSA >10 episodes per hour is associated with considerable CV mortality in both men and women under the age of sixty regardless of body habitus (Lavie, Herer et al. 2003). This is especially evident in obese women with OSA, which experience approximately an 18-fold increase in CV mortality over the general population. Direct cause for OSA-related hypertension could be either central or peripheral in nature, and evidence for both cases exists. Increased hypertrophy (Kraiczi, Peker et al. 2001) and impaired diastolic filling (Kraiczi, Caidahl et al. 2001) of the left

ventricle are present in patients regardless of hypertensive status. Recent study indicates that both systolic and diastolic function are impaired in response to hypoxic insult and that function can be partially restored by proper treatment (Alchanatis, Tourkohoriti et al. 2002). OSA patients also experience a reduction in cardiac cycle variability between the R-waves of successive myocardial contractions, commonly known as heart rate variability, compared to normal controls (Narkiewicz, Montano et al. 1998; Penzel, Bunde et al. 2000). Based on detailed study in both humans and animals, a plausible hypothesis of the pathophysiology of cardiovascular impairment in OSA has been proposed (Figure 3).

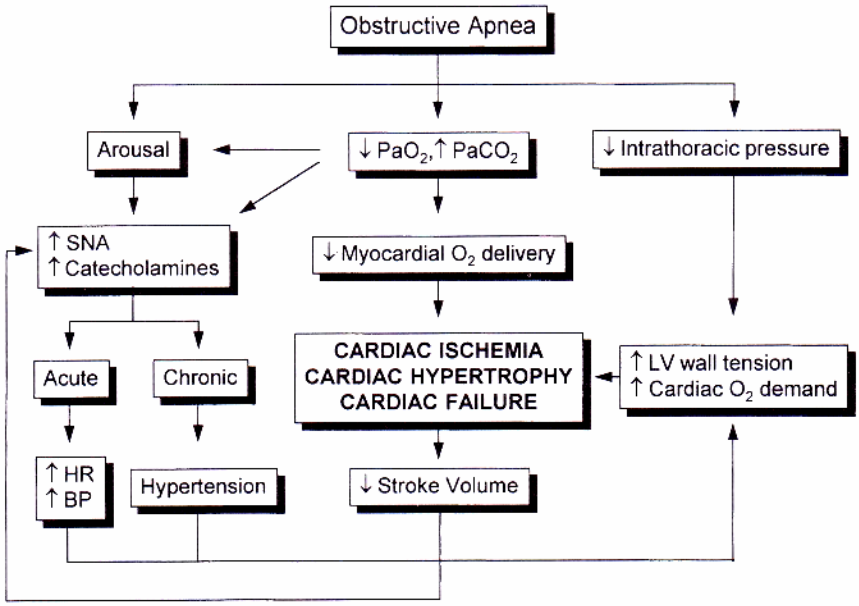

Figure 3. Proposed Mechanism of Cardiovascular Impairment in Obstructive Sleep Apnea
Obstructive sleep apnea affects the cardiovascular system through three major pathways, arousal, hypoxia, and decreased intrathoracic pressure. Through these three mechanisms, increased sympathetic activity, decreased myocardial oxygen delivery, and increased left ventricular wall tension and oxygen demand lead to lead to cardiac ischemia, hypertrophy and failure (figure from Bradley and Flores, 2000).

In spite of evidence of a central mechanism for cardiovascular impairment, the argument can also be made for a peripheral cause of hypertension manifested by vascular dysfunction. OSA patients experience significant reductions in vascular reactivity in both arterial (Anand, Remsburg-Sailor et al. 2001; Kraiczi, Caidahl et al. 2001) and venous (Duchna, Guilleminault et al. 2000) vessels. As with cardiac function, successful treatment partially restores vascular function in patients (Imadojemu, Gleeson et al. 2002). Evidence also exists for decreased vascular production of vasodilators such as nitric oxide (NO) (Kato, Roberts-Thomson et al. 2000) and enhanced sensitivity to vasoconstrictors such as endothelin (ET-1), epinephrine (EPI), norepinephrine (NE), and angiotensin II (AngII) (Kraiczi, Hedner et al. 2000). Unfortunately, the exact mechanism by which OSA leads to vascular dysfunction is incompletely understood. Impaired cardiovascular regulation in patients is believed to occur following years of intermittent hypoxia during sleep, making prospective study in humans difficult. Opportunely, the many similarities between rodents and humans in cardiovascular response allow for detailed study in animal models (Fletcher 2001).

Normal sleep patterns result in a progressive drop in both systemic blood pressure and cardiac output during non-rapid eye movement (REM) sleep (Roux, D'Ambrosio et al. 2000). The drop in blood pressure is commonly called “dipping.” In contrast, OSA patients do not experience nocturnal reductions in blood pressure compared to normals, regardless of hypertensive status (Suzuki, Guilleminault et al. 1996). As a result, OSA patients are sometimes referred to as “non-dippers.” A possible explanation of the “non-dipper” phenomenon is a sympathetic surge in response to arousal. As stated previously,

neuromuscular tone of the respiratory dilator muscles is reset by a surge in sympathetic neural discharge following an apneic/hypopneic episode (Yoon and Jeong 2001). This sympathetic surge leads to brief episodes of vasoconstriction and transient hypertension (Tilkian, Guilleminault et al. 1976).

Evidence for the proposed sympathoexcitation in humans can be seen in elevations of both EPI (Eisensehr, Ehrenberg et al. 1998) and NE (Baylor, Mouton et al. 1995) plasma levels following arousal episodes. Aggregate measures of daily catecholamine release are also elevated in the urine of OSA patients (Dimsdale, Coy et al. 1995). Direct experimental evidence corroborates the role of sympathetic activation in OSA patients. Rats exposed to 35 days of intermittent hypoxia develop hypertension in the laboratory setting (Fletcher 2000) similar to that of arousals in humans. Like humans, these animals display elevated levels of plasma EPI (Fletcher, Bao et al. 1999). Interestingly, hypertension does not occur in animals where EPI is removed through adrenal demedullation and renal artery demedullation (Bao, Metreveli et al. 1997) or in the event of catecholaminergic receptor blockade (Kirby, Pinto et al. 1995).

This stimulus has been known to also interfere with the renin-angiotensin system (Fletcher, Bao et al. 1999) as well as vasoactive substances such as ET-1 and NO (Tahawi, Orolinova et al. 2001), indicating that increased catecholamines are not the sole determinant of altered cardiovascular regulation.

Role of Nitric Oxide in Vascular Function

A proposed mechanism of chronic hypertension involves endothelial dysfunction and increased vascular tone (Tahawi, Orolinova et al. 2001). This is supported by a predominance of vasoconstrictive influences, suppression of the NO system, functionally increased vascular resistance and a reduced ability to respond to hemodynamic stress (Hedner 1996). Under physiologic conditions, the vascular endothelium plays a key role in modulating blood pressure, vascular smooth muscle proliferation, platelet adhesion and aggregation, coagulation, and monocyte adhesion (Luscher and Noll 1995). A primary means by which the vascular endothelium exerts its effects is through NO. In the systemic vasculature, resting tone is maintained by basal release of NO (Li and Forstermann 2000), and blockade of NO synthesis subsequently leads to profound systemic vasoconstriction (Aisaka, Gross et al. 1989; Gardiner, Compton et al. 1990).

NO is a relatively stable reactive species created in the conversion of the amino acid L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS) (Maxwell 2002) and is normally produced in response to the substances bradykinin, ET-1, circulating catecholamines, and acetylcholine (Luscher and Noll 1995) and by vascular shear stress (Fisher, Al-Mehdi et al. 2002). In endothelial cells, NO is produced constitutively to maintain vascular tone by the enzyme endothelial NOS (eNOS). The importance of this enzyme is apparent in animals deficient in the eNOS gene. These animals develop hypertension and exhibit an impaired vasodilatory capacity (Huang, Huang et al. 1995). Under times of cellular stress, inducible NOS (iNOS) is expressed to increase NO production. Vascular smooth muscle and neuronal cells also express the constitutive

isoform neuronal NOS (nNOS) (Stuehr 1999). Once formed, NO gas diffuses rapidly into surrounding tissue and nitrosylates free thiol groups of cysteine amino acid residues, causing conformational protein changes (Gaston 1999). These conformational changes are especially important in vascular smooth muscle cells, where the enzyme soluble guanylate cyclase facilitates vasodilation (Koesling and Friebe 1999). Mechanistically, conformational modification of this enzyme activates a number of protein kinases and decreases intracellular concentrations of calcium, resulting in vasodilation (Gewaltig and Kojda 2002).

Since the discovery of NO as endothelial derived relaxing factor in 1987 (Palmer, Ferrige et al. 1987), literally thousands of research articles have been published regarding the role of NO in hypertension. In this wealth of available information, it has become apparent that blockade of NO synthesis leads to hypertension. Pharmacologically, use of the L-arginine analog N^{ω}-nitro-L-arginine methyl ester (L-NAME) inhibits the formation of NO by competing with L-arginine. Exogenous administration of L-NAME consequently leads to profound vasoconstriction (Aisaka, Gross et al. 1989) and elevations in resting blood pressure (Stamler, Loh et al. 1994; Gewaltig and Kojda 2002). Further evidence for the pivotal role of NO in vascular function stems from findings that mice lacking the constitutive eNOS gene exhibit hypertension (Huang, Huang et al. 1995). Dietary reductions in the substrate L-arginine also lead to decreased NO production (Wu, Flynn et al. 1999). Dietary supplementation of L-arginine has been shown to improve flow-mediated dilation in humans (Lekakis, Papathanassiou et al. 2002), prompting many to

believe increasing L-arginine intake may be a simple means of treating essential hypertension (Maxwell 2002).

Unfortunately, large scale clinical trials on NO deficiency in humans have been limited by the transient nature of this gaseous signal molecule. NO has a short half-life of approximately 3 seconds (Palmer, Ferrige et al. 1987), making direct *in vivo* assessment difficult. It therefore becomes necessary to sensitively measure NO production through alternative means. In aqueous solutions, NO is rapidly broken down into the metabolite nitrite, which is further broken down into nitrate in the presence of hemoglobin (Kelm 1999). Therefore, it has been proposed that serum nitrate and nitrite concentrations may be a sensitive marker of systemic NO production (Kelm, Preik-Steinhoff et al. 1999). Essential hypertensives experience depressed urinary excretion of both nitrates and nitrites with a positive relationship to resting blood pressure (Forte, Copland et al. 1997). Because dietary intake can affect excretion, control of nitrate ingestion in this study indicates a reduction in basal NO synthesis. In concert with urinary excretion, aberrant plasma levels of NO metabolites has also been found (Sagnella, Markandu et al. 1997).

Role of Endothelin in Vascular Function

To counterbalance the vasodilatory properties of NO, endothelial cells have the ability to synthesize powerful vasoconstrictors when necessary. A prominent vasoconstrictor substance implicated in the cardiovascular impairment of OSA is ET-1 (Lavallee, Takamura et al. 2001). ET-1 is a 21 amino acid peptide encoded on chromosome 6p23-24 (Webb, Monge et al. 1998). ET-1 is primarily produced in response to elevated blood

pressure, Ang II, vasopressin, and low density lipoprotein cholesterol (Hunley and Kon 2001). Because no appreciable intracellular stores have been found, ET-1 regulation occurs primarily at the transcription level in the form of the mRNA preproendothelin (Wagner, Christ et al. 1992). Genetically, the 3' untranslated region contains 2 AUUUA sequences that mediate selective breakdown and contribute to a short half life of the preproendothelin mRNA (Inoue, Yanagisawa et al. 1989). Functional ET-1 is formed by the catalytic cleavage of the precursor big endothelin (BE) by endothelin converting enzyme (ECE). The conversion of preproendothelin to active ET-1 is illustrated in Figure 4. BE itself has mild vasoconstrictory properties, albeit approximately 1% that of ET-1 (Pacher, Bergler-Klein et al. 1993).

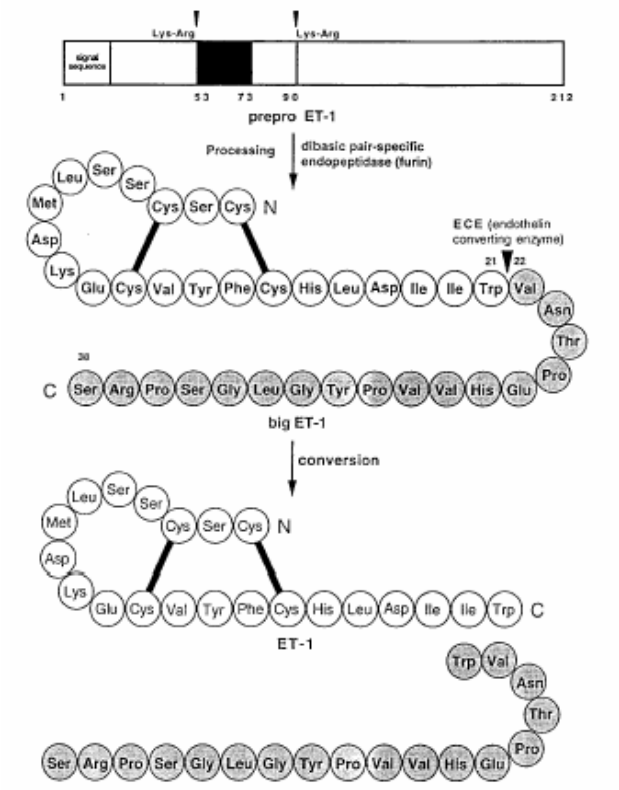

Figure 4. Biosynthesis of Endothelin-1
Production of bioactive endothelin-1 occurs following the cleavage of big endothelin by endothelin converting enzyme (Hunley and Kon 2001).

The actions of ET-1 are mediated by a cell surface receptor of which there are two general subtypes: ET-1_A and ET-1_B. ET-1_A receptors are found primarily on vascular smooth muscle cells, where they facilitate vasoconstriction (Arai, Hori et al. 1990). ET-1_B receptors locate both on vascular smooth muscle cells and on the luminal side of the endothelium, where they exert respective constrictor and dilator functions (Masaki, Miwa et al. 1999). The subcellular mechanism of ET-1 action is primarily through regulation of intracellular calcium levels by the second messenger cyclic adenosine monophosphate (cAMP), with constriction coupled to increases in cAMP and calcium and dilation promoted by decreased levels (Rubanyi and Polokoff 1994). Clearance and inactivation of mature ET-1 occurs primarily through the ET-1_B receptor subtype (Burkhardt, Barton et al. 2000).

Under physiologic conditions, low levels ET-1 released by vascular endothelial cells into the circulation promote vasodilation through stimulation of the NO pathway in an autocrine or paracrine fashion (Bassenge 1995). Further, study has shown that blockade of both ET-1 receptor types initially causes peripheral vasoconstriction (Haynes, Ferro et al. 1996). In this manner, endothelin appears to contribute to basal vascular tone and blood pressure along with NO. The role of ET-1 in basal vascular tone can be seen in Figure 5. Under physiological stress, such as exercise, blood flow is actively redistributed from the non-working to working tissues in a manner not completely understood. However, recent evidence has pointed to the paramount role of ET-1 in this process. In the rat model, regional blood flow in the internal organs was significantly increased following pharmacologic blockade of the ET-1_A receptor (Maeda, Miyauchi et

al. 2002). Elevations in renal preproendothelin mRNA was also demonstrated following acute exercise stress (Maeda, Miyauchi et al. 1998). Because ET-1 expression is normally regulated at the genetic level, transcriptional increases of preproendothelin indicate enhanced production of mature ET-1. Evidence in human subjects has also illustrated a positive relationship between exercise tolerance and the amount of ET-1 found in the circulation (Tanabe, Yamamoto et al. 2000).

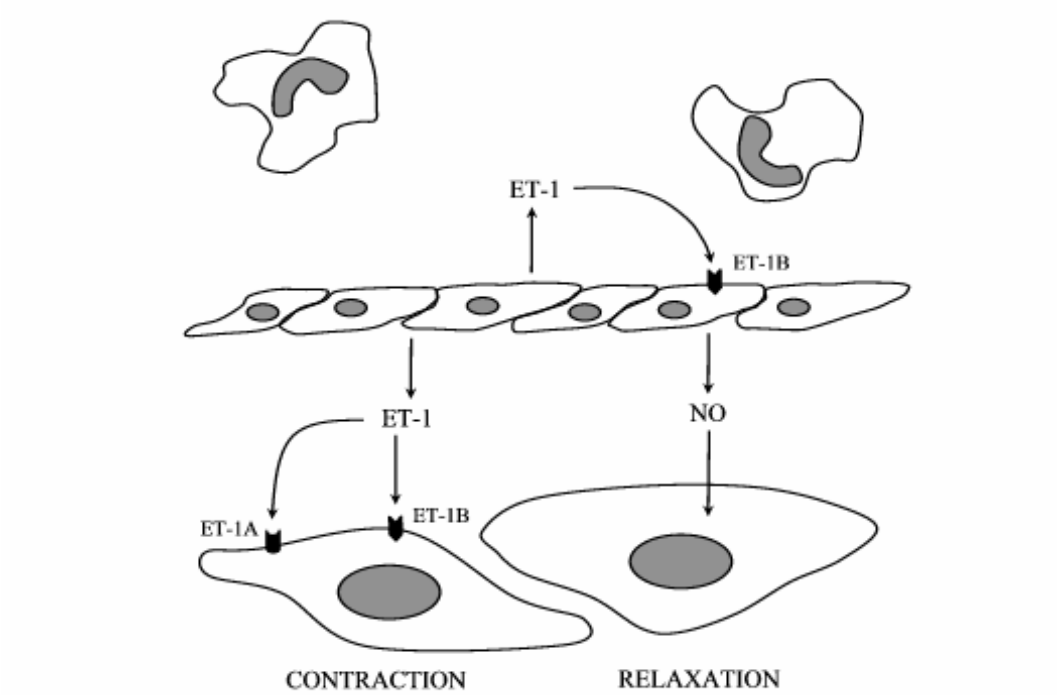

Figure 5. Role of Endothelin-1 in Basal Vascular Tone
Endothelin is predominately released into the vascular cell wall where it facilitates vascular smooth muscle cell contraction via the type A and B receptors. Small amounts of endothelin are acutely released into the vessel lumen where binding to the B receptor leads to the production of nitric oxide causes vasodilation. Nitric oxide in turn inhibits further release of endothelin. Ultimately, acute release of endothelin leads to vasodilation and contributes to basal vascular tone.

Under pathophysiologic conditions such as essential hypertension, excess plasma ET-1 production induces marked vasoconstriction and increased blood pressure (Cardillo, Kilcoyne et al. 1999). Borderline hypertensive patients experience elevated levels of ET-1 in the circulation (Lemne, Lundeberg et al. 1994). Consequently, 4 wk of treatment with the ET-1 receptor antagonist bosentan significantly decreased resting blood pressure in essential hypertensives (Krum, Viskoper et al. 1998). Coronary artery disease patients also demonstrate increased vascular smooth muscle cell production of preproendothelin and ECE, which converts the precursor BE into the biologically active form ET-1 (Rossi, Colonna et al. 1999). Plasma levels also may be indicative of the degree of ischemia experienced in unstable coronary patients (Lubov, Marmor et al. 2001). The most notable example of exaggerated ET-1 levels is in heart failure, where profound elevations in plasma levels positively correspond to disease severity (Margulies, Hildebrand et al. 1990; Lerman, Kubo et al. 1992). Unfortunately, the functional importance of naturally occurring plasma ET-1 in essential hypertension is poorly understood, as conflicting studies indicate a positive relationship between plasma levels and blood flow or no role at all (Schmitz-Spanke and Schipke 2000).

During physiologic stress, the functional difference in ET-1 production between normotensives and hypertensives widens. Prior to exercise, forearm vasodilation in response to the ET-1_A receptor antagonist was identical in both normal and hypertensive subjects (McEniery, Wilkinson et al. 2002). During handgrip exercise, infusions of ET-1_A receptor antagonist did not alter vasodilation. However, patients experienced significantly greater vasodilation in response to identical doses of ET-1_A antagonist,

indicating a greater influence of plasma ET-1 in the hypertensive state. In heart failure, exaggerated increases in ET-1 were seen both prior to and following treadmill exercise (Yousufuddin, Shamim et al. 2000). However, serial measurements during leg ergometry exercise indicate a delay in ET-1 release (Ishikawa, Miyauchi et al. 1998). This blunted ET-1 response could be a possible explanation for the exercise intolerance experienced by heart failure patients. Theoretically, elevated plasma ET-1 may enhance systemic vasoconstriction in response to stress, thereby acting as an early compensatory mechanism in heart failure (Best and Lerman 2000). Further investigation is needed to clarify the role of ET-1 in both cardiovascular disease and stress-induced circulatory redistribution.

Production of ET-1 was originally believed to derive from the vessel itself in an effort to control vascular tone. However, in 1994 it was discovered that circulating mononuclear cells activated during acute long allograft rejection can cause vasoconstriction in endothelium-denuded pulmonary vessels (Cale, Recagna et al. 1994). Administration of an L-arginine analogue subsequently abolished vasoconstriction indicating that NO produced from an intact, healthy endothelium would inhibit the activity of activated mononuclear cells. Conversely, administration of an $ET\text{-}1_A$ antagonist reduced vasoconstriction, implicating ET-1 as the culprit in this pathologic state. In addition to canine models, human trials have indicated that circulating leukocytes contribute to vascular tone during activated states. Chronic immunologic activation is commonly observed in heart failure patients as well as elevated levels of plasma ET-1. In a novel idea, mononuclear cells from heart failure patients were isolated and cultured *in vitro*.

Compared to mononuclear cells from normal individuals, mononuclear cells from heart failure patients spontaneously produced physiologically relevant levels of ET-1 (Krum and Itescu 1994). The possible contribution of circulating leukocytes to plasma ET-1 levels was also seen in dialysis patients (Ebihara, Nakamura et al. 1997), a condition characterized by chronic immunologic activation. Subsequent investigations have verified that ET-1 is synthesized by leukocytes in the presence of low density lipoproteins (Haug, Schmid-Kotsas et al. 2001) and inflammatory proteins such as tumor necrosis factor alpha (TNF-α) (Cambiaggi, Mencarelli et al. 2001). While no definitive evidence has been established, it appears that chronic immunologic activation may occur in OSA patients (Yokoe, Minoguchi et al. 2003). Further, documented elevations in TNF-α have been shown in diagnosed OSA patients that strongly correlate with disease severity (Liu, Liu et al. 2000). Based on this finding, the potential contribution of circulating leukocytes to plasma ET-1 levels can not be discounted in diseases where immunologic activation may be present, including OSA.

Nitric Oxide and Endothelin Dysfunction in Obstructive Sleep Apnea

As stated previously, OSA patients experience significant cardiovascular co-morbidity and impaired vascular function. While there is ample evidence for the role of both NO and ET-1 in vascular control, their respective importance in OSA is incompletely understood. However, the considerable crosstalk between the two vasoactive substances warrants their concomitant discussion (Lavallee, Takamura et al. 2001). Physiologically, ~80% of endothelin is released abluminally into the vascular wall (Wagner, Christ et al. 1992). The ~20% of ET-1 released into circulation principally binds to ET-1_B receptors

to induce NO release and vasodilation. Subsequently, newly released NO inhibits further ET-1 secretion (Boulanger and Luscher 1990). Under normal conditions, acute vascular shear stress increases NO production and reduces ET-1 release and activity. However, prolonged shear stress, as would occur in conditions of decreased NO synthesis, enhances ET-1 synthesis (Lavallee, Takamura et al. 2001). Under NO blockade, exogenous administration of ET-1 to double normal levels increased vascular resistance three to fourfold over normal NO conditions (Lerman and Burnett 1992). In OSA, the proposed mechanism of vascular dysfunction is a reduction in NO production, with observable decreases in serum NO metabolites seen during untreated disease (Ip, Lam et al. 2000). Therefore, plasma levels of ET-1 would become more important in vascular control in this disease state.

Evidence for diminished NO production and activity in OSA has been demonstrated in both human and animal models. In the rat model, 35 days of exposure to 8 hr/day of intermittent hypoxia results in diurnal blood pressure elevation. In these animals, NO synthesis blockade by L-NAME resulted in attenuated vasoconstriction versus controls (83% of baseline vessel diameter vs. 63% of baseline vessel diameter, respectively) (Tahawi, Orolinova et al. 2001), indicating a reduced basal release of NO. Human trials have established that NO metabolite levels are depressed in OSA patients. In a recent study, it was determined that OSA directly causes a reduction in serum nitrate and nitrite that is promptly reversed following successful therapy (Schulz, Schmidt et al. 2000). Long term follow-up of this group revealed that the marked improvement in NO production can be maintained by habitual nCPAP therapy. Unfortunately, this study did

not control for body habitus or cardiovascular disease status. Although the results of the aforementioned investigation are subject to discussion, other research has verified the general statement of NO dysfunction in OSA. Moderate to severe OSA patients experience decreased serum nitrate and nitrite concentrations following one night of untreated OSA that negatively correlated to disease severity, oxygen desaturation, and systolic blood pressure (Ip, Lam et al. 2000). Following successful nCPAP therapy, NO metabolite levels partially returned to non-diseased levels. By controlling for age and body habitus in this study, the claim can be made for an independent effect of OSA on overall NO production. Functionally, OSA patients experience reduced forearm vasodilation in response to Acetylcholine, a known stimulus for NO release (Kato, Roberts-Thomson et al. 2000). Exogenous administration of a NO donor revealed no differences in vascular reactivity between control and OSA subjects. This finding indicates the inherent ability of the endothelium to produce NO is impaired while the signaling pathway remains intact.

Concurrent with a decrease in NO production, OSA patients appear to exhibit an enhanced production of vasoconstrictors, namely ET-1. The first published study implicating ET-1 dysfunction in OSA revealed increased plasma ET-1 levels in OSA patients versus controls regardless of hypertensive status (Saarelainen, Seppala et al. 1997). Successful treatment of these patients did not yield appreciable differences in circulating peptide levels. Elevated ET-1 production was confirmed during serial blood sampling in untreated OSA patients. Four hours of untreated disease resulted in significant increases in bioactive ET-1 that positively related to mean arterial pressure

and the degree of oxygen desaturation (Phillips, Narkiewicz et al. 1999). No relationship between mean arterial pressure and ET-1 was observed in controls. During the split-night approach employed in this study, 5 hours of nCPAP therapy successfully reduced both mean arterial pressure and plasma ET-1 levels. However, recent evidence has surfaced to shed doubt on the potential role of ET-1 in OSA. In a study of 29 male OSA patients, similar plasma ET-1 levels were observed in comparison to controls (Grimpen, Kanne et al. 2000). Further, plasma ET-1 levels did not correspond to either disease severity or 24 hour blood pressure measurements. Both short and long term follow-up revealed no variation in ET-1 production. Unfortunately, this study group utilized hypertensive controls as well as medications that may affect ET-1 synthesis (e.g. Angiotensin Converting Enzyme Inhibitors). Therefore, more study is needed to assess the purported importance of ET-1 in the cardiovascular control of OSA patients.

Exercise and Obstructive Sleep Apnea

The harmful effects of OSA are commonly reversed through standard first-line therapy, namely nCPAP. However, many of the favorable changes seen following therapy are negated by poor compliance over time and viable low cost alternatives are needed to enhance long-term treatment outcomes. The addition of regular aerobic physical activity to standard treatment for OSA would be of great interest as it may promote favorable alterations in vascular function and ET-1 production. Recently, one study has demonstrated dramatic improvements in endothelial function following exercise training in heart failure patients (Hambrecht, Gielen et al. 2000). To date, three studies have attempted to examine the potential role of exercise in the treatment of OSA. The first

published report detailed a 6 month exercise program consisting of 2 sessions per week (Netzer, Lormes et al. 1997). While no improvements in oxygen consumption, body weight, lactate profile, or blood pressure occurred, there was a significant decrease in RDI, signifying a reduction in disease severity. The authors hypothesize that the potential benefit of exercise in conjunction with nCPAP therapy is an increase in respiratory muscle tone. However, no evidence supporting this hypothesis was provided and the use of a non-traditional exercise regimen could be considered suspect. Significant improvements in AHI of ~30% were also seen employing a similar exercise regimen in a group of moderate to severe OSA patients (Giebelhaus, Strohl et al. 2000). The dramatic reduction in disease severity in this study can primarily be attributed to the exercise stimulus as no improvements in sleep architecture, body habitus, blood pressure, or exercise tolerance were noted. A reduction in AHI was further observed in research utilizing a more standard exercise intervention of 3 sessions per week (Norman, Von Essen et al. 2000). Comparable to the previous investigations, no appreciable improvements in physical fitness or body weight resulted from the exercise training. Unfortunately, the results of this study are suspect in that a heterogeneous group of OSA patients was chosen without regard to nCPAP usage, medication, or dietary consultation. These three studies ultimately implicate a beneficial effect of exercise intervention as an adjunct therapy to standard nCPAP treatment. However, widely disparate group selection and exercise methods limit the impact of these findings. Measures were also not considered to explain the potential mechanism whereby exercise exerts a positive influence in disease management.

The reduction in OSA severity presents exercise training as a beneficial component of disease treatment. However, physical training may play an important role in reducing the cardiovascular impairment commonly seen in OSA patients. Animal models suggest that repetitive exercise-induced vascular shear stress, as would occur during training, increases NO substrate uptake, enhances eNOS activity and expression, and upregulates antioxidant mechanisms that prevent premature NO breakdown (Gielen, Erbs et al. 2002). Experimentally, chronic exercise training is associated with significant increases in basal nitrate and nitrite production over sedentary controls (Vassalle, Lubrano et al. 2003). Interestingly, the increase in NO production appears to be systemic, as vasodilation of non-trained muscle tissue is similar to that of trained muscle (Green, Cheetham et al. 2002). In healthy young volunteers, 8 weeks of exercise training increased NO metabolites and reduced plasma ET-1 concentrations that persisted through 4 weeks of follow-up inactivity (Maeda, Miyauchi et al. 2001). The negative correlation between these measures indicates that activity increases NO production, while reducing circulating ET-1. Older healthy women also experience reductions in plasma ET-1 following exercise training (Maeda, Tanabe et al. 2003).

Despite the potential benefits of exercise training in altering vascular function via the ET-1 and NO pathways, few studies to date have attempted to verify the positive effects of exercise in cardiovascular disease patients. In rats, a common means of inducing heart failure is through ligation of the coronary artery. In this model, 12 weeks of exercise training partially restored flow mediated vasodilation in isolated, perfused gracilis muscle (Varin, Mulder et al. 1999). This improvement in blood flow coincided with an increase

in eNOS transcript number, which is reduced in sedentary disease rats. Exercise training also increases forearm endothelium-dependent vasodilation in hypertensive humans (Higashi, Sasaki et al. 1999). Animal models have suggest that repetitive exercise-induced vascular shear stress, as would occur during training, increases NO substrate uptake, enhances eNOS activity and expression, and upregulates antioxidant mechanisms that prevent premature NO breakdown (Gielen, Erbs et al. 2002). No studies to date have attempted to assess the role of exercise training on ET-1 production in cardiovascular disease patients.

Preliminary Research

There is a relative paucity of research demonstrating the benefits of CPAP therapy on functional performance. Initially, our laboratory discovered that 4 wk of PAP therapy reduced heart rate at a similar submaximal workload in severe OSA patients (Shifflett, Walker et al. 2001). From this information, it was hypothesized that adjunct exercise training may improve the outcomes of CPAP therapy. Following a substantial literature review, case study data and the potential efficacy of exercise training on cardiovascular co-morbidity in OSA were published in a series of review articles (Chittenden 2002; Kaleth, Chittenden et al. 2002). Based on these findings, private research funding has allowed for the continued study of exercise testing and training in OSA patients. In particular, this laboratory has presented preliminary research indicating that exercise training may improve cardiovascular autonomic function (Kaleth, Chittenden et al. 2003). This trial has further indicated that cardiopulmonary stress testing may provide a means of disease screening or assessing cardiovascular dysfunction in OSA patients

(Chittenden, Kaleth et al. 2002). Most recently, research has indicated that circulating biomarkers of vascular function may provide a simple means to evaluate cardiovascular function and treatment in conjunction with cardiopulmonary stress testing (Chittenden, Kaleth et al. 2003).

Summary

Obstructive sleep apnea is a condition manifested by repetitive episodes of apnea/hypopnea and arousal during sleep. While the exact etiology of OSA is unknown, patients experience a circadian-dependent withdrawal of neuromuscular drive to the upper airway. Predictors for the presence of OSA include both inherent factors such as snoring, pharyngeal fat deposition, facial morphology, age, gender, obesity, and race and modifiable factors such as alcohol and tobacco consumption. Patients with OSA unfortunately experience numerous social and physiological consequences, one of which is hypertension. The hypothetical link between OSA and hypertension is impaired vascular function, which is normally controlled through an intricate balance between NO and ET-1 production. Of these substances, constitutive NO production is primarily responsible for basal vascular tone, which can be rapidly altered through the actions of ET-1. Acutely, ET-1 synthesis stimulates NO production, whereas chronic production induces profound vasoconstriction. OSA is associated with a reduction in NO production that can be reversed through successful nCPAP treatment. Conversely, OSA may be associated with enhanced ET-1 production. Exercise training significantly alters both NO and ET-1 production in normal, aging individuals and a few cardiovascular disease subpopulations. However, no studies to date have attempted to determine the effect of

exercise training on NO and ET-1 production in OSA patients. Therefore, the potential utility of exercise training in modifying vascular function via the NO and ET-1 systems may have important implications in OSA patients.

Chapter IIIa. Journal Manuscript I

The Relationship between Circulating Markers of Nitric Oxide and Endothelin-1 and Hemodynamic Function in Obstructive Sleep Apnea Patients

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ABSTRACT

PURPOSE: The purpose of this study is to comprehensively assess circulatory markers of nitric oxide (NO) and endothelin-1 (ET-1) in newly diagnosed obstructive sleep apnea patients (OSA) (respiratory disturbance index = 26.3 ± 4.4 events/hr) and their relationship to hemodynamic function. The potential immune contribution to plasma ET-1 levels was investigated through measurement of preproendothelin gene expression in circulating mononuclear cells. **METHODS:** Thirteen newly diagnosed OSA patients (9 male, 4 female, age 45.0 ± 3.0 yr) were age and body mass index (BMI) matched with 4 apparently healthy control subjects (2 male, 2 female, age 44.75 ± 3.15 yr) screened for disease by a portable diagnostic system. Blood pressure was recorded over one complete day and prior to, during, and following maximal exercise testing on a cycle ergometer. Blood samples were taken prior to exercise testing and assessed for the nitrate and nitrite by HPLC and for big endothelin-1 (BE) and ET-1 by ELISA. Relative gene expression of preproendothelin-1 was measured by real-time RT-PCR. **RESULTS:** 24-hr ambulatory systolic and diastolic blood pressures were elevated in OSA patients vs. control subjects (systolic: 128.9 ± 3.8 mmHg vs. 108.8 ± 1.3 mmHg, respectively; diastolic: 97.5 ± 2.0 mmHg vs. 82.1 ± 1.9 mmHg, respectively). Patients experienced significant elevations in systolic (OSA 209.7 $\pm$ 5.7 mmHg; Control 174.5 $\pm$ 6.2 mmHg) and mean arterial pressures (OSA 125.8 $\pm$ 3.2 mmHg; Control 109.05 $\pm$ 4.5 mmHg) at peak exercise. No differences in nitrate, nitrite, or BE were noted. Plasma ET-1 concentrations were below assay detection limit. BE levels were significantly correlated with BMI in OSA patients ($r = 0.96$; $p = 0.001$) and control subjects ($r = 0.80$; $p = 0.0454$). Relative gene expression was not elevated in OSA patients. **CONCLUSIONS:** Moderate OSA is associated with elevated blood pressure that is exacerbated during exercise stress. Circulating biomarkers are not indicative of cardiovascular dysfunction in moderate OSA patients without diagnosed hypertension.

Introduction

Obstructive sleep apnea (OSA) is a disorder may affect up to 25% of the middle-aged population (Henderson and Strollo 1999). Patients exhibit recurring episodes of upper airway obstruction during sleep that decrease blood oxygen concentration (hypoxia) and are terminated by brief arousals (McNicholas 1997). This pattern fragments sleep and has numerous physiological consequences, including pulmonary hypertension (Weitzenblum, Krieger et al. 1988; Marrone and Bonsignore 2002; Yamakawa, Shiomi et al. 2002), cerebrovascular complications (Wessendorf, Teschler et al. 2000; Franklin 2002; Hermann and Bassetti 2003), and hypertension (Lavie, Herer et al. 1995; Peppard, Young et al. 2000; Peker, Hedner et al. 2002). In general, OSA patients experience a cardiovascular mortality rate over three and a half times that of the general population between the fourth and fifth decades of life (Lavie, Herer et al. 1995).

A proposed mechanism of cardiovascular dysfunction in OSA patients is chronic sympathoexcitation and increased vascular tone. Patients experience significant reductions in vascular reactivity in both arterial (Anand, Remsburg-Sailor et al. 2001; Kraiczi, Caidahl et al. 2001) and venous (Duchna, Guilleminault et al. 2000) vessels that can be reversed by continuous positive airway pressure therapy (Imadojemu, Gleeson et al. 2002). One possible mechanism for this phenomenon is a reduction in the vasodilator nitric oxide (NO). Under physiologic conditions, resting tone is maintained by the basal release of NO (Aisaka, Gross et al. 1989; Gardiner, Compton et al. 1990) and evidence exists for diminished NO production and activity in OSA (Ip, Lam et al. 2000; Kato, Roberts-Thomson et al. 2000; Schulz, Schmidt et al. 2000; Tahawi, Orolinova et al.

2001). In concert with a reduction in NO production may be an enhanced synthesis of the vasoconstrictor endothelin-1 (ET-1) in OSA patients (Saarelainen, Seppala et al. 1997; Phillips, Narkiewicz et al. 1999).

Endothelin-1 is regulated at the transcriptional level (Wagner, Christ et al. 1992) and secreted into the circulation in minute quantities with a relatively short half-life (Levin 1995). Conversely, circulatory levels of the ET-1 precursor big endothelin are relatively stable and provide a means to rapidly increase plasma ET-1 levels when needed (Plumpton, Ferro et al. 1996). Elevated big endothelin levels raise vascular resistance and blood pressure by augmenting active ET-1 concentrations (Bohm, Johansson et al. 2002). Increased levels of both ET-1 (Letizia, Barilla et al. 1996; Mangiafico, Malatino et al. 2000) and big endothelin (Pacher, Bergler-Klein et al. 1993; Hulsmann, Stanek et al. 1998) have been observed in patients with various forms of cardiovascular disease, and may be attributed to either increased synthesis or decreased clearance (Schmitz-Spanke and Schipke 2000). While the exact source is debatable, chronic immune activation may contribute to plasma ET-1 levels (Krum and Itescu 1994; Ebihara, Nakamura et al. 1997; Haug, Schmid-Kotsas et al. 2001). Functionally, plasma ET-1 has demonstrated an important role in blood flow redistribution during exercise (Maeda, Miyauchi et al. 1998; Tanabe, Yamamoto et al. 2000; Maeda, Miyauchi et al. 2002), and a positive relationship exists between exercise tolerance and the amount of ET-1 found in the human circulation (Tanabe, Yamamoto et al. 2000).

The contrasting effects of NO and ET-1 play an important role in vascular function and blood pressure regulation, and may be a primary cause of the observed cardiovascular impairment in OSA. Unfortunately, concomitant measurement of ET-1, big endothelin, and NO in OSA patients has not been attempted to date. Additionally, OSA patients may experience chronic immune activation (Yokoe, Minoguchi et al. 2003), implicating a potential role of circulating leukocytes in ET-1 production. Owing to its importance in circulatory redistribution, blood pressure assessment during exercise stress may provide valuable information on the functional importance of NO, ET-1, and big endothelin in OSA patients. The purpose of this study was to assess comprehensively the circulatory markers of NO production (through its metabolites nitrate and nitrite), ET-1, and big endothelin in newly diagnosed OSA patients. Additionally, the potential immune contribution to plasma ET-1 levels is investigated through preproendothelin gene expression measurement in circulating mononuclear cells. Cardiopulmonary exercise testing was used to profile the functional importance of NO, ET-1, and big endothelin in hemodynamic regulation.

Methods

Subjects. Thirteen patients (9 male, 4 female) were recruited from volunteers referred to the Southwest Virginia Sleep Disorders Center (SVSDC) in Christiansburg, VA and subsequently diagnosed with OSA (Respiratory Distress Index: RDI >5.0). The study protocol and informed consent was approved by the Virginia Tech IRB for Human Subjects' Research. Patients were excluded from study based on the presence of co-existing cardiovascular, respiratory, metabolic, orthopedic, musculoskeletal, and/or

neuromuscular disabilities. Further exclusion criteria included current use of anti-hypertensive medication or the presence of severe hypertension, current tobacco use, or recent history of moderately vigorous physical activity ($\geq$3 d/wk, $\geq$ 30 min/session) within 6 months. In addition, four (2 male, 2 female) apparently healthy volunteers matched for age and body mass index were recruited to serve as controls from the surrounding community. Absence of OSA was confirmed using a health history questionnaire, standardized sleep questionnaire (Epworth Sleepiness Scale), sleep-related symptom review, clearance from their primary care physician, and preliminary diagnostic screening using the Ebletta® portable diagnostic system (ResMed®, Inc., San Diego, CA) during a normal sleep cycle at the subject's home. Exclusion criteria for control subjects were identical to that of OSA patients.

Ambulatory Blood Pressure Measurement. Daytime ambulatory blood pressure was obtained via an automated digital blood pressure device (Omron HEM-705CP). According to manufacturer recommendations, multiple recordings were taken at four daily time points; morning awakening, noon, afternoon, and evening. Blood pressure was measured in a seated position after 5 min of rest. Subjects were instructed to abstain from caffeine and nicotine products and refrain from vigorous physical activity for a time period of 12- and 24-hr, respectively, prior to all measurements. Repeated recordings were taken at each time interval until 2 systolic measures were within 5 mmHg. The overall daytime mean systolic, diastolic, and mean arterial pressures were used for analysis.

Exercise Stress Testing. Maximal exercise stress testing was completed on an electronically braked cycle ergometer (CardioO$_2$®, MedGraphics, St. Paul, MN). Anthropometric measurements (height, weight, neck, waist, and hip circumference) were obtained prior to exercise testing. Resting blood pressure was taken following 2 min of seated rest. Following a 1 min warm-up period at an initial rate of 25 W, the ergometer was ramped at a rate of 15 W/min to achieve a total duration of 8-12 min. Cardiopulmonary measurements were obtained throughout the ramped exercise test (RXT). Blood pressure was acquired by manual auscultation at the antecubital fossa of the left forearm by trained personnel at baseline, 2 min, 5 min, and at 2 min intervals until test termination. Blood pressure at peak exercise was also measured prior to exercise termination. Heart rate and rhythm were assessed by continuous electrocardiographic (ECG) monitoring throughout exercise. Exercise was terminated at maximal subject effort (RPE > 17). A licensed physician or EMT was present for every test and an American College of Sports Medicine (ACSM) Certified Exercise Specialist^{SM} supervised the RXT. Test termination criteria were in accordance with American Heart Association and ACSM standards. Participants completed an active recovery phase at 25 W that was terminated upon subject request. Blood pressure was recorded at 1 min intervals until 6 min into recovery.

Blood Collection. Blood was drawn prior to RXT by antecubital venipuncture with the subject in a seated position from the arm opposite to that used for blood pressure measurements. Blood was collected in glass tubes containing the anticoagulant agent EDTA (total volume ≤ 20ml). Circulating mononuclear cells were separated from whole

blood prior to centrifugation. Collection tubes were centrifuged (2500 rpm) at room temperature for 15 min no longer than 2 hr post blood draw. Plasma samples were stored in 1.8 ml cryogenic tubes at -80° C.

Measurement of Endothelin-1 and Big Endothelin. Following centrifugation, plasma samples were assessed for ET-1 and BE by ELISA (Assay Designs®, Inc., Ann Arbor, MI) following manufacturer instructions. Protein concentrations were determined using a fluorescent plate reader at 450 nm (uQuant® Universal Microplate Spectrophotometer, Bio-Tek Instruments, Inc., Winooski, VT).

Measurement of Nitrate and Nitrite. Analysis of the nitric oxide metabolites nitrite and nitrate was performed with an HPLC system using anion-exchange chromatography. Briefly, plasma proteins were removed by filter centrifugation at 14,000 g for 10 min at 27°C. Twenty microliters of filtrate was injected into a 100 x 4.6 mm Wescan Anion/S IC Column (Alltech Associates, Deerfield, IL). The mobile phase consisted of 1.5 mM sulfuric acid at a flow rate of 1 ml/min. Nitrate and nitrite were detected by an UV detector at 210 nm. Sample detection and analysis was completed on the Beckman-Coulter System Gold HPLC System (Fullerton, CA).

Mononuclear Cell Isolation and RNA Extraction. Mononuclear cells were isolated from whole blood. Briefly, 3 ml of whole blood was layered onto 3 ml of HISTOPAQUE® -1077 (Sigma, St. Louis, MO) in a 15 ml conical centrifuge tube and centrifuged at 400 x g for 30 min at room temperature. Following removal of the plasma

layer, the opaque interface was transferred to a clean 15 ml conical centrifuge tube and washed with 10.0 ml 1X PBS and centrifuged at 250 x g for 10 min. The supernatant was removed and the cell pellet resuspended in 5.0 ml of 1X PBS and centrifuged at 250 x g for 10 min. A third wash with 5.0 ml of 1X PBS under identical centrifugation was then completed. The supernatant was aspirated and 600 µl of RLT lysis buffer (RNeasy Mini Kit, Qiagen, Bothell, WA) and 6 µl of 1% β-mercaptoethanol were added to the cell pellet, mixed using repeat pipetting, and stored at -80°C until processed. Total RNA was purified from mononuclear cell preparations using RNeasy® Mini Kit (Qiagen®, Bothell, WA) according to manufacturer instructions. RNA samples were assessed for quality and genomic contamination prior to further analysis via RNA 6000 Nano Assay (Agilent 2100 Bioanalyzer®, Agilent Technologies, Palo Alto, CA).

Reverse Transcription. Reverse transcription of 0.1 µg total RNA was completed in a total volume of 20 µl reaction mixture (iScript® cDNA synthesis kit, Bio-Rad Laboratories, Hercules, CA) using both random hexamer and oligo(DT) primers. Samples were incubated at 25°C for 5 min, at 42°C for 30 min, and then at 85°C for 5 min, followed by a hold at 4°C. cDNA samples were analyzed for quality prior to further analysis via DNA 1000 ASSAY (Agilent 2100 Bioanalyzer®, Agilent Technologies, Palo Alto, CA).

Quantitative Real-Time RT-PCR Assay. Relative gene expression of preproendothelin-1 was conducted on the Bio-Rad® iCycler System (Bio-Rad Laboratories, Hercules, CA). Briefly, the assay detects the formation of double-stranded

PCR product through the binding of the double-stranded binding protein SYBR Green. During the annealing phase, SYBR® Green binds to double-stranded product that can be detected by the iCycler System. The threshold cycle (C_T) is defined as the fractional cycle number where sample fluorescence reaches a certain level. The C_T was determined by user definition at the mid log phase of fluorescence following baseline level subtraction. Control patient C_T values were averaged prior to OSA relative expression calculation to account for variability in the sample set. Constitutive β-Actin was used as the housekeeping gene. To amplify both the 71 bp preproendothelin-1 and 110 bp β-Actin products, 1 µl of cDNA diluted 1:10 was added to a SYBR® Green Supermix reaction mixture (Bio-Rad Laboratories, Hercules, CA) containing 100 mM KCl, 40 mM Tris-HCl (pH 8.4), 0.4 mM of each dNTP, iTaq DNA polymerase (50 unit/ml), 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, and stabilizers. Amplification primers were assessed and sequenced previously (Gan, Selin-Sjogren et al. 2000; Fries, Roth et al. 2003) and are described in Table 1. Each sample was assessed in triplicate with a no template control tube on a proprietary optical plate in one assay run. Thermal cycler conditions included an initial denaturation of 95°C for 3 min for DNA polymerase activation. A 50-cycle three step PCR was then employed consisting of a 30 sec denaturation at 95°C, a 1 min annealing step at 60°C, and a 1 min extension at 72°C. A 10 min final extension was completed at 72°C. Following amplification, a melt curve was completed with a 10 sec hold every 0.5°C from 95°C to 58°C to assess the PCR product for primer dimerization.

Statistical Analysis. Data were analyzed with JMP 4.0 statistical software (SAS Institute Inc., Cary, NC). Data are expressed as mean ± standard error of the mean (SE). Mean differences between OSA patients and control subjects were determined by the Student's t-test. Correlations were assessed between all biochemical and hemodynamic measures. Significance level was set at $P<0.05$ *a priori* (two-tailed test).

Results

Descriptive and polysomnographic (PSG) data for both OSA patients and controls are presented in Table 2. In general, patient sample size was limited by multiple exclusion criteria and the viability of biological samples. Screening of control patients by the portable Embletta® system eliminated 3 additional subjects due to suspicion of underlying OSA. Screening results were evaluated independently by a certified sleep technician and physician. The OSA group suggested moderate disease with an overall RDI of 25.29 (± 4.41) events/hr and percent time below 90% oxygen saturation value of 2.69% (± 0.98). However, there was wide variation in individual disease severity with a range of 6.7 to 55.1 events/hr. OSA patients and control subjects were similar in age and displayed similar body type and exercise tolerance. While no statistically significant difference was noted, OSA patients and control subjects are clinically disparate in that OSA patients would be classified as class I obese and control subjects simply overweight based on group mean. The waist to hip ratio was significantly greater in OSA patients than control subjects (0.94 ± 0.02 versus 0.80 ± 0.06, respectively), indicating a differing pattern of fat distribution between groups. Clinically, OSA patients exhibited increased neck circumferences 41.42 (± 0.94) cm compared to control subjects 35.10 (± 2.45)cm (P

< 0.05). Functionally, OSA patients subjectively indicated highly significant elevations in daytime sleepiness compared to controls, with Epworth Sleepiness Scores of 12.85 (± 1.13) vs. 2.75(± 0.48), respectively (P < 0.01).

All measures of ambulatory daytime blood pressure were elevated in OSA patients compared to controls with systolic values of 130.5 (± 3.96) mmHg vs. 107.3 (± 1.53) mmHg, diastolic values of 84.0 (± 1.25) mmHg vs. 69.5 (± 1.38) mmHg, and mean arterial values of 97.5 (± 1.95) mmHg vs. 80.6 (± 1.58) mmHg, respectively. Systolic, diastolic, and mean arterial hemodynamic values were elevated prior to exercise, at peak exercise, and at 1 min recovery intervals through minute 6 (Tables 3, 4, and 5). However, a significant group difference was noted only at peak exercise in systolic (OSA 209.7 (± 5.65) mmHg; Control 174.5 (± 6.18) mmHg) (Figure 1) and mean arterial pressures (OSA 125.8 (± 3.24) mmHg; Control 109.1 (± 4.46) mmHg) (Figure 2). No relationship was observed between disease severity (RDI and SaO2 < 90%) and any hemodynamic measurement, although a positive trend was noted between RDI and Systolic Blood Pressure at 1 min into recovery (r = 0.571; P = 0.052).

Plasma ET-1 levels normally reside in the low pg/ml range, but were below the assay detection limit of 0.78 pg/ml (Table 5). No differences were noted in plasma BE-1 and nitrite values between OSA patients and controls. A highly significant relationship was found between BMI and BE-1 in both OSA patients (r = 0.80; P = 0.001; Figure 3) and control subjects (r = 0.96; P = 0.045). Nitrate levels were undetectable in 1 control and 11 OSA patients and were therefore not included in further analyses. Relative gene expression of preproendothelin-1 was not elevated in OSA patients versus control

subjects, with only a 0.40 (± 0.16) fold increase noted. Overall, preproendothelin-1 relative gene expression was positive for all subjects, ranging from a 0.08 to 1.74 fold increase. Interestingly, the two highest values, 1.74 and 1.50 belonged to 2 of the 3 highest RDI values and a trend toward a positive relationship between RDI and Relative ET-1 expression ($r = 0.54$, $p = 0.059$) was noted.

Discussion

Over half of OSA patients experience systemic hypertension (Hla, Young et al. 1994) and OSA is widely recognized as an independent risk factor in the development of hypertension (Peppard, Young et al. 2000; Peker, Hedner et al. 2002). Conversely, approximately 25% of hypertensives were diagnosed with OSA by subsequent PSG (Fletcher, DeBehnke et al. 1985). Evidence increasingly points to vascular dysfunction as the primary cause of hypertension in OSA (Anand, Remsburg-Sailor et al. 2001; Kraiczi, Caidahl et al. 2001). Unfortunately, the exact etiology of vascular dysfunction remains unclear. In an attempt to elucidate the development of vascular dysfunction in humans, this study investigated circulating markers of the vasoactive substances NO and ET-1 and their relationship to hemodynamic function at rest, during, and following exercise stress.

In the present study, moderate OSA patients with no previous history of cardiovascular disease or anti-hypertensive medication were invited to participate. These criteria limited overall recruitment due the high prevalence of hypertension in OSA. However, it is believed that this eliminated the possibility that patients would have hypertension

independent of OSA. Studies with similar exclusion criteria have previously demonstrated impaired vascular function in OSA (Kraiczi, Hedner et al. 2000; Kraiczi, Caidahl et al. 2001). In the present study, ambulatory systolic and diastolic blood pressure were significantly elevated in OSA patients compared to controls and similar to published results (Duchna, Guilleminault et al. 2000; Kraiczi, Hedner et al. 2000; Kraiczi, Caidahl et al. 2001). OSA patients consistently recorded higher systolic and diastolic blood pressures before, during, and after exercise stress. However, values were considered normal and both groups demonstrated a similar overall response. The lone difference between the hemodynamic response of OSA patients and control subjects occurred at peak exercise. At peak, OSA patients exhibited a significantly greater systolic blood pressure than control subjects. While not uncommon, this value may have prognostic implications in non-hypertensive OSA. Previous study has indicated an exaggerated systolic blood pressure response at maximal exercise is indicative of future hypertension (Miyai, Arita et al. 2002). Because heart rates between groups were similar at peak exercise (OSA: 154.4 ± 4.00 beats per min; Control: 156.8 ± 7.47 beats per min), the exaggerated blood pressure response observed in OSA patients may potentially indicate the future development of hypertension. Unfortunately, exercise blood pressure responses are difficult to reproduce (Sharabi, Almer et al. 2001), and follow-up with greater subject numbers is warranted before definitive conclusions are drawn. No significant relationships were noted between disease severity and any blood pressure measurement. A single trend was discovered between RDI and systolic blood pressure at 1 min.

Nitric oxide is formed continuously by the vascular endothelium in the low nM concentration range, but is rapidly broken down into its metabolites nitrite and nitrate (Kelm, Preik-Steinhoff et al. 1999). Plasma NO has a half-life of approximately 3 sec (Palmer, Ferrige et al. 1987), making *in vivo* measurement difficult. In red blood cells, nitrite is rapidly converted to nitrate. However, plasma nitrite levels are stable for up to several hours in plasma and are a sensitive measure of endothelial NO production (Kelm, Preik-Steinhoff et al. 1999). In this study, plasma nitrite levels were similar between OSA patients and control subjects. Physiologically, control subject nitrite levels were similar to OSA patients in a previous study (Ip, Lam et al. 2000). However, lack of dietary control may play a primary role in the observed plasma nitrite levels in a small sample set (Mochizuki, Toyota et al. 2000). Patient nitrite levels were similar to those documented in previous work prior to CPAP therapy (Ip, Lam et al. 2000; Schulz, Schmidt et al. 2000).

It was thought that ET-1 levels would be elevated in OSA patients compared to control subjects. Endothelin-1 has a short half-life in plasma of 4-7 min, but is present in most individuals in the low pg/ml range (Levin 1995). In a previous study of OSA patients, apparently normal control subjects had approximately 3.0 pg/ml of plasma ET-1 with patient values between approximately 6-8 pg/ml (Saarelainen, Seppala et al. 1997). It was assumed ET-1 study values would approach those obtained in the Saarelainen study. Unfortunately, ET-1 levels were below the detection limit (<0.78 pg/ml) for all study samples, indicating the need to precipitate plasma ET-1 prior to analysis. Protein purification was employed in a study by Grimpen and others where similar ET-1 levels

were found in OSA and control subjects (Grimpen, Kanne et al. 2000). Since the inception of this study, additional study has supported the findings of Grimpen et al. that ET-1 levels are not elevated in OSA patients (Diefenbach, Kretzchmer et al. 2003).

Patients and control subjects exhibited similar plasma levels of the ET-1 precursor, BE-1 that was highly correlated to BMI. This result was expected due to the recent finding that plasma ET-1 production is enhanced in obese individuals regardless of hypertensive diagnosis (Tiret, Poirier et al. 1999) . Direct activation of the ET-1 system also occurs in experimental models of obesity (Barton and Dubey 2002). This was the first known study to assess BE-1 levels in OSA patients. Prior studies have shown increased ET-1 levels in OSA patients but failed to measure plasma BE-1 levels (Saarelainen, Seppala et al. 1997; Phillips, Narkiewicz et al. 1999). As plasma BE-1 is fairly time-stable and physiologically serves to rapidly increase ET-1, use of OSA groups with high BMI may be suspect. Increased ET-1 in these patients may stem from augmented cleavage of active protein from obesity-elevated plasma BE-1 levels. As for the source of BE-1 and ET-1, it does not appear that circulating mononuclear cells substantially contribute to plasma protein levels. However, relative preproendothelin-1 expression was non-significantly upregulated in all patients and weakly correlated with RDI. Therefore, patient samples with severe disease and higher RDI may exhibit increased immune preproendothelin-1 production that contributes to plasma ET-1 and BE-1 levels.

Conclusions

The results of this study should be interpreted cautiously due to small sample size. OSA patients displayed a wide range of disease severity that may confound study findings. Increased sample size with a more homogeneous sample set is warranted for future investigations. Overall, moderate OSA is associated with elevated blood pressure that is exacerbated during exercise stress. Circulating biomarkers of NO and ET-1 are not indicative of cardiovascular dysfunction in moderate OSA patients without diagnosed hypertension. An obese body habitus may affect measurement of circulating vasoconstrictors and vascular function in OSA patients.

Table 1. Quantitative Real-Time PCR Oligonucleotide Primers

Gene	Oligonucleotide	Sequence	Product Size
Preproendothelin-1	Sense Primer	5'-TGG ACA TCA TTT GGG TCA ACA –3'	71 bp
	Antisense Primer	5'-TCT CTT GGA CCT AGG CT TCC-3'	
B-Actin	Sense Primer	5'-TTG CAA TGA GCG GTT CCG CT-3'	110 bp
	Antisense Primer	5'-TAC AGC TGT TTG CGG ATG TCC-3'	

Table 2. OSA Patient and Control Subject Characteristics

Patient Characteristics	OSA Group (N = 13)	Control Group (N = 4)
Age	45.0 ± 3.0	44.8 ± 3.2
Male	9	2
Female	4	2
RDI	25.3 ± 4.4	N/A
SaO2 <90%	2.7 ± 1.0	N/A
Epworth Score	12.9 ± 1.1[a]	2.8 ± 0.5
BMI (kg/m^2)	34.9 ± 2.3	31.0 ± 2.4
Waist: Hip Ratio	0.94 ± 0.02[b]	0.84 ± 0.06
Neck Circumference (cm)	41.4 ± 0.9[b]	35.1 ± 2.5
Daytime Systolic Blood Pressure (mmHg)	130.5 ± 4.0[b]	107.3 ± 1.5
Daytime Diastolic Blood Pressure (mmHg)	84.0 ± 1.3[a]	69.5 ± 1.4
Daytime Mean Arterial Pressure (mmHg)	97.5 ± 2.0[a]	80.6 ± 1.6
Peak VO_2 (ml/kg/min)	21.0 ± 1.0	21.6 ± 2.1
Peak Watts	162.7 ± 9.0	146.3 ± 12.0

[a] $p < 0.01$; [b] $p < 0.05$

Table 3. Pre-Exercise, Peak Exercise, and Exercise Recovery Systolic Blood Pressure

	Group	
Measure	**OSA**	**Control**
Pre-Exercise SBP	131.6 ± 4.2	128.0 ± 1.4
Peak Exercise SBP	209.7 ± 5.7[a]	174.50 ± 6.2[a]
1 min Recovery SBP	180.8 ± 6.4	156.5 ± 10.0
2 min Recovery SBP	167.1 ± 5.9	147.5 ± 7.0
3 min Recovery SBP	153.3 ± 6.5	140.0 ± 6.5
4 min Recovery SBP	144.3 ± 5.5	130.5 ± 4.4
5 min Recovery SBP	136.7 ± 5.5	124.5 ± 3.2
6 min Recovery SBP	135.5 ± 5.3	122.0 ± 2.2

[a]$p < 0.05$; SBP = Systolic Blood Pressure (mmHg)

Table 4. Pre-Exercise, Peak Exercise, and Exercise Recovery Diastolic Blood Pressure

	Group	
Measure	**OSA**	**Control**
Pre-Exercise DBP	90.2 ± 9.5	88.0 ± 0.0
Peak Exercise DBP	89.9 ± 3.4	81.0 ± 4.5
1 min Recovery DBP	85.8 ± 3.8	77.0 ± 3.1
2 min Recovery DBP	82.0 ± 3.0	73.0 ± 4.4
3 min Recovery DBP	81.5 ± 2.6	71.5 ± 5.3
4 min Recovery DBP	82.8 ± 2.8	72.0 ± 6.9
5 min Recovery DBP	82.8 ± 2.8	75.0 ± 5.7
6 min Recovery DBP	84.5 ± 2.7	76.5 ± 4.9

DBP = Diastolic Blood Pressure (mmHg)

Table 4. Pre-Exercise, Peak Exercise, and Exercise Recovery Mean Arterial Measures

	Group	
Measure	**OSA**	**Control**
Pre-Exercise MAP	102.6 ± 2.7	100.0 ± 0.4
Peak Exercise MAP	125.8 ± 3.2[a]	109.1 ± 4.5
1 min Recovery MAP	114.3 ± 3.8	100.9 ± 3.2
2 min Recovery MAP	107.5 ± 3.4	95.4 ± 4.2
3 min Recovery MAP	103.0 ± 3.4	92.1 ± 5.0
4 min Recovery MAP	101.2 ± 3.3	89.6 ± 5.5
5 min Recovery MAP	99.0 ± 3.3	89.9 ± 4.7
6 min Recovery MAP	99.8 ± 3.1	90.2 ± 4.1

[a]$p<0.05$; MAP = Mean Arterial Pressure (mmHg)

Table 5. Nitric Oxide and Endothelin-1 Biomarker Measures

Measure	Group	
	OSA	Control
	N = 13	N = 4
Plasma BE (pg/ml)	2.84 ± 0.36	3.79 ± 0.87
Plasma ET-1 (pg/ml)	<0.78	<0.78
Relative Preproendothelin Gene Expression (versus control)	0.40 ± 0.16	N/A
Plasma Nitrite (µg/ml)	68.88 ± 7.33	51.86 ± 7.20
Plasma Nitrate (µg/ml)	0.17 ± 0.11	2.11 ± 0.17
	(n = 4)	(n = 2)

BE = Big Endothelin; ET-1 = Endothelin-1:

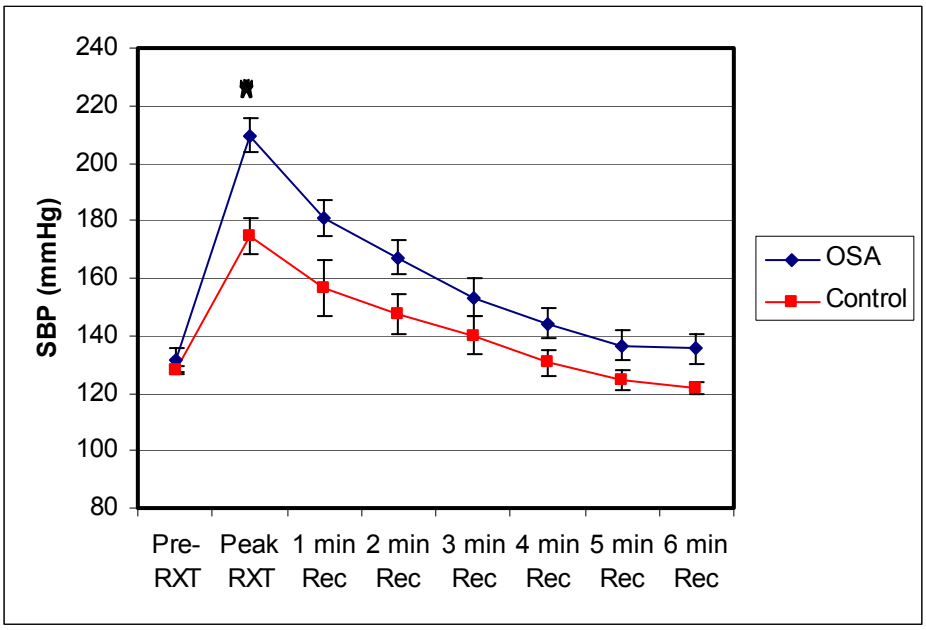

Figure 1. Systolic Blood Pressure Measurements
*p < 0.05; SBP = Systolic Blood Pressure, RXT = Ramped Exercise Testing

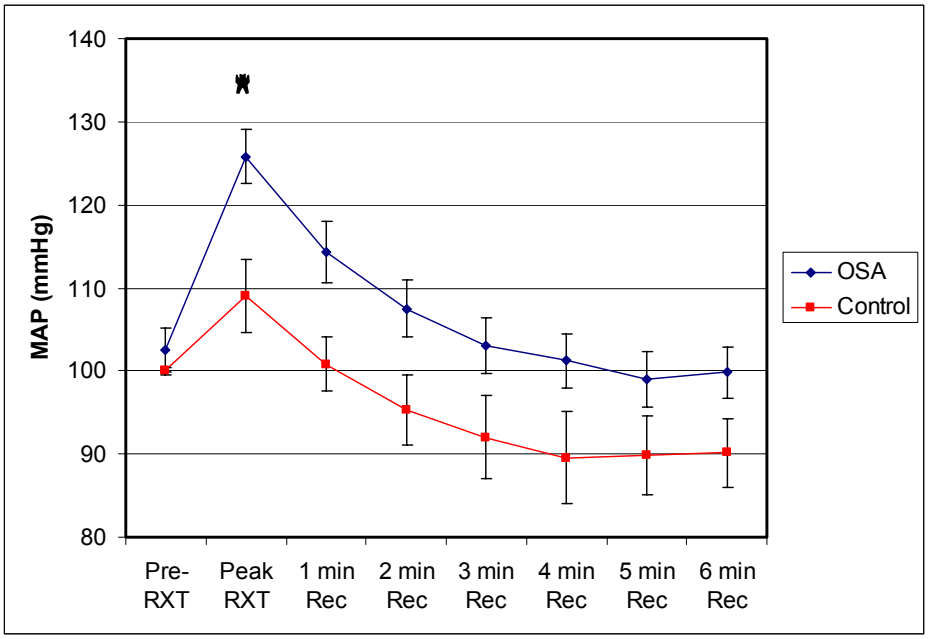

Figure 2. Mean Arterial Pressure Measurements
*p < 0.05; MAP = Systolic Blood Pressure, RXT = Ramped Exercise Testing

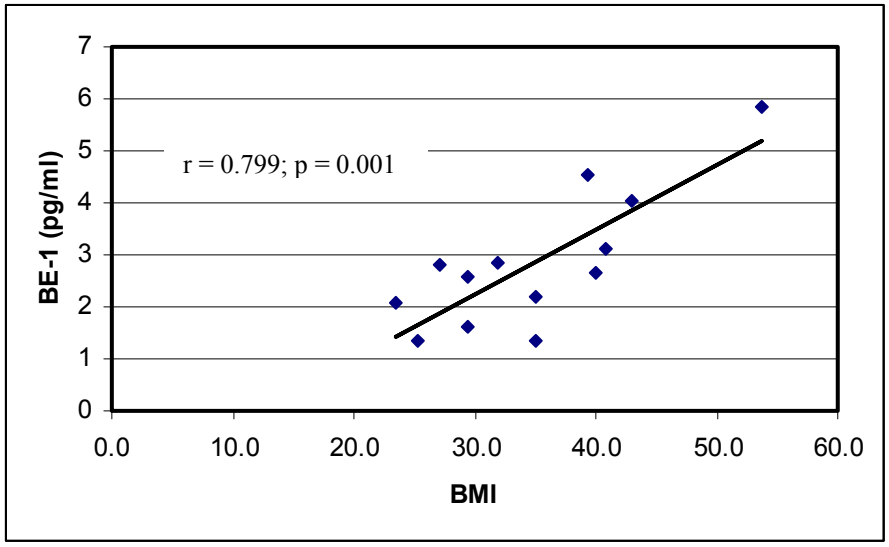

Figure 3. Relationship between BMI and BE-1 in OSA Patients
BMI = Body Mass Index; BE-1 = big endothelin-1

Chapter IIIb. Journal Manuscript II

Additive Effects of Exercise Training to Nasal Continuous Positive Airway Pressure Therapy on Resting and Exercise Blood Pressure and Biomarkers of Vascular Function in Obstructive Sleep Apnea Syndrome

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ABSTRACT

PURPOSE: The purpose of this study was to examine the effects of long-term nasal continuous positive airway pressure (nCPAP) therapy, both with and without aerobic exercise training, in altering hemodynamic function and circulating biomarkers of nitric oxide (NO) and endothelin-1 (ET-1) in newly diagnosed Obstructive Sleep Apnea (OSA) patients. Cardiopulmonary exercise testing was used to profile the functional importance of circulating biomarkers in hemodynamic regulation. **METHODS:** Ten newly diagnosed OSA patients (8 male, 2 female, age 46.3 ± 3.2 yr) were stratified by age and OSA severity and randomized into either a standard therapy group (nCPAP) or a standard therapy group with a moderate intensity aerobic training regimen (nCPAP+Ex). Blood pressure was recorded over one complete day and prior to, during, and following maximal exercise testing on a cycle ergometer. Blood samples were taken prior to exercise testing and assessed for the nitrate and nitrite by HPLC and for big endothelin-1 (BE) and ET-1 by ELISA. All measures were repeated following 12 wk of treatment. **RESULTS:** nCPAP usage was not uniform in either group, with an overall range of 182 to 495 min mean usage per night. A moderate intensity exercise training program failed to elicit a training response through standard hemodynamic or cardiopulmonary indices. No significant improvements in systolic or diastolic blood pressures were observed prior to, during, or following exercise stress. ET-1 and nitrate levels were below assay detection limits. Plasma nitrite levels rose from 55.6 ± 5.8 μg/ml to 76.5 ± 6.4 μg/ml in the nCPAP group. No reductions in BE were noted. **CONCLUSIONS:** Plasma nitrite levels rose following nCPAP therapy, indicating a possible increase in basal nitric oxide formation. nCPAP therapy failed to elicit significant alterations in hemodynamic function in moderate OSA patients without diagnosed hypertension. Circulating biomarkers of endothelin-1 production were unaltered following nCPAP therapy. Adjunct moderate intensity exercise did not improve standard nCPAP therapy. Higher intensity exercise regimens may be needed to elicit the positive benefits of exercise training in OSA patients without significant cardiovascular dysfunction.

Introduction

Obstructive sleep apnea (OSA) is a serious disorder that affects between 2% and 4% of the adult population (Henderson and Strollo 1999). Patients experience repetitive respiratory cessations throughout sleep that result in varying degrees of systemic hypoxia and/or sleep fragmentation (McNicholas 1997). These nocturnal disturbances constitute the primary pathophysiologic event in patients. However, most sufferers go unrecognized and untreated by current medical practice. As a result, potentially up to 25% of the middle aged population may have varying degrees of the disease (Henderson and Strollo 1999). Obstructive sleep apnea is associated with numerous physiological consequences, including pulmonary hypertension (Weitzenblum, Krieger et al. 1988; Marrone and Bonsignore 2002; Yamakawa, Shiomi et al. 2002), cerebrovascular complications (Wessendorf, Teschler et al. 2000; Franklin 2002; Hermann and Bassetti 2003), and hypertension (Lavie, Herer et al. 1995; Peppard, Young et al. 2000; Peker, Hedner et al. 2002). In general, OSA patients experience a cardiovascular mortality rate over three and a half times that of the general population between the fourth and fifth decades of life (Lavie, Herer et al. 1995).

A proposed mechanism of cardiovascular dysfunction in OSA patients is chronic sympathoexcitation and increased vascular tone. Patients experience significant reductions in vascular reactivity in both arterial (Anand, Remsburg-Sailor et al. 2001; Kraiczi, Caidahl et al. 2001) and venous (Duchna, Guilleminault et al. 2000) vessels that can be reversed by continuous positive airway pressure therapy (Imadojemu, Gleeson et al. 2002). One possible mechanism for this phenomenon is a reduction in the vasodilator

nitric oxide (NO). Under physiologic conditions, resting tone is maintained by the basal release of NO (Aisaka, Gross et al. 1989; Gardiner, Compton et al. 1990) and evidence exists for diminished NO production and activity in OSA (Ip, Lam et al. 2000; Kato, Roberts-Thomson et al. 2000; Schulz, Schmidt et al. 2000; Tahawi, Orolinova et al. 2001). In concert with a reduction in NO production may be an enhanced synthesis of the vasoconstrictor endothelin-1 (ET-1) in OSA patients (Saarelainen, Seppala et al. 1997; Phillips, Narkiewicz et al. 1999).

Successful OSA treatment with nasal Continuous Positive Airway Pressure (nCPAP) has been extensively shown to reduce blood pressure (Mayer, Becker et al. 1991; Wilcox, Grunstein et al. 1993; Davies, Crosby et al. 1994) and improve biomarkers of vascular function (Ip, Lam et al. 2000; Schulz, Schmidt et al. 2000). Many of the favorable changes seen following therapy are unfortunately negated by poor compliance over time and viable low cost alternatives are needed to enhance long-term treatment outcomes. The addition of regular aerobic physical activity may promote a multitude of health benefits and may decrease disease severity in OSA patients (Netzer, Lormes et al. 1997; Giebelhaus, Strohl et al. 2000; Norman, Von Essen et al. 2000).

The potential reduction in OSA severity presents exercise training as a beneficial component of disease treatment. However, physical training may play an important role in reducing the cardiovascular impairment commonly seen in OSA patients. Experimentally, chronic exercise training is associated with significant increases in basal nitrate and nitrite production over sedentary controls (Maeda, Miyauchi et al. 2001; Vassalle, Lubrano et al. 2003). Conversely, exercise training may reduce plasma ET-1

levels in healthy adults (Maeda, Miyauchi et al. 2001; Maeda, Tanabe et al. 2003). The purpose of this study was to examine the effects of long-term nCPAP therapy in altering circulating biomarkers of NO and ET-1 in newly diagnosed OSA patients. Adjunct use of 12 wk of aerobic training to nCPAP therapy may espouse the potential benefits of exercise stress in reducing the cardiovascular dysfunction normally associated with OSA. Cardiopulmonary exercise testing was used to profile the functional importance of circulating biomarkers in hemodynamic regulation.

Methods

Subjects. Ten patients (8 male, 2 female) were recruited from volunteers referred to the Southwest Virginia Sleep Disorders Center (SVSDC) in Christiansburg, VA and subsequently diagnosed with OSA (Respiratory Distress Index: RDI > 5.0). Each participant was given an informed consent prior to study inclusion. Approval for the protocol and the informed consent process and form were approved by the Virginia Tech IRB for Human Subjects' Research. Patients were excluded from study based on the presence of co-existing cardiovascular, respiratory, metabolic, orthopedic, musculoskeletal, and/or neuromuscular disabilities. Further exclusion criteria included current use of anti-hypertensive medication or the presence of severe hypertension, current tobacco use, or recent history of moderately vigorous physical activity ($\geq$3 dy/wk, $\geq$ 30 min/session) within 6 months.

Ambulatory Blood Pressure Measurement. Daytime ambulatory blood pressure was obtained via an automated digital blood pressure device (Omron HEM-705CP).

According to manufacturer recommendations, multiple recordings were taken at four daily time points; morning awakening, noon, afternoon, and evening. Blood pressure was measured in a seated position after 5 min of rest. Subjects were instructed to abstain from caffeine and nicotine products and refrain from vigorous physical activity for a time period of 12- and 24-hr, respectively, prior to all measurements. Repeated recordings were taken at each time interval until two systolic measures were within 5 mmHg. The overall daytime mean systolic, diastolic, and mean arterial pressures were used for analysis.

Exercise Stress Testing. Two identical maximal exercise stress testing sessions were completed on an electronically braked cycle ergometer (CardioO$_2$®, MedGraphics, St. Paul, MN) both prior to and following either nCPAP or nCPAP+Ex treatment. Anthropometric measurements (height, weight, neck, waist, and hip circumference) were obtained prior to exercise testing. Resting blood pressure was taken following 2 min of seated rest. Following a 1 min warm-up period at an initial rate of 25 W, the ergometer was ramped at a rate of 15 W/min to achieve a total duration of 8-12 min. Cardiopulmonary measurements were obtained throughout the ramped exercise test (RXT). Blood pressure was acquired by manual auscultation at the antecubital fossa of the left forearm by trained personnel at baseline, 2 min, 5 min, and at 2 min intervals until test termination. Blood pressure at peak exercise was also measured prior to exercise termination. Heart rate and rhythm were assessed by continuous electrocardiographic (ECG) monitoring throughout exercise. Exercise was terminated at maximal subject effort (RPE > 17). A licensed physician or EMT was present for every test and an

American College of Sports Medicine (ACSM) Certified Exercise Specialist^{SM} supervised the RXT. Test termination criteria were in accordance with American Heart Association and ACSM standards. Participants completed an active recovery phase at 25 W that was terminated upon subject request. Blood pressure was recorded at 1 min intervals until 6 min into recovery.

Exercise Training Protocol. After the initial RXT, subjects were randomized to the nCPAP or nCPAP plus exercise training groups as illustrated in Figure 1 (5 nCPAP, 5 nCPAP+Ex). Study patients in the nCPAP or nCPAP+Ex groups were prescribed an AutoSet T® or AutoSet Spirit® (ResMed Corporation, Poway, CA). Patients randomized into the nCPAP+Ex group subsequently participated in aerobic exercise-training program for 12 wk. Initially, exercise training sessions were supervised under continuous ECG monitoring in the Laboratory for Health & Exercise Sciences at Virginia Tech. Exercise was performed 3 dy/wk, 30 - 40 min/session, at an intensity range of 50 - 60% of VO_2pk. Sessions included a 5 to 10 min warm-up and cool-down. Blood pressure and RPE were monitored and recorded for each exercise session. Exercise progression was increased accordingly based on subject ability to maintain the prescribed target HR of 50 - 60% VO_2pk. Increases in exercise duration preceded those of intensity until subjects could maintain 30 - 40 min of continuous exercise. Thereafter, intensity was increased to maintain a HR at 50 - 60% VO_2pk. Two weeks after the start of the supervised exercise training program (3 dy/wk), patients transitioned to unsupervised sessions on days not scheduled for supervised exercise. At week 5, subjects fully transitioned to a schedule of unsupervised exercise 4 dy/wk. Exercise logs and heart rate

monitors were provided for documentation of all sessions. Subjects could participate in one of three local community activity centers or at the University-based adult exercise program held 3 mornings/wk in War Memorial Hall on the campus of Virginia Tech. Activities in alternative exercise facilities included walking/jogging or cycle ergometry, in accordance with individualized plans specified by the research staff. To provide flexibility for exercise sessions, subjects were offered the opportunity to participate in morning, afternoon and/or evening monitored exercise sessions at the Laboratory for Health and Exercise Sciences. Subjects in the nCPAP+Ex and nCPAP groups were contacted weekly by the research nurse to verify exercise adherence. The research nurse or an exercise staff member met monthly with each subject to review their study experience, discuss any relevant problems/issues related to compliance with nCPAP therapy and/or exercise treatments if necessary. Subjects in the Control and nCPAP groups abstained from regular exercise training for a period of 12 wk. After 12 wk, subjects in all three groups completed follow-up testing on all measures identical to baseline.

Blood Collection. Blood was drawn prior to RXT by antecubital venipuncture with the subject in a seated position from the arm opposite to that used for blood pressure measurements. Blood was collected in glass tubes containing EDTA (total volume ≤ 20 ml). Collection tubes were centrifuged (2500 rpm) at room temperature for 15 min no longer than 2 hr post blood draw. Plasma samples were stored in 1.8 ml cryogenic tubes at -80° C.

Measurement of Endothelin-1 and Big Endothelin. Following centrifugation, plasma samples were assay3ed for ET-1 and BE by ELISA (Assay Designs®, Inc., Ann Arbor, MI) following manufacturer instructions. Protein concentrations were determined using a fluorescent plate reader at 450 nm (uQuant® Universal Microplate Spectrophotometer, Bio-Tek Instruments, Inc., Winooski, VT).

Measurement of Nitrate and Nitrite. Analysis of the nitric oxide metabolites nitrite and nitrate was performed with an HPLC system using anion-exchange chromatography. Briefly, plasma proteins were removed by filter centrifugation at 14,000 g for 10 min at 27°C. Twenty microliters of filtrate was injected into a 100 x 4.6 mm Wescan Anion/S IC Column (Alltech Associates, Deerfield, IL). The mobile phase consisted of 1.5 mM sulfuric acid at a flow rate of 1 ml/min. Nitrate and nitrite were detected by an UV detector at 210 nm. Sample detection and analysis was completed on the Beckman-Coulter System Gold HPLC System (Fullerton, CA).

Statistical Analysis. Data were analyzed with JMP 4.0 statistical software (SAS Institute Inc., Cary, NC). Data are expressed as mean ± standard error of the mean (SE). Mean differences between nCPAP and nCPAP+Ex groups were determined by the Student's t-test. Due to the limited sample size, treatment effects were determined by paired t-test. Correlations were assessed between all biochemical and hemodynamic measures. Significance level was set at $P < 0.05$ *a priori* (two-tailed test).

Results

Descriptive and polysomnographic (PSG) data for nCPAP only and nCPAP+Ex groups both before and after 12 wk of treatment are presented in Table 1. Group size was limited by multiple exclusion criteria and viability of biological samples. In general, groups were similar in age, neck circumference, waist to hip ratio, and in disease severity. Mean RDI scores do not differ statistically, but placed the nCPAP and nCPAP+Ex groups into separate disease classifications of moderate (24.5 ± 8.4 events/hr) and severe (33.64 ± 4.64 events/hr) disease, respectively. Overall, a wide variation existed in the experimental groups with a range of 7.6 to 55.1 events/hr. While significance was not reached, nCPAP patients tended to display a higher degree of obesity than are considered Class II Obese versus a Class I Obese classification in nCPAP+Ex patients. Functionally, nCPAP patients tended to report increased daytime sleepiness compared to nCPAP+Ex patients, with Epworth Sleepiness Scores of 16.2 (± 1.2) vs. 11.2 (± 1.9), respectively (P = 0.031). Groups exercised to similar workloads but displayed a slight difference in overall exercise tolerance, with a peak relative oxygen consumption of 19.9 (± 1.2) ml/kg/min in nCPAP patients and 23.84 (±1.28) ml/kg/min in nCPAP+Ex patients (P = 0.058). Ambulatory blood pressure was similar between groups and within acceptable values. Groups also displayed similar systolic and diastolic responses to exercise stress, as recorded in Tables 2 and 3 respectively.

Endothelin-1 and NO biomarker values are recorded in Table 4. Patients in the nCPAP group displayed elevated BE-1 levels (3.4 ± 0.4 pg/ml) versus nCPAP+Ex patients (2.0 ± 0.3 pg/ml). Plasma BE-1 levels were positively related to the body habitus in nCPAP

patients ($r = 0.83$; $p = 0.039$). Nitrite levels were similar in both groups and comparable to previous studies (Ip, Lam et al. 2000; Schulz, Schmidt et al. 2000). Nitrate levels were absent in 8 of 10 study patients and were not included in further analysis. ET-1 levels were below the detection limit (< 0.78 pg/ml) for all study samples, indicating the necessity to precipitate plasma ET-1 prior to analysis.

Usage data downloaded from nCPAP devices following 12 weeks of therapy revealed similar therapeutic intervention between groups (Table 1). Overall mean usage exceeded the minimum therapeutic threshold of 300 min/night. However, adherence was not uniform between patients and ranged from 182 to 495 min. Groups utilized similar amounts of nCPAP therapy as recorded in Table 1. nCPAP therapy successfully reduced subjective ratings of sleepiness (Epworth Sleepiness Scores) from 16.2 (± 1.2) to 10.0 (± 2.4). The additive effects of exercise training failed to elicit improvements in either hemodynamic or subjective ratings of sleepiness in the nCPAP+Ex group. Plasma BE-1 was unaffected in either treatment group. Plasma nitrite rose significantly from 55.6 (± 5.8) μg/ml to 76.2 (± 6.4) μg/ml in the CPAP group.

Discussion

Continuous positive airway pressure maintains airway patency during sleep and alleviates the repetitive respiratory events that constitute the primary pathophysiologic event in OSA. Successful CPAP therapy lowers resting blood pressure (Mayer, Becker et al. 1991; Wilcox, Grunstein et al. 1993), increases NO production (Ip, Lam et al. 2000) and may reduce ET-1 production (Saarelainen, Seppala et al. 1997). Unfortunately, many

patients can not tolerate CPAP and the favorable changes seen following therapy. The addition of regular aerobic physical activity to standard treatment for OSA would be of great interest as it may promote favorable alterations in vascular function and ET-1 production. Recently, study has demonstrated dramatic improvements in endothelial function following exercise training in heart failure patients (Hambrecht, Gielen et al. 2000). A reduction in the cardiovascular co-morbidity and hypothesized vascular dysfunction associated with OSA would have important implications in disease treatment.

To date, three studies have attempted to examine the potential role of exercise in the treatment of OSA. Overall, the primary aim of these studies was to assess the role of exercise as a means of reducing OSA severity. The first published report detailed a 6 mo exercise program consisting of two sessions per week (Netzer, Lormes et al. 1997). While no improvements in oxygen consumption, body weight, lactate profile, or blood pressure occurred, there was a significant decrease in RDI, signifying a reduction in disease severity. The authors hypothesize an increase in respiratory muscle tone is responsible for the demonstrated improvement in OSA severity. However, no evidence supporting this hypothesis was provided and the use of a non-traditional exercise regimen should be taken into consideration. Significant improvements in AHI of ~30% were also seen employing a similar exercise regimen in a group of moderate to severe OSA patients (Giebelhaus, Strohl et al. 2000). The dramatic reduction in disease severity in this study is primarily attributed to the exercise stimulus as no improvements in sleep architecture, body habitus, blood pressure, or exercise tolerance were noted. A reduction in AHI was

further observed in research utilizing a more standard exercise intervention of 3 sessions per week (Norman, Von Essen et al. 2000). Comparable to the previous investigations, no appreciable improvements in physical fitness or body weight were realized following exercise training. Unfortunately, the results of this study are suspect in that a heterogeneous group of OSA patients was chosen without regard to nCPAP usage, medication, or dietary consultation (Norman, Von Essen et al. 2000). However, widely disparate group selection and exercise methods limit the impact of these findings. Further, cardiovascular function and potential improvement was not assessed in these studies.

Unlike prior investigations, the primary aim of this study was to assess the cardiovascular adaptations associated nCPAP therapy with or without the addition of 12 wk of moderate intensity exercise training. Initial stratification resulted in patient groups of similar age and disease severity. However, wide variation in disease severity existed in both groups. Pre-treatment patient characteristics indicate a greater degree of functional debilitation in the nCPAP only patients. nCPAP only patients demonstrated a significantly lower exercise tolerance and increased functional limitation (Epworth Sleepiness Score) than nCPAP+Ex patients. Nonetheless, exercise workloads were similar in both groups. Hemodynamic response to exercise was similar in both groups.

Patients randomized into the nCPAP+Ex group were asked to begin a moderate intensity exercise program at an initial intensity of 50-60% VO_{2pk}. This intensity is commonly employed in outpatient cardiac rehabilitation programs to reduce attrition and transition

from a sedentary to active lifestyle. OSA patients tend to display reduced functional capacity and vigor, and it was believed that a moderate intensity exercise regimen would reduce dropout and elicit the greatest benefits. Further, moderate exercise training prescribed in this study would not appreciably affect body weight. Weight loss results in significant improvements in OSA severity (Fisher, Pillar et al. 2002) and is commonly recommended in disease treatment. The major drawback to the overall exercise protocol was the reliance on independent activity. Due to geographical and facility limitations, supervised exercise was not possible for the duration of the study. Following the initial sessions, exercise adherence was verified through phone and electronic media and intermittent supervised sessions. Further, access to dedicated fitness locations was limited. It is therefore assumed that maintenance of exercise intensity and duration in all nCPAP+Ex patients was not achieved based on post-hoc analysis of exercise records. Consequently, training results were less than optimal.

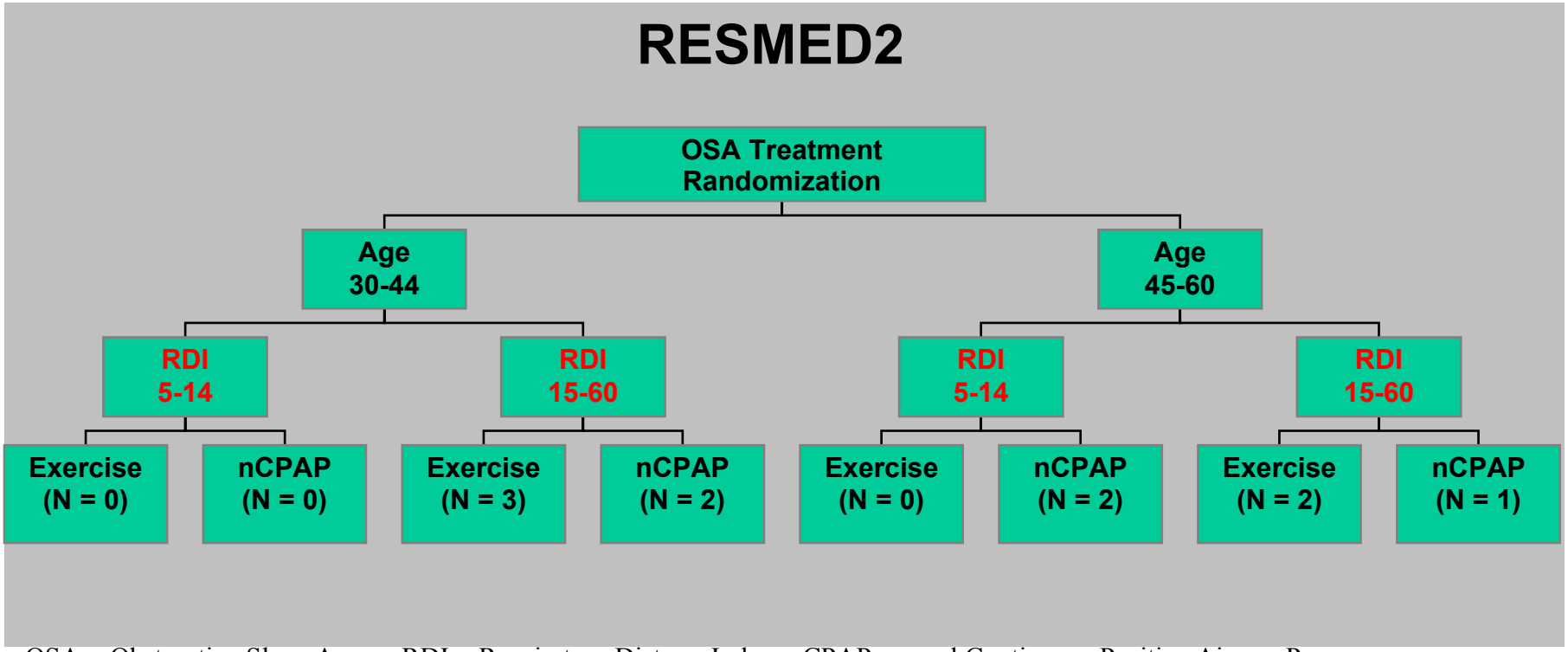
Overall, no significant improvements were seen in either group following 12 wk of treatment. nCPAP only therapy elevated plasma nitrite, possibly indicating an enhanced NO release shown in past research (Ip, Lam et al. 2000; Schulz, Schmidt et al. 2000). However, increased nitrite levels were not related to hemodynamic function. Lack of dietary control may further impact this finding (Mochizuki, Toyota et al. 2000). Patient nitrite levels were similar to those documented in previous work prior to CPAP therapy (Ip, Lam et al. 2000; Schulz, Schmidt et al. 2000). Patients on nCPAP only further experienced an improvement in overall functional ability, as witnessed by a reduction in the Epworth Sleepiness Score. Recent evidence espousing the beneficial effects of CPAP

therapy on daytime functioning in patient groups has been studied previously (Patel, White et al. 2003).

Conclusion

In this study, the hypothesized benefits of exercise training were not realized in moderate OSA patients. Overall CPAP usage was above minimum threshold, but varied tremendously among subjects and may have influenced study results. Both groups failed to significantly alter hemodynamic function in moderate OSA patients without diagnosed hypertension. Nitrite levels rose following CPAP therapy, but parallel improvements in hemodynamic function were not realized. Circulating biomarkers of endothelin-1 production were unaltered following CPAP therapy. Higher intensity exercise regimens may be needed to educe the positive benefits of exercise training in OSA patients without significant cardiovascular dysfunction.

Figure 1. Subject Randomization Protocol



OSA = Obstructive Sleep Apnea; RDI = Respiratory Distress Index; nCPAP = nasal Continuous Positive Airway Pressure

Table 1. nCPAP and nCPAP+Ex Group Characteristics

Patient Characteristics	nCPAP Group (N = 5)		nCPAP+Ex Group (N = 5)	
	Pre-TX	Post-TX	Pre-TX	Post-TX
Age	44.4 ± 3.5		48.2 ± 5.7	
Male	3		5	
Female	2		0	
RDI	24.5 ± 8.4		33.8 ± 4.6	
SaO2 <90%	1.1 ± 0.6		4.4 ± 2.0	
Epworth Score	16.2 ± 1.2[b]	10.2 ± 2.4[b]	11.2 ± 1.5	10.2 ± 1.9
BMI (kg/m^2)	36.1 ± 3.0	36.2 ± 3.0	30.1 ± 1.7	29.6 ± 1.8
Waist: Hip Ratio	0.97 ± 0.04	0.91 ± 0.03	0.97 ± 0.02	0.95 ± 0.02
Neck Circumference (cm)	41.7 ± 1.8	41.7 ± 1.5	42.0 ± 1.0	42.5 ± 1.0
Daytime SBP (mmHg)	126.0 ± 3.0	127.1 ± 6.8	127.2 ± 5.1	130.4 ± 4.5
Daytime DBP (mmHg)	81.0 ± 0.6	82.4 ± 2.0	83.0 ± 1.9	81.3 ± 1.7
Daytime MAP (mmHg)	94.0 ± 0.8	96.1 ± 1.8	96.1 ± 2.5	95.0 ± 2.7
Peak VO2 (ml/kg/min)	19.2 ± 1.3[a]	20.9 ± 2.1	23.8 ± 1.3[a]	25.9 ± 2.3
Peak Watts	150.0 ± 16.2	155.0 ± 16.7	190.0 ± 14.6	193.0 ± 19.2
% CPAP Usage		93.9 ± 4.3		87.9 ± 5.5
CPAP mean usage (min)		375.6 ± 48.6		302.6 ± 41.0

[a]$p < 0.05$ between groups; [b]$p < 0.05$ between treatments; SBP = systolic blood pressure; DBP = diastolic blood pressure, MAP = mean arterial pressure

Table 2. nCPAP and nCPAP+Ex Group Systolic Hemodynamic Measures

Patient Characteristics	nCPAP Group (N = 5)		nCPAP+Ex Group (N = 5)	
	Pre-TX	Post-TX	Pre-TX	Post-TX
Pre-Exercise SBP	126.8 ± 8.8	128.0 ± 8.0	131.2 ± 7.1	133.6 ± 6.8
55 Watt Submaximal Exercise SBP	161.2 ± 17.6	153.6 ± 11.6	149.6 ± 9.5	150.0 ± 13.3
100 Watt Submaximal Exercise SBP	172.8 ± 11.8	170.8 ± 7.9	173.6 ± 16.1	173.0 ± 18.2
Peak Exercise SBP	200.4 ± 11.1	205.2 ± 7.7	205.2 ± 15.1	209.6 ± 17.8
1 min Recovery SBP	171.6 ± 4.7	174.4 ± 8.4	178.8 ± 10.6	173.2 ± 14.4
2 min Recovery SBP	156.0 ± 8.5	163.6 ± 8.4	155.0 ± 12.7	159.2 ± 9.6
3 min Recovery SBP	140.4 ± 5.78	150.8 ± 11.8	154.0 ± 5.9	153.2 ± 8.3
4 min Recovery SBP	133.2 ± 6.8	139.2 ± 11.9	146.0 ± 5.6	143.6 ± 4.9
5 min Recovery SBP	126.4 ± 6.8	132.4 ± 8.2	137.2 ± 10.6	136.4 ± 5.0
6 min Recovery SBP	120.4 ± 17.7	128.5 ± 8.6	130.8 ± 2.7	130.4 ± 4.5

SBP = systolic blood pressure (mmHg)

Table 3. nCPAP and nCPAP+Ex Group Diastolic Hemodynamic Measures

Patient Characteristics	nCPAP Group (N = 5)		nCPAP+Ex Group (N = 5)	
	Pre-TX	Post-TX	Pre-TX	Post-TX
Pre-Exercise DBP	87.2 ± 5.6	86.4 ± 5.8	89.8 ± 6.0	86.8 ± 5.4
55 Watt Submaximal Exercise DBP	88.8 ± 7.7	87.6 ± 6.6	88.0 ± 4.6	90.0 ± 3.2
100 Watt Submaximal Exercise DBP	87.2 ± 5.0	87.6 ± 5.1	90.4 ± 4.6	91.2 ± 3.7
Peak Exercise DBP	88.4 ± 5.9	87.2 ± 5.6	92.4 ± 6.7	84.4 ± 8.0
1 min Recovery DBP	80.4 ± 5.1	78.8 ± 3.5	84.4 ± 3.9	76.4 ± 4.21
2 min Recovery DBP	79.2 ± 5.4	74.8 ± 4.1	79.2 ± 2.7	74.4 ± 6.2
3 min Recovery DBP	78.4 ± 4.8	74.4 ± 4.0	78.8 ± 3.2	81.60± 1.7
4 min Recovery DBP	81.6 ± 4.9	75.6 ± 3.9	80.4 ± 4.0	81.2 ± 1.9
5 min Recovery DBP	79.2 ± 5.4	77.2 ± 4.4	80.8 ± 4.1	80.8 ± 3.1
6 min Recovery DBP	79.6 ± 6.8	82.0 ± 5.2	82.0 ± 3.3	81.6 ± 4.1

DBP = Diastolic Blood Pressure (mmHg)

Table 4. nCPAP and nCPAP+Ex Group Nitric Oxide and Endothelin-1 Biomarker Measures

Measure	nCPAP Group (N = 5)		nCPAP+Ex Group (N = 5)	
	Pre-TX	Post-TX	Pre-TX	Post-TX
Plasma BE (pg/ml)	3.4 ± 0.4[a]	2.9 ± 0.7	2.0 ± 0.3[a]	2.6 ± 0.5
Plasma ET-1 (pg/ml)	<0.78	<0.78	<0.78	<0.78
Plasma Nitrite (μg/ml)	55.6 ± 5.8[b]	76.5 ± 6.4[b]	60.5 ± 10.1	63.9 ± 9.4

[a]$p < 0.05$ between groups; [b]$p < 0.05$ between treatments; BE = Big endothelin-1; ET-1 = endothelin-1

Chapter IV

Recommendations for Future Research

Based on the findings of this study and on published research pertaining to vascular dysfunction and exercise tolerance in obstructive sleep apnea, the following recommendations are made:

1. Two years of recruitment have yielded a total of 20 OSA patients and 13 Control subjects. Use of limited subject samples does not allow definitive conclusions to be made concerning the mechanism of vascular dysfunction in OSA. Enhanced recruitment strategies, including patient stipends, may be necessary to increase sample size. Control subject screening using a portable diagnostic system has demonstrated the potential drawbacks to clinical screening via questionnaire. However, funding for control stipends should be allocated to promote volunteerism and adherence.

2. Vascular function is indirectly assessed through cardiopulmonary stress testing and hemodynamic measurements. The overall research scope would be greatly enhanced by the addition of a direct means of vascular function, i.e. strain gauge plethysmography. Biological sampling performed in concert with strain gauge plethysmography would allow for definitive relationships to be discovered. The addition of a credentialed medical school on campus affords the opportunity for pharmacologic vascular function studies.

3. Current research attempts to circulating biomarkers to delineate the mechanism responsible for cardiovascular co-morbidity in OSA. Much of the fundamental research dealing with the pathophysiology of OSA stems from the intermittent hypoxia rat model. Mechanistic study of vascular control and exercise outcomes would be greatly enhanced by use of the intermittent hypoxia rat model or comparable animal and culture models.

4. Use of cardiopulmonary stress testing has shown promise as a means of assessing cardiovascular co-morbidity in OSA patients. However, limited sample size reduces the impact of this potential tool. Efforts should be made to increase baseline cardiopulmonary stress testing in newly diagnosed OSA patients. Definitive baseline results may direct efforts to specific areas of promise in mechanistic study, treatment options, or prognostic value.

5. Moderate intensity, unsupervised exercise training does not appear to provide an adequate stimulus for cardiovascular adaptation. Future protocols should employ higher intensity exercise under trained supervision in an effort to realize the beneficial effects of exercise in OSA patients.

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APPENDIX A

DETAILED METHODOLOGY

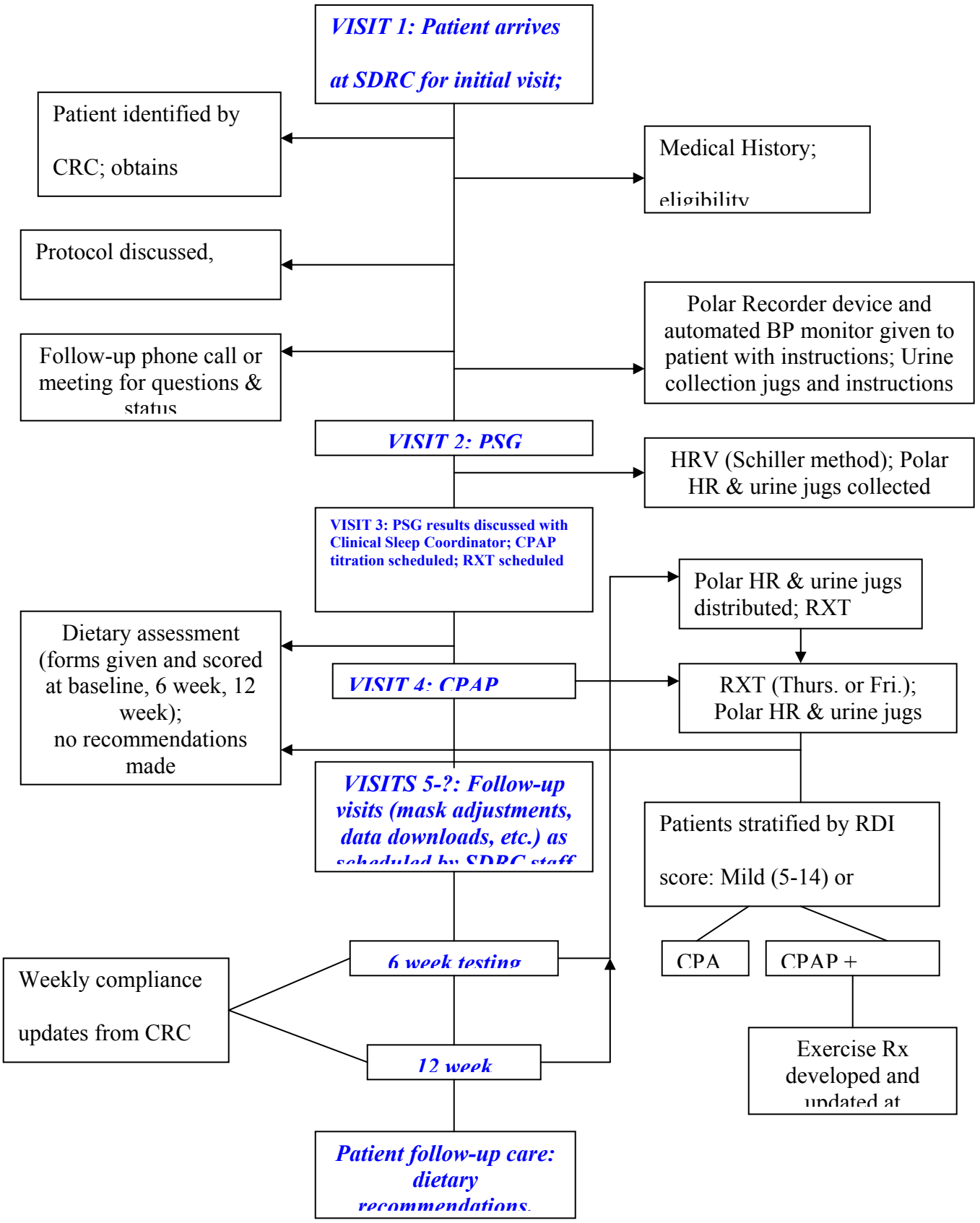
SUBJECTS

Twenty volunteer patients referred to the Southwest Virginia Sleep Disorders Center (SVSDC) in Christiansburg and subsequently diagnosed with OSA (Respiratory Distress Index: RDI >5.0) are selected for study. Each participant is given an informed consent; approval for the protocol and the informed consent process and form was approved by the Virginia Tech IRB for Human Subjects' Research. Patients are excluded from study based on the presence of co-existing cardiovascular, respiratory, metabolic, or orthopedic, musculoskeletal, and/or neuromuscular disabilities. A history of moderately vigorous physical activity ($\geq$3 d/wk, $\geq$ 30 min/session) within 6 months also constitute grounds for study exclusion. In addition, ten apparently healthy subjects are recruited to serve as controls. Absence of OSA was confirmed using a health history questionnaire, standardized sleep questionnaire (Epworth sleepiness scale), sleep-related symptom review, and clearance from their primary care physician. Exclusion criteria for control subjects is identical to that of OSA patients. None of the subjects reported a history of nocturnal gasping, apnea or witnessed apnea by a bed partner, or reported excessive daytime sleepiness. Verification of non-disease status is completed using the Ebletta® portable diagnostic system (ResMed®, Inc., San Diego, CA) during a normal sleep cycle at the subject's home.

STUDY DESIGN AND SCREENING

After initial medical evaluation, patients were briefed prior to full-overnight PSG study concerning the investigation purposes, requirements, and benefits. If the patient agreed to participate, determination was then made as to their medical eligibility. Following positive OSA diagnosis, the patient was scheduled for an experimental testing session prior to initiation of nCPAP therapy. Upon arrival to the Sleep Disorders Center, each participant completed the appropriate questionnaires and routine demographic and health history variables were collected. Subjects then performed a ramped exercise test on an electronically braked stationary ergometer (RXT). The research nurse performed venipunctures at the antecubital vein of the arm in a seated position, collecting a total amount of blood at baseline and each follow-up test interval of $\leq$ 20 ml. A flow chart of recruitment and experimental is depicted in Figure 1.

Figure 1. RESMED 2 FLOW CHART



DAYTIME RESTING BLOOD PRESSURE MEASUREMENT

Measures for daytime resting blood pressure (4/day X 2 days) were obtained on the day preceding the RXT. For each study patient, the Baseline, six, and twelve week tests were completed at the same time of day. Daytime blood pressure was recorded via multiple measurements using an automated digital blood pressure device (Omron HEM-705CP). Recordings were obtained upon awakening in the morning, around mid-day, early evening, and just prior to sleep. Subjects were instructed to obtain recordings in a seated position following a 5-min rest period and to abstain from caffeine and nicotine ingestion as well as vigorous physical activity for at least 12 and 24 hours, respectively prior to measurement. A minimum of 2 hours transpired after food consumption before blood pressure measurement. A minimum of 2 measurements were recorded at each specified time until 2 measurements were within $\leq$ 5 mmHg.

RAMP EXERCISE TESTING (RXT) PROCEDURES

The RXT was completed on an electronically braked cycle ergometer (CardioO_2®, MedGraphics, St. Paul, MN). Following a 1 min warm-up period at an initial rate of 25 W, the ergometer was ramped at a rate of 15 W/min to achieve a total duration of 8-12 min. Cardiopulmonary measurements were obtained throughout the RXT. Blood pressure was acquired by manual auscultation at the antecubital fossa of the left forearm by trained personnel at baseline, 2 min, 5 min, and at 2 min intervals until test termination. Blood pressure at peak exercise was also measured prior to exercise termination. Subjects continued exercising until a maximal effort (RPE> 17) was attained. A licensed physician or EMT was present for every test and an ACSM Certified

Exercise Specialist[SM] supervised the RXT. Test termination criteria was in accordance with AHA and ACSM standards. Participants completed an active recovery phase at 25 W that was terminated upon subject request. Blood pressure was recorded at 1 min intervals until 6 min into recovery.

DESCRIPTION OF EXPERIMENTAL TREATMENTS

After the initial RXT, subjects were randomized to the nCPAP or nCPAP plus exercise training groups as illustrated in Figure 2. Study patients in the nCPAP or CPAP+Ex groups were prescribed a Sullivan® V Elite® or other Sullivan® nCPAP system. These particular ResMed® units are capable of recording data on time, date, and actual hours of usage at pressure, which can be downloaded to a computer (ResMed®, Inc., San Diego, CA). Patients randomized into the nCPAP+Ex group subsequently participated in aerobic exercise-training program for 12 wk. Initially, exercise training sessions were supervised under continuous electrocardiographic monitoring in the Laboratory for Health & Exercise Sciences at Virginia Tech. Exercise was initially performed 3 days/wk, 30-40 min/session, at an intensity range of 50-60% of VO_2pk. Sessions included a 5 to 10 min warm-up and cool-down. Blood pressure and RPE were monitored and recorded for each exercise session. Exercise progression occurred according to the ability to maintain target HR, i.e. at 50-60% VO_2pk and perceived effort, i.e. RPE. Increases in exercise duration preceded those of intensity until subjects could maintain 30-40 min of continuous exercise. Thereafter, increases in intensity were made to maintain a HR at an intensity range of 50-60% VO_2pk. At wk 6, a peak VO_2 RXT were repeated and the exercise prescription adjusted according to the guidelines

previously mentioned. Two weeks after the start of the supervised exercise training program (3 days/wk), patients transitioned to unsupervised sessions on days not scheduled for supervised exercise. At week 5, subjects fully transitioned to a schedule of unsupervised exercise 4-days/wk. Exercise logs and heart rate monitors were provided for documentation of all sessions. Subjects could participate in one of three local community activity centers or at the University-based adult exercise program held 3 mornings/wk in War Memorial Hall on the campus of Virginia Tech. Activities in alternative exercise facilities included walking/jogging or cycle ergometry, in accordance with individualized plans specified by the research staff. To provide flexibility for exercise sessions, subjects were offered the opportunity to participate in morning, afternoon and/or evening monitored exercise sessions at the Laboratory for Health and Exercise Sciences. Subjects in the CPAP+Ex and CPAP groups were contacted weekly by the research nurse to verify exercise adherence. The research nurse or an exercise staff member met monthly with each subject to review their study experience, discuss any relevant problems/issues related to compliance with CPAP therapy and/or exercise treatments if necessary. Subjects in the Control and CPAP groups abstained from regular exercise training for a period of 12 wk. After 12 wk, subjects in all three groups completed follow-up testing on all measures identical to baseline.

BLOOD COLLECTION PROCEDURES

Blood is drawn prior to RXT by antecubital venipuncture with the subject in a seated position. Blood is collected in glass tubes containing either the anticoagulant agent EDTA or without anticoagulant (total volume ≤ 20ml). Circulating mononuclear cells are separated from whole blood prior to centrifugation. Collection tubes are centrifuged (2500 rpm) at room temperature for 15 min no longer than 2 hr post blood draw. Plasma and serum samples are stored in 1.8 ml cryogenic tubes at -80° C.

MONONUCLEAR CELL ISOLATION PROCEDURE

1. To a 15-mL conical centrifuge tube, add 3.0 mL HISTOPAQUE-1077 and bring to room temperature.
2. Layer 3.0 mL whole blood onto the HISTOPAQUE-1077. Centrifuge at 400 x g for exactly 30 min at room temperature. Centrifugation at lower temperature, such as 4°C, may result in cell clumping and poor recovery.
3. After centrifugation, aspirate, with a Pasteur pipet, the upper layer (Plasma) to within 0.5 cm of the opaque interface containing mononuclear cells. Discard upper layer.
4. Transfer the opaque interface, with a Pasteur pipet, into a clean conical centrifuge tube.
5. Add to this tube (step 4) 10 mL Isotonic Phosphate Buffered Saline Solution and mix by gentle aspiration.
6. Centrifuge at 250 x g for 10 min.
7. Aspirate the supernatant and discard.

8. Re-suspend cell pellet with 5.0 mL isotonic PBS and mix by gentle aspiration with a Pasteur pipet.
9. Centrifuge at 250 x g for 10 min.
10. Repeat steps 7, 8,and 9, discard supernatant and re-suspend cell pellet in 0.5 mL Isotonic PBS.

RNeasy PROTOCOL FOR ISOLATION OF TOTAL RNA FROM ANIMAL CELLS

1. Once cells have been harvested, loosen the cell pellet thoroughly by flicking the centrifuge tube.
2. Add 600 µl RLT and 6 µl (1%) β-mercaptoethanol to the conical centrifuge tube containing the mononuclear cell pellet. βME is toxic; dispense in a fume hood and wear protective clothing.
3. Vortex or pipet to mix.
4. Transfer both the lysed cells and buffer mixture to a microcentrifuge tube.
5. Store at -80°C.

RNA PURIFICATION PROCEDURE

1. Homogenize the sample by one of the following three methods:
    a. Pipet lysate directly onto a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed.
    b. Homogenize cells for 30 sec using a rotor-stator homogenizer.

c. Pass the lysate at least 5 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe.

2. Add 1 volume (usually 350 ul or 600 ul) of 70% ethanol to the homogenized lysate, and mix well by pipetting. DO NOT CENTRIFUGE.

   Adjust amount of ethanol accordingly

3. Apply up to 700 ul of the sample, including any precipitate that may have formed, to an RNeasy mini column placed in a 2 ml collection tube. Close the tube gently, and centrifuge for 15 sec at ≥8000 x g. Discard the flow through and reuse in the following step.

   DO NOT USE MORE THAN 1 x 10^7 CELLS IN COLUMN.

4. Add 700 ul Buffer RW1 to the RNeasy column. Close the tube gently, and centrifuge for 15 s at ≥8000 x g to wash the column. Discard the flow-through and collection tube.

5. Transfer the RNeasy column into a new 2 ml collection tube. Pipet 500 ul Buffer RPE onto the RNeasy column. Close the tube gently, and centrifuge for 15 sec at ≥8000 x g to wash the column. Discard the flow-through and re-use collection tube in the following step.

   Make sure that ethanol is added to Buffer RPE before use.

6. Add another 500 ul Buffer RPE to the RNeasy column. Close the tube gently and centrifuge for 2 min at ≥8000 x g to dry the RNeasy silica-gel membrane. **Let sit with lid open for ~5 min to remove all ethanol from membrane.**

7. To elute, transfer the RNeasy column to a new 1.5 ml collection tube. Pipet 30 ul RNase-free water directly onto the RNeasy silica-gel membrane. Close the tube gently, and centrifuge for 1 min at ≥8000 x g to elute.
8. Repeat the above step into a second collection tube to ensure all RNA is collected.
9. Determine the amount of RNA present with a spectrophotometer and run a 1% agarose gel to ensure quality.

ENDOTHELIN-1 ELISA

Sample Preparation

Plasma samples should be drawn into chilled EDTA tubes containing Aprotonin (500 KIU/mL of blood). Centrifuge blood at 1600 xg for 15 min at 0°C. Store in plastic tube at -70° C.

Procedural Notes

- Allow all reagents to warm to room temp for at least 30 min before opening
- Standards can be made in either glass or plastic tubes
- Pre-rinse the pipet tip with reagent
- Add the reagents to the side of the well to avoid contamination
- Unused plate strips must be kept desiccated at 4°C in the sealed foil bag.
- PRIOR TO ADDITION OF STANDARD, ANTIBODY OR SUBSTRATE, ENSURE THAT THERE IS NO RESIDUAL WASH BUFFER IN THESE WELLS. ANY REMAINING WASH BUFFER MAY CAUSE VARIATION IN ASSAY RESULTS.

Reagent Preparation

1. **Wash Buffer**

   Prepare wash buffer by diluting 25 ml of the supplied concentrate with 975 ml of deionized water. This can be stored at 4°C until the kit expiration date.

2. **ET-1 Standards**

   - Add 500 µl of deionized water to the ET-1 standard. Let sit at room temp for 5 min. Mix gently. This solution contains 200 pg/ml ET-1.
   - Label eight 12 x 75 mm glass tubes #1 through #8. Pipet 220 µl of Assay Buffer into tubes #1 through #8. Add 220 µl of the 200 pg/ml standard to tube #1. Vortex. Add 220 µl of tube #1 to tube #2 and vortex thoroughly. Continue for tubes #3 through #8.

     The concentration of ET-1 in tubes #1 through #8 will be 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 pg/ml, respectively. Store standard at -20 °C.

1. **Preparation of Labeled Antibody Conjugate**

   Add the entire contents of 1 bottle of Labeled Antibody Diluent to the vial of ET-1 Antibody Conjugate. Let stand at room temp for 5 min and then vortex gently.

2. **Preparation of Substrate**

   Just prior to addition of the substrate solution to the plate, prepare the substrate by adding 1 substrate tablet to 2.5 ml of Substrate Buffer and mix allowing the tablet to completely dissolve before proceeding. Ensure that the tablet has completely

dissolved before proceeding. Add 2.75 ml of the peroxide solution to this and mix well. **Use within 15 min.**

Assay Procedure

1. Wash the plate by adding 400 µl of wash solution to every well. Repeat the wash 1 more time for a total of 2 washes. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
2. Pipet 100 µl of Assay Buffer into the S0 (0 pg/ml Standard) wells.
3. Pipet 100 µl of Standards #1 through #8 into the appropriate wells.
4. Pipet 100 µl of the samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at 4°C for 18-24 hr **OR** at 37°C for 1 hr.
7. Empty the contents of the wells and wash by adding 400 µl of wash solution to every well. Repeat the wash 6 more times for a total of 7 washes. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 100 µl of the Labeled Antibody into each well, except the Blank.
9. Seal the plate and incubate at 37°C for 30 min. Prepare Substrate.

    - **Preparation of Substrate (stated above)**

    Just prior to addition of the substrate solution to the plate, prepare the substrate by adding 1 substrate tablet to 2.5 ml of Substrate Buffer and mix allowing the tablet to completely dissolve before proceeding. Ensure that the

tablet has completely dissolved before proceeding. Add 2.75 ml of the peroxide solution to this and mix well. **Use within 15 min.**

10. Empty the contents of the wells and wash by adding 400 µl of wash solution to every well. Repeat the wash 8 more times for a total of 9 washes. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 100 µl of the substrate solution to each well.
12. Incubate for 30 min at room temp in the dark.
13. Add 100 µl of Stop Solution to each well.
14. Blank the plate reader against the Blank wells, read the optical density at 450 nm, preferably with correction between 570 and 590 nm.

BIG ENDOTHELIN ELISA

Procedural Notes

- Do not mix components from different kit lots or use reagents beyond the kit expiration date.
- Allow all reagents to warm to room temperature for at least 30 minutes before opening.
- Standards can be made up in either glass or plastic tubes.
- Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
- Pipet standards and samples to the bottom of the wells.
- Add the reagents to the side of the well to avoid contamination.

- This kit uses plates with removable strips. Unused strips must be kept desiccated at 4°C in the sealed foil bag. The strips should be used in the frame provided.
- **Prior to addition of antibody or substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. Wash Buffer

Dilute the Wash Buffer Concentrate 1:40 by measuring 25 mls of the Concentrate and bring the volume up to 1,000 mL with deionized water. The diluted Wash Buffer is stable stored at 4°C until the kit's expiration date, or for 3 months, whichever is earlier.

2. human Big Endothelin-1 Standards

- Add 500 µL of deionized water to the Big Endothelin-1 Standard. Let it sit at room temperature for 5 minutes. Mix it gently. This solution contains 200 pg/mL Big Endothelin-1.
- Label eight 12 x 75 mm glass tubes #1 through 8. Pipet 220 µL of Assay Buffer into tubes #1 through #8. Add 220 µL of the 200 pg/mL standard to tube #1. Vortex. Add 220 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #8.

The concentration of Big Endothelin-1 in tubes #1 through #8 will be 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 pg/mL respectively. See Big

Endothelin-1 Assay Layout Sheet for dilution details. STORE STANDARD AT -20°C, avoid repeated freeze/thaws.

3. Preparation of Labeled Antibody Conjugate

Add the entire contents of one (1) bottle of Labeled Antibody Diluent to the vial of Big Endothelin-1 Antibody Conjugate. Let it stand at room temperature for 5 minutes and then vortex it gently. After reconstitution any unused Labeled Antibody should be aliquoted and stored at -20°C. Avoid repeated freeze-thaws of the aliquots.

4. Preparation of Substrate

Just prior to addition of the substrate solution to the plate, prepare the substrate by adding 1 substrate tablet to 2.5 mL of Substrate Buffer and mix. Ensure that the tablet has completely dissolved before proceeding. Add 2.75 mL of the peroxide solution to this and mix well. Use within 15 minutes.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the foil pouch and seal the Ziploc. Store unused wells at 4°C.
2. Pipet 100 µL of Assay Buffer into the S0 (0 pg/mL Standard) wells.
3. Pipet 100 µL of Standards #1 through #8 into the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.

6. Seal the plate and incubate at 4°C overnight.
7. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 6 more times for a total of **7 washes**. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 100 µL of the Labeled Antibody into each well, except the Blank.
9. Seal the plate and incubate at 4°C for 30 minutes. Prepare Substrate (See page 5, Section 4).
10. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 8 more times for a total of **9 washes**. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 100 µL of the Substrate Solution to each well.
12. Incubate for 30 minutes at room temperature in the dark.
13. Add 100 µL of Stop Solution to each well.
14. Blank the plate reader against the Blank wells, read the optical density at 450nm., preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the blank wells from all readings.

MONONUCLEAR CELL ISOLATION PROCEDURE

11. To a 15-mL conical centrifuge tube, add 3.0 mL HISTOPAQUE-1077 and bring to room temperature.

12. Layer 3.0 mL whole blood onto the HISTOPAQUE-1077. Centrifuge at 400 x g for exactly 30 min at room temperature. Centrifugation at lower temperature, such as 4°C, may result in cell clumping and poor recovery.
13. After centrifugation, aspirate, with a Pasteur pipet, the upper layer (Plasma) to within 0.5 cm of the opaque interface containing mononuclear cells. Discard upper layer.
14. Transfer the opaque interface, with a Pasteur pipet, into a clean conical centrifuge tube.
15. Add to this tube (step 4) 10 mL Isotonic Phosphate Buffered Saline Solution and mix by gentle aspiration.
16. Centrifuge at 250 x g for 10 min.
17. Aspirate the supernatant and discard.
18. Re-suspend cell pellet with 5.0 mL isotonic PBS and mix by gentle aspiration with a Pasteur pipet.
19. Centrifuge at 250 x g for 10 min.
20. Repeat steps 7, 8,and 9, discard supernatant and re-suspend cell pellet in 0.5 mL Isotonic PBS.
21. Once cells have been harvested, loosen the cell pellet thoroughly by flicking the centrifuge tube.
22. Add 600 µl RLT and 6 µl (1%) β-mercaptoethanol to the conical centrifuge tube containing the mononuclear cell pellet. βME is toxic; dispense in a fume hood and wear protective clothing.
23. Vortex or pipet to mix.

24. Transfer both the lysed cells and buffer mixture to a microcentrifuge tube.
25. Store at -80°C.

RNA PURIFICATION PROCEDURE (RNeasy® Mini Kit, Qiagen, Bothell, WA)

10. Pipet lysate directly onto a QIAshredder® spin column placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed to homogenize sample.
11. Add 1 volume (usually 350 ul or 600 ul) of 70% ethanol to the homogenized lysate, and mix well by pipetting. DO NOT CENTRIFUGE.

    Adjust amount of ethanol accordingly
12. Apply up to 700 ul of the sample, including any precipitate that may have formed, to an RNeasy mini column placed in a 2 ml collection tube. Close the tube gently, and centrifuge for 15 sec at ≥8000 x g. Discard the flow through and reuse in the following step.

    DO NOT USE MORE THAN 1 x 10^7 CELLS IN COLUMN.
13. Add 700 ul Buffer RW1 to the RNeasy column. Close the tube gently, and centrifuge for 15 s at ≥8000 x g to wash the column. Discard the flow-through and collection tube.
14. Transfer the RNeasy column into a new 2 ml collection tube. Pipet 500 ul Buffer RPE onto the RNeasy column. Close the tube gently, and centrifuge for 15 sec at ≥8000 x g to wash the column. Discard the flow-through and re-use collection tube in the following step.

    Make sure that ethanol is added to Buffer RPE before use.

15. Add another 500 ul Buffer RPE to the RNeasy column. Close the tube gently and centrifuge for 2 min at ≥8000 x g to dry the RNeasy silica-gel membrane. **Let sit with lid open for ~5 min to remove all ethanol from membrane.**
16. To elute, transfer the RNeasy column to a new 1.5 ml collection tube. Pipet 30 ul RNase-free water directly onto the RNeasy silica-gel membrane. Close the tube gently, and centrifuge for 1 min at ≥8000 x g to elute.
17. Repeat the above step into a second collection tube to ensure all RNA is collected.
18. Determine the amount of RNA present through Agilent 2100 Bioanalyzer®.

RNA 6000 NANO ASSAY (Agilent 2100 Bioanalyzer®, Agilent Technologies, Palo Alto, CA)

Preparing Gel Dye Mix

1. Place 400 µl of RNA gel matrix into the top receptacle of a spin filter.
2. Place the spin filter in a microcentrifuge and spin at 4000 rpm for 10 min.
3. Place 130 µl of the filtered RNA gel matrix into an RNAse free 1.5 ml microfuge tube and add 2 µl of RNA dye concentrate.
4. Cap the tube, vortex thoroughly and visually inspect proper mixing of gel and dye.

Procedure

1. Take a new RNA chip out of sealed bag.
2. Place the chip on the Chip Priming Station.
3. Draw up 9.0 µl of the gel-dye mix with a pipette.

4. Place the tip of the pipette at the bottom of the well marked G and dispense the gel-dye mix (see attached figure).

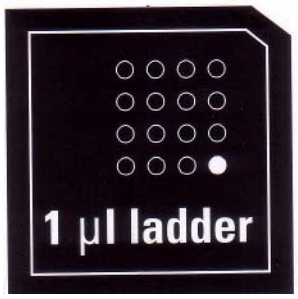
5. Make sure the plunger is at 1 ml, then close the Chip Priming Station.
6. Press plunger until it is held by the syringe clip.
7. Wait for exactly 30 sec and then release the plunger with the clip release mechanism.
8. Pull back the plunger to the 1 ml position.
9. Open the Chip Priming Station.
10. Turn over the chip to check for air bubbles.
11. Pipette 9.0 µl of the gel-dye mix in each of the wells marked G (see attached figure).

12. Draw up 5 µl of the RNA 6000 Nano Marker.
13. Place the pipette tip all the way to the bottom of the well marked with the ladder symbol. Dispense the buffer into the well.

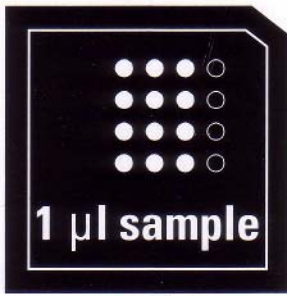
14. Dispense 5 µl of the RNA 6000 Nano Marker into each of the 12 sample wells (see attached figure).

15. Draw up 1 µl of the RNA 6000 ladder into a pipette.

16. Place the pipette tip all the way to the bottom of the well marked with the ladder symbol. Dispense the ladder into the well (see attached figure).



17. Pipette 1 µl of each sample into each of the 12 sample wells (see attached figure).



18. Place the chip in the adapter of the vortex mixer. Vortex for 1 min at the IKA vortexer set-point.

19. Place the chip in the Agilent 2100 Bioanalyzer® and start the run within 5 min. Select the Eukaryote Total RNA Nano Assay and begin analysis.

cDNA SYNTHESIS REACTION ASSEMBLY AND PROTOCOL (Invitrogen®, Carlsbad, CA)

To a 0.2ml tube, assemble:

4 ul 5X iScript Reaction Mix

1 ul iScript reverse transcriptase

x ul Nuclease-free water

x ul RNA sample

20ul final volume

Next, program the thermal cycler to run the following protocol:

5 min at 25°C

30 min at 42°C

5 min at 85°C

Hold at 4°C

DNA 1000 ASSAY (Agilent 2100 Bioanalyzer®, Agilent Technologies, Palo Alto, CA)

Preparing Gel Dye Mix

1. Add 25 µl of dye into a DNA gel matrix vial.
2. Cap the tub and vortex well.
3. Open the tube and transfer the gel-dye mix to the top receptacle of a spin filter.
4. Place the spin filter in a microcentrifuge and spin at 6000 rpm for 15 min.

5. Discard the filter.

Procedure

1. Take a new DNA chip out of sealed bag.
2. Place the chip on the Chip Priming Station.
3. Draw up 9.0 µl of the gel-dye mix with a pipette.
4. Place the tip of the pipette at the bottom of the well marked G and dispense the gel-dye mix (see attached figure).

5. Make sure the plunger is at 1 ml, then close the Chip Priming Station.
6. Press plunger until it is held by the syringe clip.
7. Wait for exactly 60 sec and then release the plunger with the clip release mechanism.
8. Pull back the plunger to the 1 ml position.
9. Open the Chip Priming Station.
10. Turn over the chip to check for air bubbles.
11. Pipette 9.0 µl of the gel-dye mix in each of the wells marked G (see attached figure).

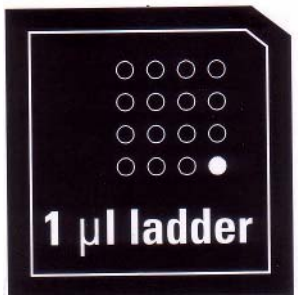
12. Draw up 5 µl of the gel-dye mix in the well marked with the ladder symbol (see attached figure).

13. Draw up 5 µl of the DNA 1000 Markers in a pipette.

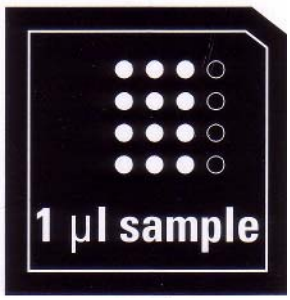
14. Dispense 5 µl of the marker into each of the 12 sample wells (see attached figure).

15. Draw up 1 µl of the DNA 1000 ladder into a pipette.

16. Place the pipette tip all the way to the bottom of the well marked with the ladder symbol. Dispense the ladder into the well (see attached figure).



17. Pipette 1 µl of each sample into each of the 12 sample wells (see attached figure).



18. Place the chip in the adapter of the vortex mixer. Vortex for 1 min at the IKA vortexer set-point.
19. Place the chip in the Agilent 2100 Bioanalyzer® and start the run within 5 min. Select the DNA 1000 Assay and begin analysis.

POLYMERASE CHAIN REACTION (Taq PCR Master Mix, Qiagen®, Bothell, WA)

1. Thaw primer solutions. Prepare primer mix of an appropriate concentration using the water provided. The final volume of diluted primer mix plus the template DNA, added at step 4, should be 50 µl per reaction.

Component	Volume/reaction	Final Concentration
Taq PCR Master Mix	50 µl	2.5 units Taq DNA poly 1x QIAGEN PCR Buffer

		200 µM of each dNTP
Diluted primer mix		
Primer A	10 µl	0.05 µM
Primer B	10 µl	0.05 µM
Distilled water	29 µl	----
Template DNA		
Template DNA, added at step 4	1 µl	≤1 µg/reaction
Total Volume	**100 µl**	----

2. Mix the Taq PCR Master Mix by vortexing briefly, and dispense 50 µl into each PCR tube (see above).
3. Distribute the appropriate volume of diluted primer mix into the PCR tubes containing the Master Mix
4. Add template DNA (≤1 µg/reaction) to the individual PCR tubes.

   For RT-PCR, add an aliquot from the reverse transcriptase reaction. The volume added should not exceed 10% of the final PCR volume.
5. Program the thermal cycler according to the manufacturer's instructions.
6. Simplified hot start: Start the PCR program. Once the thermal cycler has reached 94°C, place the PCR tubes in the thermal cycler.

Thermal cycler conditions		**NOTE**
Initial denaturation:	3 min	94°C
3-step cycling		
Denaturation:	0.5 min	94°C
Annealing:	1 min	60°C

Extension:	1 min	72°C
Number of cycles:	40	
Final extension:	10 min	72°C

REAL TIME PCR (Bio-Rad® iCycler, Hercules, CA)

cDNA samples were diluted 1:10 in molecular grade water. Aliquots of iQ® SYBR® Green Supermix were combined in PCR ready tubes in the following volumes:

Component	**Volume per reaction**	**Final Concentration**
iQ SYBR Green Supermix	100 µl	1X
Primer 1 (Sense)	1 µl	0.5 µM
Primer 2 (Antisense)	1 µl	0.5 µM
Sterile Water	295 µl	

50 µl of each aliquot were used as negative controls. 3 µl of sterile water was added to the mixture and mixed by repeated pipetting. 3 µl of diluted template cDNA was then added to each tube to bring the total volume to 300 µl. Samples were serially pipetted into a sterile optical plate. All samples were run in triplicate. SYBR Green Supermix is composed of 100 mM KCl, 40 mM Tris-HCl (pH 8.4), 0.4 mM of each dNTP, iTaq DNA polymerase (50 unit/ml), 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, and stabilizers. Optical thermal cycler conditions were as follows:

Thermal cycler conditions		**NOTE**
Initial denaturation:	3 min	95°C
3-step cycling		

Denaturation:	0.5 min	95°C
Annealing:	1 min	60°C
Extension:	1 min	72°C
Number of cycles:	50	
Final extension:	10 min	72°C

Additionally, a melt curve was completed with a 10 second hold every 0.5°C from 95°C to 58°C.

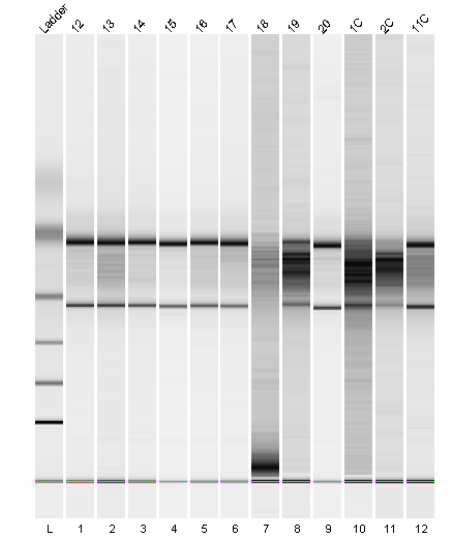
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

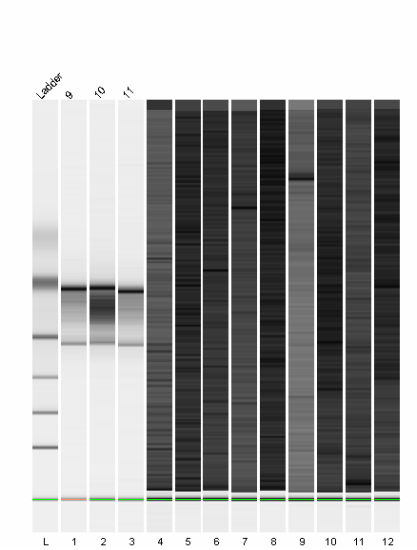
Proteins from plasma samples were removed by filter centrifugation at 14,000g for 10 min at 27°C. 20 µl of filtrate was injected into a 100 x 4.6 mm Wescan Anion/S IC Column (Alltech Associates, Deerfield, IL). Mobile phase consisted of 1.5 mM sulfuric acid at a flow rate of 1 ml/min. Nitrate and nitrite were detected by an UV detector at 210 nm. Sample detection and analysis was completed on the Beckman-Coulter System Gold HPLC System (Fullerton, CA).

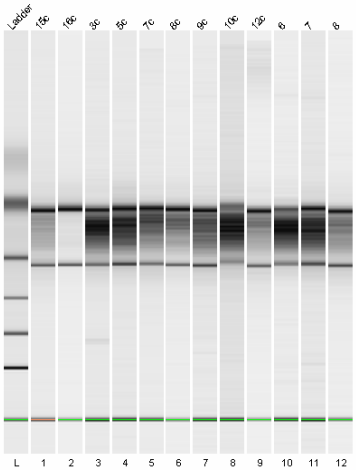
APPENDIX B

RAW DATA

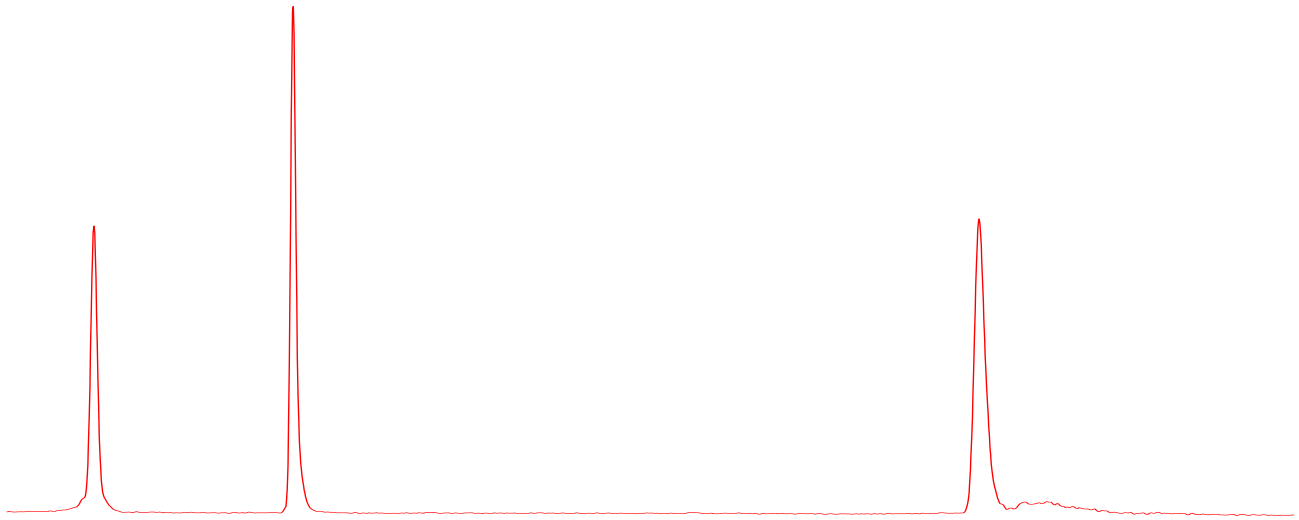
RNA Quality Analysis



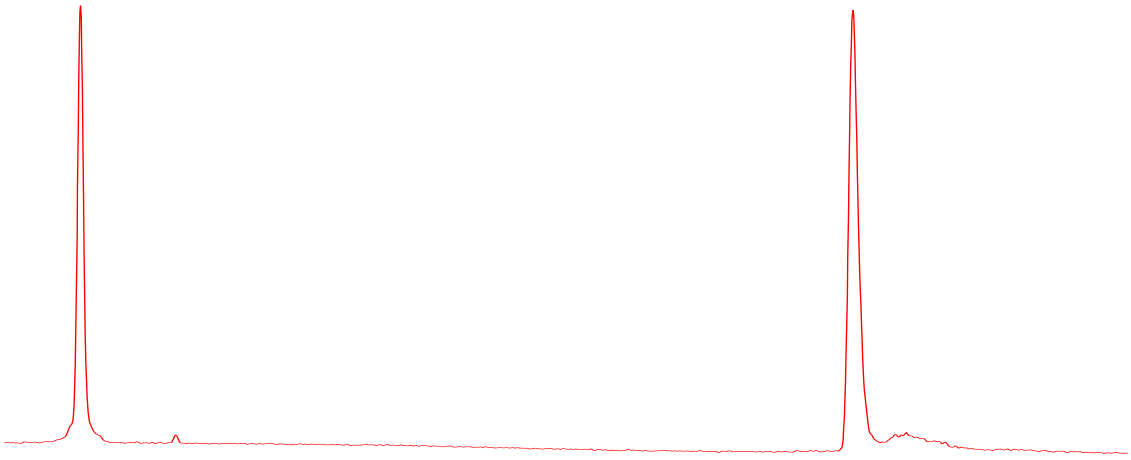




PCR Amplification of B-Actin Test Sample



PCR Amplification of Preproendothelin Test Sample



ID #	sample 1	sample 2	RT-PCR	RDI	<90% O2	low O2	Disease	post trt	CPAP
1C	wk 1	no	no				0	0	0
2C	wk 1	no	no				0	0.00	0
3C	wk 1	no	yes				0	0.00	0
5C	wk 1	no	no				0	0.00	0
6C	wk 1	wk 12	no				0	1.00	0
7C	wk 1	wk 12	yes				0	1.00	0
8C	wk 1	no	yes				0	0.00	0
9C	wk 1	wk 6	no				0	1.00	0
10C	wk 1	wk 6	no				0	1.00	0
11C	wk 1	no	yes				0	0.00	0
12C	wk 1	wk 6	yes				0	1.00	0
15C	wk 1	wk 12	yes				0	1.00	0
16C	wk 1	wk 12	yes				0	1.00	0
5	wk 1	wk 12	no	10.5	1.6	14	1	1.00	1
6	wk 1	wk 12	no	32	7.8	10	1	1.00	0
7	wk 1	no	yes	19.2	0	17	1	0.00	1
8	wk 1	wk 12	yes	55.1	0	16	1	1.00	1
9	wk 1	no	yes	43.6	3	10	1	0.00	1
10	wk 1	no	yes	20	10	8	1	0.00	0
11	wk 1	wk 12	yes	51	10	7	1	1.00	0
12	wk 1	wk 12	yes	33.2	4	15	1	1.00	0
13	wk 1	wk 12	yes	9.8	3	15	1	1.00	1
14	wk 1	wk 12	yes	7.6	2	11	1	1.00	1
15	wk 1	wk 12	yes	17.7	1	21	1	1.00	1
16	wk 1	wk 12	yes	29.6	0	15	1	1.00	1
17	wk 1	wk 12	yes	29.3	0	10	1	1.00	0
18	wk 1	wk 12	no	23.3	0	14	1	1.00	0
19	wk 1	no	yes	6.7	0	13	1	0.00	0
20	wk 1	no	yes	19	2	9	1	0.00	1

CPAP + EX	Control	Birthdate	Age	Male	Female	lbs	kg	lbs	kg
0	1	10/26/1959	42	1	0	160	72.73		
0	1	9/21/1973	28	0	1	204.25	92.84		
0	1	6/7/1955	46	0	1	193.00	87.73		
0	1	7/22/1967	34	0	1	153.25	69.66		
0	1	1/15/1965	36	1	0	229.00	104.09	226.75	103.07
0	1	4/24/1950	51	1	0	190.00	86.36	192.00	87.27
0	1	1/21/1957	44	0	1	192.25	87.39		
0	1	12/27/1966	34	0	1	127.75	58.07	130.75	59.43
0	1	5/14/1964	37	1	0	157	71.36	154	70.00
0	1	10/16/1969	32	1	0	196.00	89.09		
0	1	8/21/1955	46	1	0	225.25	102.39	227	103.18
0	1	7/23/1966	36	0	1	138.00	62.73	141.00	64.09
0	1	7/21/1944	59	0	1	193.00	87.73	197.50	89.77
0	0	2/27/1952	49	0	1	184.75	83.98	189.00	85.91
1	0	1/31/1968	33	1	0	199.25	90.57	192.00	87.27
0	0	5/26/1948	53	0	1	218.00	99.09		
0	0	5/24/1957	44	1	0	192.75	87.61	192.00	87.27
0	0	5/9/1939	62	1	0	219.25	99.66		
1	0	11/5/1962	39	0	1	233.25	106.02		
1	0	10/18/1940	61	1	0	166.50	75.68	165.10	75.05
1	0	7/21/1965	37	1	0	253.00	115.00	243.00	110.45
0	0	5/19/1955	47	0	1	247.75	112.61	252.00	114.55
0	0	2/3/1977	25	0	1	313.50	142.50	318.00	144.55
0	0	1/20/1971	31	1	0	312.50	142.05	318.00	144.55
0	0	5/15/1951	51	1	0	280.00	127.27	269.00	122.27
1	0	8/28/1951	51	1	0	264.25	120.11	268.50	122.05
1	0	10/11/1943	59	1	0	249.00	113.18	244.50	111.14
1	0	9/23/1960	42	1	0	168.50	76.59		
0	0	3/12/1961	42	1	0	207.25	94.20		

Height cm	Height m	BMI 1	BMI 2	Waist 1 (cm)	Hip 1 (cm)	Waist 2 (cm)	Hip 2 (cm)	W/H Ratio 1
176.00	1.76	23.48		88.90	101.60			0.88
169.80	1.70	32.20		96.52	121.29			0.80
162.50	1.63	33.22		94.00	120.00			0.78
167.64	1.68	24.79		82.00	107.60			0.76
177.30	1.77	33.11	32.79	111.00	114.00	111.10	113.00	0.97
169.10	1.69	30.20	30.52	98.3	109	102.5	108.70	0.90
159.40	1.59	34.39		87.9	119			0.74
169.10	1.69	20.31	20.78	71.2	94	68.8	94.41	0.76
168.50	1.69	25.13	24.65	83	99.2	75.9	100.00	0.84
171.00	1.71	30.47		104.7	110.2			0.95
178.00	1.78	32.31	32.57	113	116.6	110.3	114.00	0.97
162.00	1.62	23.90	24.42	70.5	98.5	70.50	109.50	0.72
164.00	1.64	32.62	33.38	104	107	108	114.00	0.97
164.30	1.643	31.11	31.82	94.20	116.60	90.90	116.00	0.81
181.30	1.813	27.55	26.55	107.00	106.20	96.50	107.30	1.01
168.00	1.68	35.11		110.00	132.20			0.83
179.80	1.798	27.10	27.00	109.50	105.50	98.30	105.00	1.04
184.30	1.843	29.34		112.10	110.00			1.02
161.00	1.61	40.90		118.00	132.50			0.89
172.80	1.728	25.35	25.13	93.20	99.50	90.30	99.50	0.94
189.75	1.8975	31.94	30.68	112.70	117.00	109.00	113.20	0.96
162.00	1.62	42.91	43.65	120.00	131.00	124.00	133.80	0.92
162.80	1.628	53.77	54.54	132.50	158.50	132.50	156.50	0.84
190.00	1.9	39.35	40.04	127.00	134.10	127.50	136.50	0.95
178.30	1.783	40.03	38.46	118.00	130.50	119.00	125.50	0.90
185.30	1.853	34.98	35.54	118.50	117.00	123.50	119.00	1.01
192.00	1.92	30.70	30.15	111.00	120.50	112.00	118.00	0.92
180.40	1.804	23.53		87.00	101.30			0.86
179.00	1.79	29.40		106.00	105.50			1.00

W/H Ratio 2	Neck Cir 1 (cm)	Neck Cir 2 (cm)	Epworth 1	Epworth 2	% usage	usage (min)	SBP am
	36.83		7				109.5
	36.83		14				104
	33.00		3				102.5
	35.00		4				103.5
0.98	43.50	41.10	5	4			134.5
0.94	39	39.6	5	8			123
	34.5		2				108
0.73	32.4	29	9	8			105.5
0.76	35.8		5	7			112.5
	40.3		4				128.5
0.97	42.1	41.5	4	9			112
0.64	30.8	30.5	2	3			96
0.95	36	35.8	2	2			110.5
0.78	36.50	37.50	14	7	100	495	122
0.90	40.30	40.00	10	11	100	389	108
	37.90		17				129.5
0.94	39.40	38.80	16	13	93.3	236	113.7
	44.00		10				171
	36.60		8				123
0.91	38.80	40.60	7	7	88.2	182	134
0.96	44.00	43.50	15	10	100	400	127.5
0.93	41.50	41.80	15	17	100	427	120
0.85	44.70	45.50	11	9	64.30	491.00	126
0.93	46.20	46.00	21	11	77.30	288.00	132
0.95	44.80	44.80	15	3	98.90	432.00	123.5
1.04	43.50	44.50	10	6	72.50	277.00	109.5
0.95	43.30	44.00	14	17	78.7	265	135
	36.00		13				105.5
	41.00		9				126.5

DBP am	MAP am	SBP n	DBP n	MAP n	SBP af	DBP af	MAP af	SBP pm	DBP pm	MAP pm	SBP day
69.3	81.36	119.5	80	91.85	116.8	77.5	89.29	111.3	74.3	85.4	114.275
68.5	79.15	111.5	80.5	89.8	123	74.5	89.05	117	71.5	85.15	113.875
68.5	78.7	103	72	81.3	102	71	80.3	107.5	73.5	83.7	103.75
62	74.45	101.5	69	78.75	103	65.7	76.89	102	63.4	74.98	102.5
94	106.15	128	94	104.2	116	84.5	93.95	118	81.5	92.45	124.125
81.5	93.95	124.5	75	89.85	113	69	82.2	111.5	60.5	75.8	118
65	77.9	112.5	69.5	82.4	110	71	82.7	103	63	75	108.375
69.5	80.3	104.5	68.5	79.3	113	75.8	86.96	109	71.5	82.75	108
69.5	82.4	115.5	68	82.25	106	67	78.7	108.5	61.5	75.6	110.625
90.5	101.9	133.5	84	98.85	132.5	83	97.85	135.5	85.5	100.5	132.5
75	86.1	121	71.5	86.35	109.5	72	83.25	110.5	71.5	83.2	113.25
61.5	71.85	102.5	72.5	81.5	105.5	70	80.65	97.5	65	74.75	100.375
73.5	84.6	132	84	98.4	128.5	80	94.55	127.5	79.5	93.9	124.625
78.5	91.55	125.5	80.5	94	117.5	77.5	89.5				121.6667
76	85.6	130	89	101.3	119	82	93.1	118	81	92.1	118.75
86	99.05	133	99.5	109.55	139.5	86.5	102.4	106	76	85	127
75	86.61	118.4	83.5	93.97	125.3	86.7	98.28	121.6	82.2	94.02	119.75
103.5	123.75	175.5	95	119.15	161.5	91	112.15	169	90	113.7	169.25
83	95	122	79	91.9	126	79	93.1	129	83	96.8	125
84	99	129	81.5	95.75	131.5	88.5	101.4	124.5	78.5	92.3	129.75
91	101.95	144.5	87.5	104.6	130.5	87.5	100.4	140.5	90.5	105.5	135.75
85	95.5	118	86	95.6	119	77	89.6	119	80	91.7	119
89	100.1	124.5	82	94.75	118	82.5	93.15	129.5	85.5	98.7	124.5
89.5	102.25	125	83	95.6	126.5	72.5	88.7	133.5	75.5	92.9	129.25
81	93.75	136.5	83	99.05	130.5	80	95.15	131	83.5	97.75	130.375
77	86.75	128.5	83.5	97	115.5	76.5	88.2	105.5	71.5	81.7	114.75
85	100	131.5	86	99.65	139.5	83	99.95	134	80.5	96.55	135
74	83.45	120	82	93.4	117.5	83	93.35	121.5	93.5	101.9	116.125
85.5	97.8	146.5	85.5	103.8	133	87	100.8	135.5	86.5	101.2	135.375

DBP day	MAP day	SBP am 2	DBP am 2	MAP am 2	SBP n 2	DBP n 2	MAP n 2	SBP af 2	DBP af 2	MAP af 2	SBP pm 2
75.275	86.975										
73.75	85.7875										
71.25	81										
65.025	76.2675										
88.5	99.1875							118.5	83.5	94	118.5
71.5	85.45	114.5	70	83.35	125	79	92.8	113	73.5	85.35	114
67.125	79.5										
71.325	82.3275	103.5	64.5	76.2	101.5	61.5	73.5	100	63	74.1	97.5
66.5	79.7375	118.3	72.7	86.38	119	68	83.3	121	62	79.7	119
85.75	99.775										
72.5	84.725	105	68.5	79.45	116	77	88.7	112	70	82.6	110.5
67.25	77.1875	114	69.5	82.85	109	75	85.2	107.5	72	82.65	93.5
79.25	92.8625	101	80	86.3	127	83.5	96.55				
78.83333	91.68333	122	78.5	91.55	125.5	80.5	94	117.5	77.5	89.5	
82	93.025	114	76	87.4	129	86.5	99.25	119	78	90.3	113.5
87	99										
81.85	93.22	135.5	84	99.45	132.5	84.5	98.9	137.5	87.5	102.5	134.5
94.875	117.1875										
81	94.2										
83.125	97.1125	120.5	77.5	90.4	135.5	83.5	99.1	133.5	87	100.95	130
89.125	103.1125	130.5	85	98.65	139	78.5	96.65	152	89	107.9	146
82	93.1	119	79	91	122	76	89.8	122	76	89.8	116.5
84.75	96.675	129	86	98.9	131	84	98.1	138	86	101.6	
80.125	94.8625	114	96	101.4	122.5	77.5	91	127.5	89	100.55	118.5
81.875	96.425	134	87.5	101.45	132.5	91	103.45	123.5	81.5	94.1	132
77.125	88.4125	113	77.5	88.15	124.5	76	90.55	107.5	80	88.25	107
83.625	99.0375	129	85	98.2	145.5	85	103.15	141.5	84.5	101.6	112.5
83.125	93.025										
86.125	100.9										

DBP pm 2	MAP pm 2	SBP day 2	DBP day 2	MAP day 2	Rest SBP 1	Rest DBP 1	Rest MAP 1	Rest SBP 2	Rest DBP 2	Rest MAP 2
					118	86	95.6			
					128	88	100			
					124	88	98.8			
					98	68	77			
78	90.15	118.5	80.75	92.075	132	90	102.6	122	92	101
68.5	82.15	116.625	72.75	85.9125	122	84	95.4	120	88	97.6
					130	88	100.6			
63.5	73.7	100.625	63.125	74.375	120	80	92	110	78	87.6
66.7	82.39	119.325	67.35	82.9425	132	74	91.4	104	82	88.6
					118	78	90			
68	80.75	110.875	70.875	82.875	128	88	100	110	78	87.6
62	71.45	106	69.625	80.5375	130	88	100.6	125	70	86.5
		114	81.75	91.425	132	98	108.2	130	86	99.2
		121.6667	78.83333	91.68333	148	98	113	134	78	94.8
78.5	89	118.875	79.75	91.4875	110	74	84.8	112	70	82.6
					146	106	118			
84.5	99.5	135	85.125	100.0875	118	78	90	98	72	79.8
					158	94	113.2			
					138	92	105.8			
83	97.1	129.875	82.75	96.8875	132	82	97	146	80	99.8
92.5	108.55	141.875	86.25	102.9375	142	96	109.8	132	98	108.2
74	86.75	119.875	76.25	89.3375	98	70	78.4	126	82	95.2
		132.6667	85.33333	99.53333	130	92	103.4	140	80	98
84	94.35	120.625	86.625	96.825	130	98	107.6	140	100	112
81	96.3	130.5	85.25	98.825	140	92	106.4	142	100	112.6
71	81.8	113	76.125	87.1875	122	88	98.2	128	88	100
71.5	83.8	132.125	81.5	96.6875	150	109	121.3	150	98	113.6
					118	86	95.6			

HR 55 1	SBP 55 1	DBP 55 1	RPP 55 1	MAP 55 1	HR 55 2	SBP 55 2	DBP 55 2	RPP 55 2	MAP 55 2	HR 100 1	SBP 100 1
117	142	86	16614	102.8						138	176
138	150	86	20700	105.2						158	166
132	146	84	19272	102.6						142	150
122	118	74	14396	87.2						140	138
97	142	90	13774	105.6	96	138	90	13248	104.4	102	160
95	142	80	13490	98.6	94	132	88	12408	101.2	105	158
112	162	80	18144	104.6						134	172
125	128	78	16000	93	108	126	82	13608	95.2	140	136
96	150	78	14400	99.6	101	132	82	13332	97	115	164
125	152	82	19000	103						149	168
95	152	96	14440	112.8	96	122	82	11712	94	105	160
142	144	70	20448	92.2	109	140	70	15260	91	171	150
125	165	84	20625	108.3	118	165	90	19470	112.5	136	210
126	198	90	24948	122.4	134	180	90	24120	117	152	200
106	124	74	13144	89	93	114	80	10602	90.2	117	126
97	162	94	15714	114.4						121	176
92	118	76	10856	88.6	87	122	68	10614	84.2	108	132
91	156	98	14196	115.4						105	180
129	154	84	19866	105						142	168
98	148	86	14504	104.6	105	180	90	18900	117	127	182
90	172	96	15480	118.8	92	146	90	13432	106.8	124	212
103	122	68	12566	84.2	108	132	80	14256	95.6	123	164
139	185	86	25715	115.7	135	160	80	21600	104	160	228
131	170	110	22270	128	118	176	108	20768	128.4	136	178
106	198	100	20988	129.4	108	158	92	17064	111.8	123	190
89	134	84	11926	99	86	130	90	11180	102	103	148
99	170	100	16830	121	87	180	100	15660	124	114	200
99	138	90	13662	104.4						125	160
103	140	90	14420	105						125	150

DBP 100 1	RPP 100 1	MAP 100 1	HR 100 2	SBP 100 2	DBP 100 2	RPP 100 2	MAP 100 2	HR pk 1	SBP pk 1	DBP pk 1
82	24288	110.2						183	192	82
78	26228	104.4						192	204	90
86	21300	105.2						163	188	80
80	19320	97.4						160	152	82
90	16320	111	116	166	92	19256	114.2	132	192	92
90	16590	110.4	102	166	90	16932	112.8	136	194	100
80	23048	107.6						153	176	82
80	19040	96.8	128	146	82	18688	101.2	162	154	76
78	18860	103.8	121	150	82	18150	102.4	169	174	72
84	25032	109.2						172	184	84
94	16800	113.8	108	160	84	17280	106.8	138	176	92
72	25650	95.4	154	140	70	21560	91	173	158	70
104	28560	135.8	131	190	98	24890	125.6	142	220	108
86	30400	120.2	165	192	90	31680	120.6	171	168	84
74	14742	89.6	111	124	80	13764	93.2	155	158	74
88	21296	114.4						140	196	90
82	14256	97	107	146	72	15622	94.2	139	190	100
100	18900	124						135	242	104
80	23856	106.4						164	188	78
88	23114	116.2	113	212	96	23956	130.8	141	204	80
100	26288	133.6	115	196	88	22540	120.4	171	244	98
72	20172	99.6	128	162	80	20736	104.6	163	194	68
70	36480	117.4	156	218	70	34008	114.4	160	228	70
100	24208	123.4	132	180	98	23760	122.6	173	220	90
96	23370	124.2	124	174	98	21576	120.8	140	230	100
92	15244	108.8	102	134	90	13668	103.2	148	190	100
98	22800	128.6	99	199	102	19701	131.1	167	230	110
98	20000	116.6						158	200	90
100	18750	115						175	200	100

RPP pk 1	MAP pk 1	Load pk 1	T Time pk 1	HR pk 2	SBP pk 2	DBP pk 2	RPP pk 2	MAP pk 2	Load pk 2	T Time pk 2	rel VO2 55 1
35136	115	190	11.5								12.8
39168	124.2	160	9								9.909
30644	112.4	155	8.37								7.409
24320	103	130	7								12.777
25344	122	160	9	133	178	88	23674	115	145	8	8.742
26384	128.2	160	9	150	232	104	34800	142.4	175	10.1	12.042
26928	110.2	135	7.17								10.070
24948	99.4	145	8	165	172	78	28380	106.2	170	8.5	11.538
29406	102.6	190	11	172	212	80	36464	119.6	205	12	11.210
31648	114	130	7								12.448
24288	117.2	175	10	144	186	88	26784	117.4	175	10	11.584
27334	96.4	120	5.5	170	150	60	25500	87	145	7	16.229
31240	141.6	115	6	137	218	88	29866	127	115	6	13.063
28728	109.2	120	6.28	165	192	90	31680	120.6	100	5.2	7.621
24490	99.2	205	12	161	144	72	23184	93.6	205	11.85	11.814
27440	121.8	130	7								7.064
26410	127	160	9	141	208	80	29328	118.4	175	10	12.669
32670	145.4	185	10.48								11.339
30832	111	145	7.85								10.941
28764	117.2	135	7.33	146	232	100	33872	139.6	130	7	11.231
41724	141.8	200	11.5	163	242	60	39446	114.6	190	10.5	9.591
31622	105.8	145	8	144	186	78	26784	110.4	130	7.5	7.432
36480	117.4	100	5	162	235	60	38070	112.5	130	6	11.186
38060	129	215	12.5	180	210	108	37800	138.6	205	13	9.018
32200	139	160	9	158	230	80	36340	125	190	10.5	9.248
28120	127	190	11	142	200	90	28400	123	190	11	8.184
38410	146	220	11.5	155	230	100	35650	139	250	15	10.867
31600	123	160	9								13.918
35000	130	190	11								9.310

abs VO2 55 1	rel VO2 55 2	abs VO2 55 2	rel VO2 100 1	abs VO2 100 1	rel VO2 100 2	abs VO2 100 2	rel VO2 pk 1	abs VO2 pk 1
0.93			18.1	1.31			31.09	2.26
0.92			11.417	1.06			20.896	1.94
0.65			12.425	1.09			18.466	1.62
0.89			18.519	1.29			21.246	1.48
0.91	10.158	1.047	12.585	1.31	14.738	1.519	20.655	2.15
1.04	10.439	0.911	17.021	1.47	17.256	1.506	24.316	2.1
0.88			15.449	1.35			17.508	1.53
0.67	12.401	0.737	21.010	1.22	20.646	1.227	27.037	1.57
0.8	13.343	0.934	17.936	1.28	18.871	1.321	32.790	2.34
1.109			18.038	1.607			20.541	1.83
1.186	10.719	1.106	13.820	1.415	13.791	1.423	25.687	2.63
1.018	12.357	0.792	23.722	1.488	19.504	1.25	24.806	1.556
1.146	9.390	0.843	17.680	1.551	14.826	1.331	18.877	1.656
0.64	9.068	0.779	12.265	1.03	14.562	1.251	16.314	1.37
1.07	10.989	0.959	15.237	1.38	15.274	1.333	26.941	2.44
0.7			12.514	1.24			15.440	1.53
1.11	11.985	1.046	18.034	1.58	18.757	1.637	23.512	2.06
1.13			15.453	1.54			23.480	2.34
1.16			12.073	1.28			17.449	1.85
0.85	11.047	0.829	17.441	1.32	17.163	1.288	20.745	1.57
1.103	12.530	1.384	9.591	1.53	17.138	1.893	22.609	2.6
0.837	9.184	1.052	7.432	1.563	12.100	1.386	18.737	2.11
1.594	10.377	1.5	11.186	2.19	14.383	2.079	15.368	2.19
1.281	8.039	1.162	9.018	1.804	12.564	1.816	21.542	3.06
1.177	11.196	1.369	9.248	1.839	13.781	1.685	19.486	2.48
0.983	8.685	1.06	8.184	1.561	12.725	1.553	22.062	2.65
1.23	9.592	1.066	13.447	1.522	13.704	1.523	26.824	3.036
1.066			17.195	1.317			24.324	1.863
0.877			15.647	1.474			28.321	2.668

rec_hr3	rec_sbp3	rec_dbp3	rec_map3	rec_hr4	rec_sbp4	rec_dbp4	rec_map4	rec_hr5	rec_sbp5	rec_dbp5	rec_map5
129	122	76	89.8	126	128	76	91.6	125	112	74	85.4
157	146	88	105.4	150	142	88	104.2	147	146	86	104
123	140	74	93.8	115	136	76	94	113	130	78	93.6
118	116	70	83.8	118	116	72	85.2	108	120	70	85
100	142	90	105.6	91	128	84	97.2	84	132	90	102.6
80	142	78	97.2	80	158	78	102	71	124	78	91.8
92	158	76	100.6	83	140	76	95.2	80	120	82	93.4
117	130	76	92.2	115	118	78	90	111	116	76	88
127	152	70	94.6	115	140	76	95.2	109	122	76	89.8
123	162	72	99	105	142	72	93	105	142	72	93
96	134	80	96.2	96	124	84	96	97	130	82	96.4
125	128	56	77.6	118	122	52	73	108	118	58	76
125	122	78	91.2	125	126	84	96.6	123	126	82	95.2
112	124	68	84.8	102	118	74	87.2	100	110	72	83.4
114	142	68	90.2	106	144	66	89.4	102	138	66	87.6
124	134	86	100.4	115	132	88	101.2	110	130	90	102
110	130	74	90.8	94	138	78	96	98	128	72	88.8
93	210	98	131.6	93	190	98	125.6	84	186	96	123
131	[redacted]			124	130	62	82.4	115	120	68	83.6
101	164	78	103.8	99	158	82	104.8	91	146	80	99.8
126	146	80	99.8	120	142	84	101.4	117	136	86	101
120	144	70	92.2	108	116	70	83.8	97	112	68	81.2
[redacted]				122	170	90	114	[redacted]			0
128	154	90	109.2	133	146	96	111	126	144	96	110.4
100	150	90	108	95	148	90	107.4	91	138	88	103
114	146	80	99.8	109	128	80	94.4	102	120	82	93.4
123	172	88	113.2	117	158	90	110.4	113	146	90	106.8
121	146	74	95.6	114	128	78	93	108	132	86	99.8
131	162	76	101.8	127	150	80	101	125	148	82	101.8

rec_hr6	rec_sbp6	rec_dbp6	rec_map6	rec_hr1	rec_sbp1	rec_dbp1	rec_map1	rec_hr2	rec_sbp2	rec_dbp2	rec_map2
123	112	74	85.4								
149	142	88	104.2								
110	122	80	92.6								
99	118	72	85.8								
73	130	90	102	117	174	84	111	103	170	88	112.6
75	126	78	92.4	107	182	90	117.6	100	144	80	99.2
84	124	80	93.2								
104	114	76	87.4	137	152	78	100.2	128	142	78	97.2
98	126	80	93.8	155	178	80	109.4	134	170	74	102.8
99	136	74	92.6								
92	126	84	96.6	124	156	74	98.6	109	140	76	95.2
113	116	62	78.2	151	150	60	87	125	120	64	80.8
123	126	86	98	129	180	88	115.6	124	186	84	114.6
97	100	68	77.6	139	190	80	113	127	160	68	95.6
99	126	70	86.8	142	130	78	93.6	128	128	78	93
105	124	88	98.8								
96	112	70	82.6	118	142	68	90.2	115	134	66	86.4
84	168	96	117.6								
111											
93	136	84	99.6	113	188	84	115.2	101	176	84	111.6
115	124	84	96	141	208	60	104.4	120	180	50	89
93	112	68	81.2	138	178	78	108	123	180	72	104.4
124	170	84	109.8	123	180	60	96	113	146	64	88.6
126	140	100	112	155	182	90	117.6	146	164	88	110.8
91	138	92	105.8	142	180	78	108.6	131	180	80	110
109	130	82	96.4	115	150	80	101	105	148	80	100.4
117	138	90	104.4	132	190	80	113	117	164	80	105.2
115	130	84	97.8								
120	142	82	100								

rec_hr3	rec_sbp3	rec_dbp3	rec_map3	rec_hr4	rec_sbp4	rec_dbp4	rec_map4	rec_hr5	rec_sbp5	rec_dbp5	rec_map5
92	144	84	102	71	138	86	101.6	81	128	86	98.6
87	132	80	95.6	85	132	80	95.6	86	120	82	93.4
121	132	78	94.2	120	128	72	88.8	110	122	70	85.6
119	150	74	96.8	117	132	74	91.4	114	128	76	91.6
99	128	74	90.2	96	128	95	104.9	95	118	78	90
116	118	58	76	111	112	60	75.6	106	110	62	76.4
115	156	80	102.8	110	132	78	94.2	112	132	82	97
106	140	70	91	104	110	70	82	103	118	68	83
124	126	76	91	111	128	78	93	106	124	74	89
105	112	70	82.6	107	112	70	82.6	105	110	74	84.8
101	160	82	105.4	94	148	80	100.4	94	146	78	98.4
112	176	86	113	103	158	88	109	101	150	90	108
111	170	68	98.6	106	168	70	99.4	99	134	70	89.2
108	144	62	86.6	103	132	62	83	106	130	62	82.4
133	154	90	109.2	129	150	90	108	129	148	92	108.8
123	178	74	105.2	107	156	78	101.4	101	152	82	103
97	146	80	99.8	91	142	78	97.2	107	134	76	93.4
110	158	84	106.2	107	142	82	100	103	128	86	98.6

rec_hr6	rec_sbp6	rec_dbp6	rec_map6	BE 1	BE 1	BE 1 avg	BE 2	BE 2	BE 2
				2.321	1.827	2.074			
				4.437	4.72	4.5785			
				4.155	4.269	4.212			
				0.557	0.98	0.7685			
70	128	92	102.8	5.496	5.919	5.7075	5.143	5.355	5.2
88	120	82	93.4	3.591	3.591	3.591	4.72	4.79	4.7
				5.707	6.06	5.8835			
109	112	72	84	2.885	3.097	2.991	2.109	2.18	2.1
107	126	80	93.8	2.532	2.462	2.497	2.956	3.167	3.0
				3.661	3.52	3.5905			
96	114	80	90.2	3.379	3.379	3.379	5.072	5.284	5.1
107	108	60	74.4	1.262	2.109	1.6855	4.226	4.085	4.1
110	124	80	93.2	1.474	1.474	1.474	1.686	2.109	1.8
99				2.806	2.903	2.8545	1.306	1.106	1.2
103	122	72	87	2.225	2.225	2.225	2.806	2.903	2.8
				1.983	2.419	2.201			
105	104	74	83	2.709	2.903	2.806	3.919	3.968	3.9
				2.613	2.516	2.5645			
				3.193	3.048	3.1205			
110	140	78	96.6	1.209	1.451	1.33	0.87	0.87	0.
99	140	96	109.2	2.855	2.855	2.855	4.016	4.016	4.0
96	132	74	91.4	4.21	3.871	4.0405	4.133	3.193	3.6
107	128	62	81.8	6.92	4.742	5.831	4.452	4.5	4.4
129	144	96	110.4	4.839	4.258	4.5485	4.742	4.549	4.6
104	134	84	99	2.613	2.661	2.637	1.258	1.064	1.1
87	118	78	90	1.016	1.693	1.3545	2.951	3.435	3.1
100	132	84	98.4	2.225	2.322	2.2735	1.79	2.032	1.9
				2.18	1.968	2.074			
				1.686	1.545	1.6155			

NO3 2	NO2 1	NO2 2	ET-1 Expression
	1.115		
	0.078		
	0.195		
	1.083		
104.729	0.411	0.726	
90.916	0.843	0.648	
	0.477		
28.742	0.711	2.173	
55.306	1.538	0.178	
	0		
126.229	0	3.933	
42.33	7.441	3.881	
88.47	6.125	0.363	
59.493	2.595	0.225	
44.384	0	0	
	0.618		0.0904
68.649	0	0	1.741
	0.157		1.498
	0.08		0.0625
60.996	0	0	0.13398
43.799	0	0	0.11136
84.648	0	0	0.7667
43.216	0	0	0.1688
96.327	0	0.853	0.0947
73.493	0	0	0.0806
90.85	0	9.328	0.1166
79.501	1.059	2.009	
	1.366		0.1788
	0		0.1469

Subject	B-actin 1	B-actin 2	B-actin 3	ET1	ET2	ET3	B-actin avg	ET avg	CT Target	Control Avg	CT Exp	Rel Exp
3c	21.4	22	21.4	28.5			21.60	28.50	6.90			
7c	21	20.4	20.8	29.5	29.5	29.9	20.73	29.63	8.90			
8c	19.5	21.4	19.6	27.2	27.2		20.17	27.20	7.03			
11c	20.3	19.9	21.6	28.3	28.6	32.8	20.60	29.90	9.30			
12c	19.6	19.3	20.3	27.1	26.6		19.73	26.85	7.12			
15c	21.5	21.4	20.6	31.5	30.6		21.17	31.05	9.88			
16c	18.7	18.6	18.5	31.3	31		18.60	31.15	12.55			
7	24.1	24	22.8	35.3			23.63	35.30	11.67	8.2	3.467	0.090
8	20.2	20.3	20.1	27.6			20.20	27.60	7.40	8.2	-0.800	1.741
9	20.4	20.7		27.8	28.9	27.8	20.55	28.17	7.62	8.2	-0.583	1.498
10	22.4	22.3	21.3	34.2			22.00	34.20	12.20	8.2	4.000	0.063
11	20.7	21.2	19.6	30.8	31.9	32.1	20.50	31.60	11.10	8.2	2.900	0.134
12	20.2	20.6	19.9	31.5	31.7		20.23	31.60	11.37	8.2	3.167	0.111
13	22.7	21.6	21.9	29.3	32		22.07	30.65	8.58	8.2	0.383	0.767
14	20.3	20.8	20.3	29	32.3	32.4	20.47	31.23	10.77	8.2	2.567	0.169
15	20.7	19.9	20	31.8			20.20	31.80	11.60	8.2	3.400	0.095
16	20.1	20	19.8	31.8			19.97	31.80	11.83	8.2	3.633	0.081
17	19.3	19.3	18.1	28.8	31.1	30.7	18.90	30.20	11.30	8.2	3.100	0.117
19	22.9	22.2	19.9	32.8	31.9		21.67	32.35	10.68	8.2	2.483	0.179
20	19.3	19.6	19.3	30.4	30.6	30.1	19.40	30.37	10.97	8.2	2.767	0.147

APENDIX C

INSTRUCTIONS AND FORMS

OBSTRUCTIVE SLEEP APNEA: RESMED 2

GRADED EXERCISE TEST DATA SHEET

Name _____ **Age** ____ **Wt** _____ **lb/kg** **Ht** _____ **in/cm** **Date** _____

Subject # _____ **Test (circle)**: **BASELINE** **6 WEEK** **12 WEEK**

Neck Circ: _____ **Waist Circ:** _____ **Hip Circ:** _____ **BMI:** _____

MEDICATIONS:

Name Dose Time last taken Name Dose Time last taken

_____

_____

_____

_____

Rest HR _____ / _____ Rest BP _____ / _____ Rest Qc (L/min) _____
standing / sitting standing / sitting sitting

Protocol: 15 W/minute Ramp (25 W warm-up for 1 min)

Time (min)	Watts	HR (bpm)	BP (mmHg)	RPE	Qc	ECG Δ/Symptoms/Comments
0 – 1	40		/			
1 – 2	55		/			
2 – 3	70		/			
3 – 4	85		/			
4 – 5	100		/			
5 – 6	115		/			
6 – 7	130		/			
7 – 8	145		/			
8 – 9	160		/			
9 – 10	175		/			
10 – 11	190		/			
11 – 12	205		/			
12 – 13	220		/			
13 – 14	235		/			
14 – 15	250		/			
15 – 16	265		/			
16 – 17	280		/			
17 - 18	295		/			

Immediate Post-Test Symptoms: Chest Discomfort Shortness of Breath Lightheadedness Other:

RECOVERY	HR	BP	ECG Δ/Symptoms/Comments
IPE		/	
1		/	
2		/	
3		/	
4		/	
5		/	
6		/	
7		/	
8		/	
9		/	
10		/	

Total Test Time: _____ Peak Watts: _____

REASON FOR STOPPING TEST: _____

Comments: _____

_____

_____

Instructions for Blood Pressure Measurements

General Guidelines

1. All measurements should be taken in a quiet place and in a relaxed state.

2. All measurements should be taken in a seated position after a 5-minute rest period.

3. A total of **4 measurements** will be taken (Immediately upon awakening; mid-day, i.e. noon; early evening, i.e. 5-6pm; and just prior to sleep).

4. A minimum of 2 measurements should be taken at each time period until 2 measurements are recorded that are $\leq$ 5 mmHg (systolic or first number) apart.

5. Repeated measurements should be separated by a minimum of 2 minutes.

6. Remain still while measurement is being taken.

7. Abstain from caffeine and nicotine products for a minimum of 12 hours prior to taking measurements.

8. Abstain from vigorous exercise for 24 hours prior to taking measurements.

9. In addition to pressing the "MEMORY SET" button after each measurement, please record the time, blood pressure, and heart rate values (from blood pressure device) on the log sheet provided.

Blood Pressure Measurement Recording Log

Patient Name:
Date:

Upon Awakening	Time	Systolic	Diastolic	Pulse
Measurement #1				
Measurement #2				
Measurement #3				
Measurement #4				
Measurement #5				

Mid-Day	Time	Systolic	Diastolic	Pulse
Measurement #1				
Measurement #2				
Measurement #3				
Measurement #4				
Measurement #5				

Early Evening	Time	Systolic	Diastolic	Pulse
Measurement #1				
Measurement #2				
Measurement #3				
Measurement #4				
Measurement #5				

Before Sleep	Time	Systolic	Diastolic	Pulse
Measurement #1				
Measurement #2				
Measurement #3				
Measurement #4				
Measurement #5				

**Note: Measurements 2 through 5 not necessary if first readings are $\leq$ 5 mmHg apart systolic. See attached sheet for more instructions.*

Please take a few moments to record what you believe to be your overall physical demands, as well as your overall emotional demands since you awakened in the morning. This information is important to help us understand how your daily routine today may have affected your blood pressure, heart rate, and stress hormones.

Physical Demands (please circle)

1- Sitting most of the day and very little walking or other activity for the rest of the day.
If other activity, please explain_____

2- Sitting 2/3 of the day, with walking or other light activity at least 1/3 of the day.
If other activity, please explain_____

3- Sitting 1/3 of the day, with walking or other light physical activity 2/3 of the day.
If other activity, please explain_____

4- Little or no sitting during the day, with walking or other physical activities most of the day.
If other activities, please explain_____

I would rate this day's physical activity as: (please circle)

Less than normal Normal More than normal Much more than normal

Please explain:

_____

_____

Emotional Demands (please circle)

1- Less stress than normal
2- Normal amount of stress
3- A little more than normal amount of stress
4- Much more than normal amount of stress

Please comment about any unusual work related or non-work related events that may have happened while you were being monitored for blood pressure, heart rate and urine output.

_____

_____

_____

_____

Virginia Tech – OSA Research Project
Exercise Log

Name: ________________________ **Target Heart Rate Range: _____________** **10 sec HR:**

Date	Time	Weight	Pre-Exercise HR/BP	Exercise Mode	Workload	Duration	RPE	Exercise HR/BP	Post-Exercise HR/BP
			/					/	/
			/					/	/
			/					/	/
			/					/	/
			/					/	/
			/					/	/
			/					/	/
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			/					/	/

Virginia Tech Obstructive Sleep Apnea Exercise Test Results

Exercise Program Recommendations

Dear,

Thank you for volunteering to participate in this study evaluating the effects of nasal continuous positive airway pressure (nCPAP), with or without exercise, on various measures of heart rate, blood pressure, exercise capacity, blood vessel function, and quality of life. Several of the key variables measured are presented in the table below. Since you were chosen for the exercise group, we hope that you'll continue to be active! The plan below is a step in that direction.

Researchers still have much to learn about obstructive sleep apnea (OSA). However, much is known about some of its potential precursors and outcomes. In addition to being a risk factor for the development of OSA, excess body weight is associated with an increased risk for the development of type 2 diabetes, hypertension (high blood pressure), dyslipidemia (abnormal blood cholesterol values), and heart disease.

Fortunately, aerobic exercise (walking, cycling, etc.) has been shown to reduce the age-related rise in blood pressure in persons at increased risk for hypertension. In addition, regular aerobic exercise *may* help prevent further weight gain and other obesity-related diseases and *may* have long-term potential to forestall or even reverse signs and symptoms associated with OSA. To date, there is very little research data available regarding the effects of exercise on OSA and OSA symptoms. Therefore, exercise and weight loss, combined with the primary treatment, ***nasal CPAP***, is a well-rounded approach to leading a healthier lifestyle. Furthermore, all treatment options should be discussed with your doctor. Please contact us if you have any questions regarding your exercise test results or exercise plan.

Good luck!

Virginia Tech OSA Research Team

Based on your exercise test results, the following *recommendations* are provided:

What types of exercise should I do?
The greatest improvements in physical fitness will occur when exercise involves the use of large muscle groups, i.e. legs, over prolonged periods in activities that are rhythmic in nature. Walking, hiking, jogging, swimming, cycling, dancing, skating, and cross-country skiing are just some examples. You can choose one or combinations of your own preferred activities.

How hard should I exercise?
We suggest performing activity that elevates your heart rate to a specific "training range." This range may change as your fitness level improves, however the activity should be perceived as "somewhat hard." Use your own judgment and decrease the intensity if the activity is too hard. Our recommendation is that you train at a heart rate range of: bpm. This is approximately % of your peak exercise capacity performed on the cycle exercise test.

How long should I exercise?
Duration and intensity go "hand-in-hand." The primary goal of an exercise program is to burn a sufficient number of calories to achieve health, fitness, and weight management goals. We suggest 15-30 minutes to start with. As your fitness level improves, you can exercise longer. The duration can be progressively increased over time until the goal (30-60 minutes of continuous aerobic activity) is achieved.

How many days per week should I exercise?
To start, we suggest 3-4 days per week. The number of sessions per week varies depending on the individual's goals, limitations, and lifestyle. Ultimately, you should strive to perform some form of physical activity every day.

Further information may be obtained from:

Brian Hawkins, MS, ACSM ES_{SM}
540-231-6374

William G. Herbert, PhD
540-231-6565

Carol Haskell, MD
540-382-1165 ext. 350

Epworth Sleepiness Scale

How likely are you to doze off or fall asleep in the following situations? Use the following scale and indicate the most appropriate number for each situation.

0 = would never doze
1 = slight chance of dozing
2 = moderate chance of dozing
3 = high chance of dozing

	Situation	**Chances of Dozing**
1.	Sitting and reading	_____
2.	Watching T.V.	_____
3.	Sitting, inactive in a public place (ex. Theatre or meeting)	_____
4.	As a passenger in a car for an hour without a break	_____
5.	Lying down to rest in the afternoon when circumstances permit	_____
6.	Sitting and talking with someone	_____
7.	Sitting quietly after a lunch without alcohol	_____
8.	In a car, while stopped for a few minutes in the traffic	_____

APPENDIX D

INFORMED CONSET

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Informed Consent for Control Subjects Participating in Investigative Project

Title of research Project: **The short-term effects of exercise training in conjunction with CPAP therapy on cardiovascular function, exercise tolerance, and quality of life in obstructive sleep apnea patients**

Principal Investigators: William G. Herbert, Ph.D., Don Zedalis, MD, John Gregg, DDS, Ph.D., William Huckle, Ph.D., Sharon Nickols-Richardson, RD, Ph.D., Thomas W. Chittenden, MA, Anthony S. Kaleth, MS, Brian J. Hawkins, MS, Lawrence Cross, Ph.D.

I. Purpose of This Research

The purpose of your participation is to provide information on how apparently healthy adults respond to a special set of fitness, cardiovascular, and biochemical tests which are being used in an investigation of patients with a sleep disorder. Thus, your results will help the investigators understand how the test results from patients with Obstructive Sleep Apnea (OSA) differ from those of people who do not have a diagnosis of this disorder. In the study, the OSA patients are taking the same tests, but doing so in conjunction with therapeutic treatment. You will not be asked to do tests to evaluate your sleep status, but most of your tests will be done at the Sleep Disorders Clinic in Christiansburg, Virginia. Before you may enter the study, you will be interviewed and complete questionnaires to be certain that you do not have any unusual sleep problems. The scientific purposes of this overall study are: 1) to determine how the OSA disorder affects the heart and circulation, physical fitness, and risk factors for heart disease; and 2) to see if treatment for the OSA patients can be improved by combining nighttime positive pressure-breathing (CPAP) with a moderate exercise program.

As part of determining your eligibility for this study, you and your doctor will be asked to verify that you do not have a known health history that includes any of the following:

- Heart problems, including heart attack, chest pain that may be related to heart problems (this is called angina pectoris), surgery for your heart or its blood vessels (coronary revascularization), or congestive heart failure;
- Chronic lung diseases;
- Uncontrolled high blood pressure or use of blood pressure medications or antihistamines;
- Uncontrolled diabetes mellitus;
- Orthopedic (bone or joint) problems, musculoskeletal conditions, and/or neuromuscular conditions that would prevent you from doing vigorous exercise;
- Use of tobacco products use (only non-smokers can participate)

In addition, to be eligible you must not have been following a program of regular moderate physical activity. If you have been exercising within the past 6 months at a moderate intensity or more, 30 minutes/day for 3 or more days a week, you are not eligible. Finally and in addition to the above, to be eligible you must have seen your personal physician at least once in the past 4 years and they must provide the investigators with written confirmation that they know of no health condition that would make vigorous exercise inadvisable for you.

II. Procedures

You will be asked to complete the following procedures at six (6) different time points over a 12-week period:

1) **Orientation Session**: A 45-minute orientation at the Sleep Center in which study procedures are explained and you complete interviews and study questionnaires. You will also be given a device to take home for one day and night. This device has a small sleeve which fits over your finger (similar to a thimble), a small battery powered box, and lead wires connecting the sleeve to the box. This device is non-invasive, harmless and painless means to measure whether you have normal blood oxygen levels during sleep. You will be asked to wear this during one night at home and given instructions on how to return it to the research staff the next day. The information about your blood oxygen levels will help determine if you qualify for this study. Dr. Zedalis, medical director of the Sleep Disorders Network will interpret the results for this blood oxygen level test. If within normal limits, you will be eligible to continue in the study. If there is a question about these results possibly being out of the normal range, you will not be eligible to continue in the study and Dr. Zedalis may recommend to you that you discuss the results with your primary care physician;

2) **Baseline Exercise Test**: Within 1 week of the Orientation Session, you will be asked to do a resting heart rate and an exercise test at the Sleep Center; the day before this test, you will carry small devices throughout your normal day to assess your heart rate and

blood pressure and you will collect all your urine output over a 24-hour period. The Sleep Center part of this will last ~90 minutes;

3) **Baseline Heart Rate Monitor Test**: Within 2 days after your Baseline Exercise Test, you will have another 30-minute resting heart rate test at the Sleep Center again. On the day before or after this Sleep Center test, you will repeat the daytime measures for heart rate, blood pressure, and urine collection. No exercise test will be done during this period.

4) **Week 3 Exercise Test**: This test will be done at the Sleep Center, in the same way as the Baseline Exercise Test, but no resting heart rate test will be done. This procedure will last ~60 minutes;

5) **Week 6 Exercise Test:** Procedures performed exactly as for the Baseline Exercise Test. This will last ~90 minutes;

6) **Week 12 Exercise Test:** Procedures performed exactly as in Baseline Exercise Test. This will last ~90 minutes.

Information about the **specific test procedures** you will do is presented below:

- Three or four questionnaires that request your opinion about the quality of your sleep, your current quality of life, and daily physical activities;
- A questionnaire that asks about your eating habits;
- Measurements of height, weight, waist and hip circumference, blood pressure, heart rate;
- During your Orientation Session, you will be asked to practice breathing techniques while wearing a special mouthpiece and nose clip; this practice will help you perform the actual exercise tests which you will do at Baseline, and Weeks 3, 6, and 12;
- Immediately after your Orientation Session, you will be asked to take a device home that will measure your blood oxygen levels overnight; instructions about how to set up and wear the device will be provided to you at the Orientation and the results will help determine your eligibility to continue in the study;

- Collect a 24-hour urine sample in a jug that the investigators will provide, as indicated in the above schedule of test sessions;
- Collect a small venous blood samples (10mL) immediately before and after each exercise test (Baseline, 6 weeks, and 12 weeks) to measure the ability of your blood vessels to relax, as well as how your nervous and cardiovascular systems are adjusting. Part of the blood sample collected before the exercise tests also will be assayed for blood fats. Slight bruising may occur around the area of the needle stick;
- Measurements of your daytime heart rate and blood pressure that you will do with small portable monitoring devices that you carry with you throughout your normal day;
- Measurements of resting heart rate and blood pressure during test sessions at the Sleep Center in Christiansburg. This will involve having electrodes placed on your chest and then lying down and breathing quietly for about 20 minutes. During this procedure, you will pace your breathing to match a signal that you hear through a stereo headset. During this period, your heart rate will be measured with a small recorder to evaluate the nervous control of your heart.
- Measurements of your maximal exercise performance on a stationary cycle, including evaluation of your heart's pumping ability and your body's oxygen requirements. The exercise test will last ~14 minutes and include assessments of oxygen consumption, heart rate, blood pressure, and cardiac output (pumping ability of your heart). To measure how much oxygen you use, we will ask you to breathe into a lightweight rubber mouthpiece. During the bicycle test, you will breathe only through the mouthpiece and may experience some dryness in your mouth. You will be asked to perform several exercise cardiac output measurements that require you to slowly exhale a special mixture of oxygen and tiny amounts of a harmless gas that doesn't interact with the body; the time you exhale for this measurement will last only ~5 seconds. You may experience more difficulty completing this procedure during higher intensities of exercise, but the investigators will only ask that you do your best to accomplish this.

The total time involved to complete all of the above procedures over the 12 weeks you are in the study will include ~7 hours of activities at the Sleep Center plus the time and some limited time for receiving and returning the portable monitoring devices and urine jugs required when you collect daytime heart and blood pressure and 24-hour urine volumes at three different days during the study.

After your Baseline Exercise Session, you must agree not to initiate any formal exercise programs. Your participation in this study will be deemed as non-exercise or sedentary

control group. The initiation of vigorous exercise on your part would greatly compromise the integrity of the research data. By agreeing to participate in this study, you are also agreeing to not participate in vigorous exercise for 12 weeks.

III. Risks

It is my understanding and I have been informed that there exists the possibility during exercise of adverse changes during the actual test. I have been informed that these changes could include abnormal blood pressure, fainting, disorders of heart rhythm, and in very rare instances, heart attack or death (~1 death in 10,000 exercise tests). Every effort will be made to minimize these possibilities for you by preliminary examination and by precautions and observations taken during the test. The intensity of the cycling exercise will increase as you pedal, over about 14 minutes. At first it will be very easy and then become harder; during the last few minutes, the work will become very intense and will represent a maximal effort on your part. It may be as hard as any exercise that you remember doing.

Qualified medical personnel will be available to perform CPR and contact the community Emergency Medical Services by telephone to deal with unusual situations, should these occur during your exercise tests. Emergency equipment and defibrillation are available at this facility and a medical professional with training in advanced cardiac life support will be onsite for all exercise testing. However, a thorough screening for signs of active heart disease and a review of your medical records from your primary healthcare provider will be done before you are allowed to take the exercise test. This will further reduce the chance of heart problems during the exercise testing procedure.

I understand that there is a very small risk of injury, heart attack, or death as a result of my performance in these maximal exercise tests, but knowing these risks, it is my desire to proceed to participate and be a subject in this research project. I understand that the results of this test will be sent to my primary care physician, if I so request. These results may help to determine my ability to safely perform certain types of physical work or exercise.

I understand that my participation in this research project is voluntary and that I may withdraw at any time, without penalty of any kind. Furthermore, I also understand that there is no guarantee that I will benefit from this research project.

IV. Benefits of Your Participation in This Project

- Both you and your physician will be provided with your individual results from your exercise test. This test is also be used for evaluating the condition of your heart and lungs. If your physician notes a concern after reviewing the results of this procedure, you and your physician may decide that you should consult with an appropriate healthcare specialist. However, any and all costs related to such a referral and medical care will by borne by you and not by Virginia Tech, nor any of its agents, including the investigators.
- A licensed physician will be in the facility and available to assist with monitoring your status during all exercise tests.
- A trained medical professional will act as a research coordinator stay in contact with you to monitor and manage your progress throughout the study and when you may request information related to your participation in the study.
- A trained nutritionist or dietitian will evaluate and make general recommendations to you about the type and amount of foods that you are eating. This information may be beneficial for your health and controlling risk factors for chronic diseases, such as coronary heart disease. Were you not in the study, this type of analysis normally costs $50 per evaluation; you will receive three such evaluations, without charge.

V. Extent of Anonymity and Confidentiality

The results of this study will be kept strictly confidential. At no time will the researchers release my results of this study to anyone other than the individuals working on the

project without your written consent. However, if the need arises, I give my permission for Dr. Zedalis' office to obtain my medical records from my primary healthcare physician. Furthermore, I understand that the information I provide will have my name removed and only a subject number (excluding social security numbers) will identify me during analyses and written reports of this research.

VI. Compensation

I can expect the following compensation for my participation in this 3-month study:

- I will receive three maximal exercise tests and resting and exercise cardiovascular assessments (blood pressure, blood flow, ECG, heart rate, etc), along with reports that I may provide to my personal physician. These tests typically cost from $250 to $300 per test in a healthcare facility. This equates to ~ $600; and
- I will receive three nutritional analyses over the 3-month study. This type of analysis normally costs $50 per session. This equates to ~$150.

VII. Freedom to Withdraw

I understand that, if I decline to participate in this research study or choose to discontinue my participation at anytime, there will be no penalties or loss of benefits that have been promised to me and described in this consent form.

VIII. Approval of Research

This research project has been approved, as required, by the Institutional Review Board for projects involving human subjects at Virginia Polytechnic and State University and the Department of Human Nutrition, Foods, and Exercise.

IX. Subject's Responsibilities

I know of no reason why I cannot participate in this study. I accept that it is my responsibility to:

- Accurately report my medical history or changes in my health during the 12-week study duration.
- Refrain from regular participation in vigorous physical activity for the 12-week period I am enrolled in the study.
- Consume no food during the 12-hour period before arriving at the testing lab for a scheduled exercise tests; and consume no foods during the 1-hour before an exercise training sessions;
- Refrain from caffeine, and nicotine products for 24 hours prior to the exercise tests;
- Remain in the testing and/or exercise area 30 minutes after each of the exercise testing periods.

Report any physical or medical problems that might occur outside the lab during the period of testing, even if I feel it is not related to the testing to: Carol Haskell (951-8814), Tony Kaleth (231-6469/951-1136), Tom Chittenden (231-6469/953-1941) or Dr. William Herbert (231-6565/951-0974).

X. Subject's Permission

I have read and understand the informed consent and conditions of this research study. I agree to undergo all screening procedures described above prior to acceptance into the study. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent for participation in this project.

If I participate, I may withdraw at any time without penalty. I agree to abide by all the rules of the project.

Questions/Response: _____

_____

_____

_____

_____

_____ _____

Signature Date

_____ _____

Witness (Research Coordinator) Date

[ ] Please check the box if you would like the information from these tests sent to your primary care physician.

Physician's Name and Telephone: _____

Should I have any questions about this research or its conduct, I will contact:

Carol Haskell, MD 951-8814
Research Coordinator

William G. Herbert, Ph.D. 231-6565
Principal Investigator
Human Nutrition, Foods, & Exercise

Brian Hawkins 231-6374
Investigator

Dr. David M. Moore. 231-4991
Chair, IRB, Research Division

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Informed Consent for Participant in ResMed2 Project – Experimental Group

Title of research Project: **The short-term effects of exercise training in conjunction with CPAP therapy on cardiovascular function, exercise tolerance, and quality of life in obstructive sleep apnea patients**

Principal Investigators: William G. Herbert, Ph.D., Don Zedalis, MD, John Gregg, DDS, Ph.D., William R. Huckle, Ph.D., Sharon Nickols-Richardson, Ph.D, R.D., Thomas W. Chittenden, MA, Anthony S. Kaleth, MS, Brian J. Hawkins, MS, Lawrence Cross, Ph.D.

I. Purpose of This Research

Your doctor has asked you to come to the Sleep Disorders Center and remain overnight to obtain measurements about the medical aspects of your sleep. He will evaluate the results of this study and inform you whether certain treatments may be recommended. We are conducting a research study to determine the possible treatment advantages of 1) a new generation nighttime positive pressure-breathing device (CPAP) that is prescribed for patients who have obstructive sleep apnea and 2) a moderate exercise program that is added to treatment with the new CPAP device.

V. Procedures

If your initial overnight sleep study shows that you have sleep apnea and you agree to participate in this study, you will be asked to complete the following procedures at three different time points 1) before starting CPAP treatment; 2) after 6 weeks of CPAP treatment; and 3) after 12 weeks of CPAP treatment:

- Allow us to use certain physical and health history information from the medical records at the Sleep Disorders Center.
- Complete questionnaires that request your opinion about the quality of your sleep, your current quality of life, mood, and physical activity status.
- Allow us to complete nutrition evaluations over the course of the study.
- Allow us to complete measurements including height, weight, body circumferences blood pressure, heart rate, and cardiac output.
- You will also be asked to practice a few of the breathing techniques while wearing a special mouthpiece and nose clip; this practice will be needed during you're exercise test, which we will ask you to perform at the Sleep Disorders Center in Christiansburg, VA.
- Allow us to obtain a 24-hour urine sample the day prior to each exercise test and a small venous blood sample (10mL) immediately before and after each exercise test to measure the ability of your blood vessels to relax, as well as how your

nervous and cardiovascular systems are adjusting. Part of the blood sample collected before the exercise tests, will be assayed for blood fats. Slight brushing may occur around the area of the needle stick.

- Allow us to obtain measurements of your heart rate and blood pressure collected two consecutive days before your exercise test using small portable devices and chest electrodes that you may keep with you for this period; these measurements will help the investigators evaluate the nervous system control of your heart.
- Complete a bicycle exercise test of approximately 12 minutes and allow us to measure your maximal fitness level at your peak effort, as well as your oxygen consumption, heart rate, blood pressure, and cardiac output. To measure how much oxygen you use during this exercise, we will ask you to breathe into a lightweight rubber mouthpiece. During exercise, you will breathe only through the mouthpiece and may experience some dryness in your mouth. You will be asked to perform several exercise cardiac output measurements that require you to slowly exhale a special mixture of oxygen and tiny amounts of a harmless gas that doesn't interact with the body; the time you exhale for this measurement will last only 5 to 6 seconds. You may experience more difficulty completing this procedure during higher intensities of exercise, but the investigators will only ask that you do your best to accomplish this.

The total time involved to complete these procedures will be ~1hour and 30 minutes per session. There will be 3 sessions 1) before starting CPAP treatment; 2) after 6 weeks of CPAP treatment; and 3) after 12 weeks of CPAP treatment.

After your first exercise test, you must agree to be assigned to one of two groups for treatment; the investigators will be unable to tell you which group this will be, until after you agree to participate in the study. The groups are:

- A group that will receive CPAP therapy with the new generation device (CPAP alone).
- A group that will receive CPAP therapy with the new device and an organized moderate exercise program; the exercise program will involve physical activities such as brisk walking or similar stationary cycling for 20-40 minutes/day, 3-4 days/week.
- Appropriate use of the CPAP system is detailed in the information and explanation given to you by the sleep staff at the time of OSA diagnosis. If you have any questions regarding the proper use of this device please contact the Research Coordinator.

Details of the Exercise Program: Exercise training will last for 12 weeks. For the first 2 to 4 weeks, exercise sessions will be held at the Health and Exercise Science Laboratory (H&ESL) on the Virginia Tech campus or The Town of Blacksburg Senior Citizen Exercise Facility (TBSCF) located on Patrick Henry Drive near Blacksburg High School. Two weeks after the start of the supervised exercise training program (3 days/wk), you will be requested to increase the frequency of exercise $\geq$ 3days/wk, and be given the option to exercise unsupervised on one of your scheduled 3 days/wk. Between months 1 to 3, you will be asked to exercise at least 4 days per week and report to the H&ESL or

TBSCF for at least two supervised exercise sessions/month. Exercise logs and heart rate monitors will be distributed to document the additional quantity of exercise over this period. You will be allowed to participate at the local community activity center or at the University-based adult exercise program that is held three mornings/wk in the same Virginia Tech building as the H&ESL (War Memorial Hall). Activities in the local community center will include walking/jogging or cycling, in accordance with individualized plans specified by the research staff. To provide flexibility for exercise sessions, subjects will be offered the opportunity to participate in morning, afternoon and/or evening monitored exercise sessions at the H&ESL or TBSCF. Your exercise level will be based on your initial exercise test and will be adjusted, as necessary, after the 6-week test. After completion of the first 6 weeks and at 3 months, all participants will be asked to return for a second and third exercise test, respectively.

VI. Risks

It is my understanding and I have been informed that there exists the possibility during exercise of adverse changes during the actual test and/or exercise sessions. I have been informed that these changes could include abnormal blood pressure, fainting, disorders of heart rhythm, and in very rare instances, heart attack or death (~1 death in 10,000 exercise tests). Every effort will be made to minimize these possibilities for you by preliminary examination and by precautions and observations taken during the test. The intensity of the cycling exercise will increase as you pedal, over about 12 minutes. At first it will be very easy and then become harder; during the last few minutes, the work will become very intense and will represent a maximal effort on your part. It may be as hard as any exercise that you remember doing.

Qualified medical personnel will be available to perform CPR and contact the community Emergency Medical Services by telephone to deal with unusual situations, should these occur during your exercise tests. Emergency equipment and defibrillation are available at this facility and a medical professional with training in advanced cardiac life support will be onsite for all exercise testing. However, a thorough screening for signs of active heart disease and a review of your medical records from your primary healthcare provider will be done before you are allowed to take the exercise test. This will further reduce the chance of heart problems during the exercise testing procedure.

I understand that there is a very small risk of injury, heart attack, or death as a result of my performance of this test and participation in exercise training, but knowing these risks, it is my desire to proceed to take the take and be a subject in this research project. I understand that the results of this test will be sent to my primary care physician, if I so request. These results may help to determine my ability to safely perform certain types of physical work or exercise.

I understand that my participation in this research project is voluntary and that I may withdraw at any time, without penalty of any kind. Furthermore, I also understand that there is no guarantee that I will benefit from this research project.

VII. Benefits of this Project

- Both you and your physician will be provided with your individual results from your exercise test. This test is also be used for evaluating the condition of your heart and lungs. If your physician notes a concern after reviewing the results of this procedure, you and your physician may decide that you should consult with an appropriate healthcare specialist. However, any and all costs related to such a referral and medical care will by borne by you and not by Virginia Tech, nor any of its agents including the investigators.
- You will be provided with and allowed to keep, as a result of participating in the study, a state of art AutoSet T$^{\rightarrow}$ breathing device from the ResMed Corporation. This breathing device costs ~$4,500 and it will be supplied to you without any cost to you or your health insurance provider, other than what would be paid were you to receive the less-advanced CPAP device usually prescribed by Dr. Zedalis for patients not in this study. .
- Participation in this study will result in increased communication about your obstructive sleep apnea care between you, Dr. Zedalis' staff, and the research team. A trained medical professional will act as a research coordinator stay in contact with you to monitor and manage your progress throughout the study, usually on a weekly basis or whenever you request assistance for advice or needs related to your participation in the study.
- A trained nutritionist or dietitian will evaluate and make general recommendations to you about the type and amount of foods that you are eating. This information may be beneficial for your health and controlling risk factors for chronic diseases, such as coronary heart disease. Were you not in the study, this type of analysis normally costs $50 per evaluation; you will receive three such evaluations, without charge.
- By agreeing to take part in this study, physicians may gain a better understanding of how the heart and lungs function during sleep disordered breathing. This may also result in better care for all sleep apnea patients.

V. Extent of Anonymity and Confidentiality

The results of this study will be kept strictly confidential. At no time will the researchers release my results of this study to anyone other than the individuals working on the project without your written consent. However, if the need arises, I give my permission for Dr. Zedalis' office to obtain my medical records from my primary healthcare physician. Furthermore, I understand that the information I provide will have my name removed and only a subject number (excluding social security numbers) will identify me during analyses and written reports of this research.

VI. Compensation

I can expect the following compensation for my participation in this 3-month study:

- I will receive three maximal exercise tests and resting and exercise cardiovascular assessments (blood pressure, blood flow, ECG, heart rate, etc), along with reports that I may provide to my personal physician. These tests typically cost from $250 to $300 per test in a healthcare facility. This equates to ~ $600;
- I will also receive a $4,500 AutoSet T breathing device from the ResMed Corporation. The added value of this system will depend on the type of standard CPAP coverage and co-payment plan that pertains to your individual health insurance;
- I will receive three nutritional analyses over the 3-month study. This type of analysis normally costs $50 per session. This equates to ~ $150; and
- If I am in the exercise-training group, I will receive 12 hours of exercise training in a supervised setting. These sessions typically cost from $15 to $20 per session. This equates to ~$240.

VII. Freedom to Withdraw

I understand that, if I decline to participate in this research study or choose to discontinue my participation at anytime, there will be no penalties or loss of benefits in my health care that will be provided by the attending physician or physicians who are providing care for me at the Sleep Disorders Center of Southwest Virginia.

VIII. Approval of Research

This research project has been approved, as required, by the Institutional Review Board for projects involving human subjects at Virginia Polytechnic and State University and the Department of Human Nutrition, Foods, and Exercise.

IX. Subject's Responsibilities

I know of no reason why I cannot participate in this study. I accept that it is my responsibility to:

- Accurately report my medical history;
- Consume no food during the 12-hour period before arriving at the testing lab for a scheduled exercise tests; and consume no foods during the 1-hour before an exercise training sessions;
- Refrain from caffeine, and nicotine products for 24 hours prior to the exercise tests;
- Remain in the testing and/or exercise area 30 minutes after the exercise testing period.
- Attend all exercise sessions for the duration of the study;

- Inform the investigators if I am not able to attend an exercise session at least one day prior to the session;
- Refrain from vigorous physical activity for 12 hours on all exercise testing days.

Report any physical or medical problems that might occur outside the lab during the period of testing, even if I feel it is not related to the testing to: Tony Kaleth (231-6469/951-1136), Tom Chittenden (231-6469/953-1941) or Dr. William Herbert (231-6565/951-0974).

X. Subject's Permission

I have read and understand the informed consent and conditions of this research study. I agree to undergo all screening procedures described above prior to acceptance into the study. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent for participation in this project.

If I participate, I may withdraw at any time without penalty. I agree to abide by all the rules of the project.

Questions/Response: _____

_____

_____

_____

_____

_____ _____

Signature Date

_____ _____

Witness (Research Coordinator) Date

[ ] Please check the box if you would like the information from these tests sent to your primary care physician.

Physician's Name and Telephone: _____

Should I have any questions about this research or its conduct, I will contact:

_____382-1165 William G. Herbert, Ph.D. 231-6565

Research Coordinator Principal Investigator

Human Nutrition, Foods, & Exercise

Tony Kaleth 231-6469
Investigator

Dr. David M. Moore. 231-4991
Chair, IRB, Research Division

Tom Chittenden 231-6469
Investigator

APPENDIX E

IRB APPROVAL

Virginia Tech
VIRGINIA POLYTECHNIC INSTITUTE
AND STATE UNIVERSITY

Institutional Review Board

Dr. David M. Moore
IRB (Human Subjects) Chair
Assistant Vice Provost for Research Compliance
CVM Phase II - Duckpond Dr., Blacksburg, VA 24061-044
Office: 540/231-4991; FAX: 540/231-6033
e-mail: moored@vt.edu

June 28, 2002

MEMORANDUM

TO: William Herbert HNFE 0430
Sharon Nickols-Richardson HNFE 0430
Lawrence Cross ELPS 0302
William Huckle DBSP 0442

FROM: David M. Moore

SUBJECT: IRB EXPEDITED CONTINUATION "The Short-Term Effects of Exercise Training in Conjunction with CPAP Therapy on Cardiovascular Function, Exercise Tolerance, and Quality of Life in Obstructive Sleep Apnea Patients" – IRB # 02-350 ref 01-281

This memo is regarding the above referenced protocol which was previously granted expedited approval by the IRB on July 16, 2001. The proposed research is eligible for expedited review according to the specifications authorized by 45 CFR 46.110 and 21 CFR 56.110. Pursuant to your request, as Chair of the Virginia Tech Institutional Review Board, I have granted approval for extension of the study for a period of 12 months, effective as of July 16, 2002.

Approval of your research by the IRB provides the appropriate review as required by federal and state laws regarding human subject research. It is your responsibility to report to the IRB any adverse reactions that can be attributed to this study.

To continue the project past the 12-month approval period, a continuing review application must be submitted (30) days prior to the anniversary of the original approval date and a summary of the project to date must be provided. Our office will send you a reminder of this (60) days prior to the anniversary date.

cc:File

VITA

Brian John Hawkins was raised outside of Oley, Pennsylvania by his parents Barry and Elsie Hawkins along with his two brothers, Scott and David. Following in his older brother David's footsteps, Brian attended the Pennsylvania State University upon graduation from high school in 1993. Involved in athletics his entire life, he decided to pursue a degree in Exercise Science with a minor in Business to prepare him for employment upon commencement. Internship experiences in the non-invasive cardiology laboratory and cardiac rehabilitation program at Geisinger Medical Center in Danville, PA peaked Brian's interest in cardiovascular system and prompted him to pursue graduate education at Bloomsburg University, the alma mater of his younger brother Scott. At Bloomsburg, Brian continued his development as a cardiovascular researcher through a curriculum in exercise physiology. It was here that he began to focus on the biology of the cardiovascular system and its control under exercise stress.

Following graduation from Bloomsburg in 2000, Brian enrolled in the doctoral program in Human Nutrition, Foods, and Exercise at Virginia Tech. Early in doctoral education, Brian began to realize the importance of utilizing an integrative approach to cardiovascular disease, and his career direction began to shift from teaching to research. The need for mechanistic study in cardiovascular research prompted him to alter his coursework and preparation to include relevant laboratory skills and the graduate curriculum in Molecular Cell Biology and Biotechnology. The focus of his doctoral research employed these skills to investigate the cardiovascular co-morbidity associated with obstructive sleep apnea. In particular, Brian attempted to delineate the relationship

between circulating biomarkers of vascular function to blood pressure regulation in mild-moderate patients.

Upon receiving his doctorate in August of 2003, Brian plans to continue his development through postdoctoral training. In particular, he wishes to focus on the mechanism of vascular function as it relates to the development of cardiovascular disease. Following adequate postdoctoral training, Brian hopes to secure a position as an instructor and researcher at a recognized institution and continue his study into the contol of the vasculature. He hopes to eventually establish a laboratory to investigate the mechanism by which physiologic stress, e.g. exercise, protects the cardiovascular system at the cellular level. He further wishes to mentor and instruct the next generation of developing scientists.