ABSTRACT

Title of Document: INFLUENCE OF AT1R POLYMORPHISMS AND AEROBIC EXERCISE TRAINING ON ANGIOTENSIN II, OXIDATIVE STRESS AND NITRIC OXIDE

Nicola Melissa Fenty, Doctor of Philosophy, 2007

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Oxidative stress is implicated in the pathogenesis of cardiovascular disease (CVD) and angiotensin II (AngII), via the type 1 receptor (AT1R), is a major factor contributing to oxidative stress. Protection against oxidative injury is provided by several antioxidants, including superoxide dismutase (SOD). Aerobic exercise training (AEX) is a non-pharmacological intervention that reduces the risk of CVD, partly through reducing levels of oxidative stress. We investigated whether the AT1R A1166C and -825 T/A polymorphisms and AEX influence oxidative stress, urinary NOx and plasma AngII. One hundred sedentary, hypertensive individuals underwent 6 months of standardized AEX. Plasma levels of AngII and SOD, and urinary excretion of NOx and 8-iso-$PGF_{2\alpha}$ were measured before and after AEX. Subject characteristics and baseline values of outcome variables were similar among all genotype groups. Overall, there was a significant increase in 8-iso-$PGF_{2\alpha}$ ($p = 0.002$)

and a significant decrease in NOx excretion ($p = 0.0001$) however, there were no significant changes in SOD activity or AngII levels with AEX. Neither oxidative stress markers nor urinary NOx were significantly different between genotype groups with AEX. There was a significant difference in AngII levels with AEX between A1166C genotype groups ($p = 0.04$) resulting in a significant interactive effect of the A1166C polymorphism and AEX on the change in AngII ($p < 0.05$). The TT genotype group of the -825 T/A polymorphism had a significant reduction ($p = 0.02$) in plasma AngII, while there was no change in carriers of the A allele. Risk allele analysis revealed that there was a significant reduction in plasma AngII ($p = 0.04$), a significant increase in 8-iso-$PGF_{2\alpha}$ ($p = 0.01$) and a significant decrease in urinary NOx ($p = 0.0001$) with AEX in individuals with 2 risk alleles. Our findings suggest that variation in the AT1R gene is associated with differential changes in plasma AngII but not with oxidative stress. Furthermore, our results may have clinical implications for the prescription of AEX in a population at risk for CVD as exercise intensities that surpass moderate intensity, may attenuate some of the beneficial effects of regular exercise by leading to increased oxidative stress.

INLFUENCE OF AT1R POLYMORPHISMS AND AEROBIC EXERCISE TRAINING ON ANGIOTENSIN II, OXIDATIVE STRESS AND URINARY NITRIC OXIDE.

By

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DEDICATION

To my mother, Angela M. Fenty.

You truly have been my inspiration throughout this entire process and I would never have had the opportunity to succeed without your hard work and whispers of encouragement and motivation during my undergrad years. Words cannot express how much I miss you and wish you were here to share these moments with me. I love you forever and may you continue to rest in peace.

To my father, David L. Fenty.

Thank you for your continued support and encouragement. You are my backbone of strength and determination. Just knowing you're here makes all the difference.

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LIST OF ABBREVIATIONS

8-iso-PGF$_{2\alpha}$ – 8 isoprostane prostaglandin $F_{2\alpha}$

ACE – angiotensin converting enzyme

AGT - angiotensinogen

AHA – American Heart Association

AngII – angiotensin II

AT1R – angiotensin II type 1 receptor

AT2R – angiotensin II type 2 receptor

BH_4 – tetrahydrobiopterin

BP – blood pressure

Cu/ZnSOD – copper/zinc superoxide dismutase

CV – cardiovascular

CVD – cardiovascular disease

DNA – deoxyribonucleic acid

ec-SOD – extracellular superoxide dismutase

eNOS – endothelial nitric oxide synthase

GFR – glomerular filtration rate

GPX – glutathione peroxidase

HDL – high-density lipoprotein

iNOS – inducible nitric oxide synthase

LDL – low-density lipoprotein

MDA – malonyldialdehyde

Mn-SOD – manganese superoxide dismutase

MPO – myeloperoxidase

mRNA – messenger ribonucleic acid

NADPH oxidase – nicotinamide adenine dinucleotide phosphate oxidase

nNOS – neuronal nitric oxide synthase

NO – nitric oxide

NOx – nitrates/nitrites

p47phox – a cytosolic subunit of NADPH oxidase

PCR – polymerase chain reaction

PKC – protein kinase C

RAS – renin-angiotensin system

ROS – reactive oxygen species

SHR – spontaneously hypertensive rat

SNP – single nucleotide polymorphism

SOD – superoxide dismutase

TBARS – thiobarbituric acid reactive substances

TG – triglycerides

UTR – untranslated region

VO_{2max} – maximal oxygen consumption

INTRODUCTION

Hypertension is a major risk factor for congestive heart failure, coronary artery disease and renal failure. It has a multifactorial etiology with a strong genetic component and is associated with an increase in reactive oxygen species (ROS) and a reduction in endogenous antioxidant defenses termed oxidative stress.[1] Oxidative stress may directly or indirectly contribute to the progression of end-organ injury by promoting hypertension, atherosclerosis or by inducing glomerular damage and renal ischemia.[2]

Oxidative Stress

ROS have recently been identified as important signaling molecules.[3] They are highly reactive molecules that have undergone a univalent reduction such that they react readily with other biological products.[4] Superoxide, one member of the ROS, has been implicated in the inactivation of nitric oxide (NO), leading to the generation of the pro-oxidant peroxynitrite and promoting endothelial dysfunction in the process.[2, 5] However, under normal physiological conditions, the rate of superoxide production is balanced by the rate of elimination.[6, 7] Superoxide acts as a second messenger that activates several redox sensitive transcription factors[8] and as a result, it is involved in vascular smooth muscle cell growth and migration, expression of adhesion molecules and modification of the extracellular matrix.[8] In addition, there is increasing evidence to support the role of superoxide and other ROS in the development of the organ damage associated with hypertension and cardiovascular disease (CVD). [2, 5, 8-10]

Endothelial Dysfunction

The endothelium plays a critical role in the regulation of vascular function by controlling the contractile and thrombotic state of the vessel. NO synthesized from L-arginine, is recognized as one of the central factors involved in the maintenance of vascular tone and thus, is important in the modulation of blood pressure (BP).[11] In addition, NO is particularly important for the prevention of platelet aggregation, leukocyte adhesion and smooth muscle cell proliferation.[12, 13] Endothelial dysfunction is a characteristic feature of patients with hypertension and atherosclerosis, and studies indicate that it may predict disease progression and cardiovascular (CV) event rate.[14-17] Impaired endothelium-dependent relaxation may be caused by several factors however, it is thought that the degradation of NO via interaction with ROS reduces NO availability and subsequently leads to endothelial dysfunction. Although the precise mechanisms responsible for endothelial dysfunction are unclear, the consensus is that increased production of ROS may contribute significantly to this event.[14] This observation is consistent with evidence indicating that oxidative stress may be a central feature of the hypertensive process. The *in vivo* concentration of NO cannot be measured directly because of its very short half-life; therefore, the stable end-products of NO metabolism, nitrates/nitrites (NOx) offer a useful index of NO generation. Evidence indicates that NOx levels are lower in disease states including hypertension, further indicating a link between oxidative stress, NO bioavailability, endothelial dysfunction, and hypertension.[18, 19]

Angiotensin II

Angiotensin II (AngII) is a potent vasoconstrictor that is associated with hypertension, opposes NO action and increases the production of ROS.[9] It has been shown to regulate the growth and migration of smooth muscle cells and fibroblasts, apoptosis of endothelial cells, differentiation of monocytes, and extracellular matrix remodeling via activation of metalloproteinases.[20] The overwhelming complexity of cellular responses to AngII is further complicated by the multiple pathways for generating AngII. In addition to the classical source of plasma AngII (circulating plasma-derived AngII), the existence of a local tissue-based production of AngII has been established.[20, 21] The tissue-based production of AngII appears to be involved in the long term changes in response to AngII including remodeling of blood vessels and kidney function.[21] Therefore, not only is AngII important for the normal regulation of arterial tone, but it is potentially critical to the pathogenesis of CVD.[20] There are at least two G-protein coupled receptors that mediate the actions of AngII, specifically the angiotensin II type 1 receptor (AT1R) and the angiotensin II type 2 receptor (AT2R). The AT1R is known to mediate most of the physiological and pathophysiological actions of AngII including increasing superoxide production by stimulating a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-containing cytochrome subunit.[4, 22] AngII-induced ROS production plays a critical role in several pathological conditions affecting vascular cells and renal cells including hypertension, endothelial dysfunction, atherosclerosis and cellular remodeling.[23-25]

Antioxidants

Superoxide dismutase (SOD) is the frontline defense against ROS-mediated injury. Under normal physiological conditions, dismutation of superoxide by SOD results not only in the scavenging of superoxide and increased availability of NO but also in the formation of water and the more stable ROS, hydrogen peroxide.[4 6] Superoxide may also spontaneously give rise to hydrogen peroxide.[25] Hydrogen peroxide has a longer half-life than superoxide, is lipid soluble and can cross cell membranes, but under normal physiological conditions it is generally scavenged by catalase and glutathione peroxidase.[6, 26] In the presence of excess superoxide or transition metals such as iron or copper, hydrogen peroxide can be converted to hydroxyl radical which is considered to be the most cytotoxic ROS known.[6, 26] In general, administration of antioxidants has been shown to improve arterial pressure and reduce oxidative stress in disease states,[26-28] substantiating a role for oxygen radicals in the maintenance of hypertension and suggesting that free radical defense via antioxidants is important in protecting against dysfunction that occurs in the diseased state.

AT1R gene polymorphisms

AngII via the AT1R has been shown to exacerbate oxidative stress by promoting the formation of F_2 isoprostanes, which are considered to be the most sensitive and specific marker for lipid peroxidation.[29-31] In humans the AT1R is predominately present in vascular smooth muscle cells[32] and the pro-oxidant effects of AngII are inhibited by various AT1R blockers, suggesting that the effects of AngII are dependent on the AT1R.[33, 34] The AT1R is a G-protein coupled receptor that,

when activated by AngII, leads to G-protein mediated signaling in a tissue-specific manner. The AT1R gene represents a good candidate gene for the development of both oxidative stress and hypertension. An A→ C substitution at position 1166 in the 3' untranslated region (UTR) of the gene, has been extensively studied and has been found to be associated with hypertension in various populations.[32, 35] More recently, this polymorphism was also found to be associated with oxidative stress in heart failure patients.[36] The -825 T/A polymorphism is located in the promoter region of the AT1R gene and has been found to be almost completely concordant with several other single nucleotide polymorphisms (SNPs) in the promoter.[37] It is reported to destroy a binding site for GATA transcription factors[37] and thus, may alter the expression of the AT1R and the influence of AngII on oxidative stress.

Aerobic Exercise Training

Physical activity reduces the morbidity and mortality associated with CVD and favorably modifies the oxidant/antioxidant balance.[38] It is also advocated for the reduction and control of CV risk factors including hypertension.[39, 40] In fact, a lifestyle of physical activity can reduce the risk of developing hypertension by 30-50 %[39] with reductions in systolic and diastolic BP averaging 11 and 8 mmHg respectively.[40] In addition, reports show that moderate physical activity leads to reductions in markers of oxidative stress including plasma isoprostanes and malonyldialdehyde (MDA).[41] Regular physical activity is also reported to reduce the mRNA and protein expression of AT1R with a concomitant reduction in ROS production,[42] significantly increase SOD activity and significantly decrease MDA, in aortic endothelial cells.[43] Finally, aerobic exercise training has been shown to result in

increased antioxidant capacity and reduced oxidative stress in previously sedentary men and women.[38]

PURPOSE OF THE STUDY

Oxidative stress is a pathological condition that increases the risk of developing hypertension, and in both animal models and in humans with essential hypertension, oxidative stress is increased. Several studies have indicated that the local concentration of superoxide is the major limiting factor for the availability of NO, therefore the interaction between NO and superoxide may account, in part, for the impairment of endothelial-dependent vasodilation. As a matter of fact, many of the CV risk factors that predispose an individual to the development of atherosclerosis, such as hypertension, diabetes and hypercholesterolemia, are also associated with endothelial dysfunction.

AngII via the AT1R is a potent vasoconstrictor that increases the production of ROS, reduces NO bioavailability, and indirectly stimulates lipid peroxidation thereby increasing oxidative stress. Behavioral factors such as aerobic exercise training and genetic factors may influence the development of oxidative stress by promoting an atheroprotective state in the vasculature.

Therefore, the purpose of this study is to determine the influence of the AT1R A1166C and -825 T/A polymorphisms and aerobic exercise training on AngII levels, urinary NOx excretion, and oxidative stress in pre- and stage 1 hypertensives.

HYPOTHESES

Hypothesis #1: The AT1R A1166C and -825 T/A polymorphisms will be associated with baseline levels of AngII, oxidative stress and urinary NOx.

- Compared to AA homozygotes, individuals carrying the C allele of the A1166C polymorphism will exhibit:
  - Greater AngII levels
  - Greater excretion of urinary 8-iso-$PGF_{2\alpha}$
  - Lower SOD activity
  - Lower excretion of urinary NOx
- Compared to A allele carriers individuals who are TT homozygotes of the -825 T/A polymorphism will exhibit:
  - Greater AngII levels
  - Greater excretion of urinary 8-iso-$PGF_{2\alpha}$
  - Lower SOD activity
  - Lower excretion of urinary NOx

Hypothesis #2: The AT1R A1166C and -825 T/A polymorphisms will influence the training-induced changes in AngII levels, oxidative stress and urinary NOx.

- AEX will elicit greater improvements in individuals carrying the C allele of the A1166C polymorphism compared with those having the AA genotype. C allele carriers will exhibit:
  - Greater reduction in AngII
  - Greater reduction in urinary 8-iso-$PGF_{2\alpha}$

- Greater increase in SOD activity
- Greater decrease in urinary NOx

- AEX will elicit greater improvements in TT homozygotes of the -825 T/A polymorphism compared with those carrying the A allele. TT homozygotes will exhibit:
  - Greater reduction in AngII
  - Greater reduction in urinary 8-iso-$PG_{2\alpha}$
  - Greater increase in SOD activity
  - Greater decrease in urinary NOx

METHODS

Participants

Men and women responding to mailed brochures and to advertisements in newspapers and on the radio were initially contacted by telephone to determine their interest and eligibility. To be eligible for participation, participants had to be 50 to 75 years of age, sedentary (physical activity < 20 minutes < 2 days per week), hypertensive (BP < 160/90 mmHg), non-diabetic, non-smokers and free of CV, renal, liver and lung disease. In addition, participants had to have a body mass index (BMI) < 37 Kg/m^2 and have no medical conditions that would preclude vigorous exercise. Women had to be postmenopausal (absence of menstrual cycle for > 2 years) and agree to maintain their hormone replacement therapy (HRT) regime, either on or not on, for the duration of the study. Participants were informed of the study procedures and requirements and provided their written informed consent. This study was approved by the University of Maryland at College Park Institutional Review Board.

Screening

Participants completed two screening visits to ensure that they were eligible to take part in this study.

Screening Visit #1

Blood was collected after a 12-hour overnight fast for blood chemistries and DNA analysis. After the fasting blood draw, participants underwent a 2-hour 75 g oral glucose tolerance test to assess diabetes status. Individuals were excluded from the study if they had a hematocrit < 35 %, evidence of renal (GFR < 60 ml/min/1.73

m^2) or liver disease, triglyceride (TG) levels > 400 mg/dL, fasting blood glucose levels > 126 mg/dL or 2-hour glucose levels > 200 mg/dL.

Screening Visit #2

Qualified participants were required to undergo a physical examination by a physician to ensure that there was no evidence of CV, pulmonary, or other chronic diseases that would preclude exercise testing or training.[44] In addition, a maximal graded treadmill test was performed and the test was terminated when the participant could no longer continue or CV signs or symptoms occurred. [44] Participants who had > 2 mV ST-segment depression or CV signs or symptoms were excluded from the study.

Dietary Stabilization

Participants were stabilized on what was previously known as the American Heart Association (AHA) Step 1 diet for 6 weeks. This diet consists of ~55 % of total daily calories from carbohydrates, < 30 % from fat, and 15 % from protein. Weight and BP were recorded at each visit (2 days/week) to the laboratory and participants were required to remain within 5 % of their study entry body weight for the entirety of the study. Individuals taking anti-hypertensive medications were tapered off of their medication during dietary stabilization after obtaining written approval from their personal physician. Participants with BP consistently < 120/80 mmHg or > 159/99 mmHg were excluded from the study.

Baseline Testing

Percentage of body fat and lean body mass were measured by dual energy X-ray (DEXA) as previously described.[45] A second maximal treadmill test, supervised by the study physician, was used to determine participants' CV fitness and to develop individualized exercise prescriptions. The test began at 70 % of the peak heart rate achieved during the screening exercise test and the treadmill grade was increased by 2 % every 2 minutes. BP, ECG and oxygen consumption (VO_2) were measured continuously throughout the test. Standard criteria were used to determine if true maximal VO_2 (VO_{2max}) was achieved.[46]

Casual Blood Pressure Measurement

Casual BP was measured in all participants on three separate days according to the JNC VII guidelines.[47] Briefly, participants were fasted for ≥ 12-hours before measurement. They sat quietly in a seated position with feet flat on the floor for 15-20 minutes and BP was measured at least twice until systolic values were within 4 mmHg and diastolic values were within 4 mmHg. When these criteria were met, the average BP was recorded. The average of the three separately recorded BP values was used as the outcome variable in data analyses.

24-hour Urine Collection

24-hour urine collection was used to measure urinary 8-iso-$PGF_{2\alpha}$ and urinary NOx. Participants were required to follow a low nitrate diet two days prior to and on the day of urine collection to eliminate the potential effect of dietary nitrate intake on urinary nitrate levels.

Measurement of Plasma Angiotensin II

All plasma samples were drawn in the morning after a 12-hour overnight fast. Solid phase extraction using phenyl cartridges (Phenomenex, Torrance, CA) and an Alltech Vacuum Manifold (Deerfield, IL) were used to isolate AngII peptides. The cartridges were pre-washed with 1 ml of methanol followed by 1 ml of deionized water. The plasma sample (1 ml) was then passed through the cartridge followed once more by 1 ml of deionized water. AngII peptides were eluted with 0.5 ml of methanol, evaporated to dryness using vacuum centrifugation and stored at -20°C until use. The extracted samples were reconstituted with 0.5 ml of EIA buffer (Cayman Chemicals, Ann Arbor, MI) and plasma AngII levels were measured using a commercially available enzyme immunoassay kit according to the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI). Baseline and final samples were assayed on the same plate and all samples were assayed in duplicate. The inter-assay and intra-assay coefficient of variation (CV) were 5 % and 20 % respectively.

Measurement of Superoxide Dismutase Activity

Blood samples for plasma SOD analysis were drawn into heparinized tubes, centrifuged at 3000 rpm for 20 minutes at 4°C, transferred to plastic microtubes (1.5 µL) and stored at -80°C until use. SOD activity was measured using a commercially available assay kit according to the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI). Baseline and final samples were assayed on the same plate and all samples were assayed in duplicate. The inter-assay and intra-assay CV were 7 % and 22 % respectively.

Measurement of Urinary Nitrates/Nitrites

Urinary NOx levels were measured using a modified Griess assay. This assay relies on a diazotization reaction that was previously described by Griess in 1879. Prior to performing the assay, urine samples were centrifuged at 3000 rpm for 15 minutes at 4°C. The assay was performed as previously described.[48] Briefly, the assay involved the reduction of nitrate to nitrite using nitrate reductase (Aspergilus species) and measurement of nitrite via the magenta-colored azo dye formed when nitrite reacts with the Griess reagents; 0.1 % N-[1-naphthylethylenediamine dihydrochloride (NED) and 1 % sulfanilamide in 5 % phosphoric acid. The concentration of urinary NOx represents the total amount of urinary NO end-products (nitrate and nitrite), and absorbance was determined by using an Emax Maxline Microplate Reader (Sunnyvale, CA) which was read at 541 nm. Baseline and final samples were assayed on the same plate and all samples were assayed in duplicate. The inter-assay and intra-assay CV were 4 % and 13 % respectively.

Measurement of Urinary 8-iso-$PGF_{2\alpha}$

Urinary levels of 8-iso-$PGF_{2\alpha}$ were measured in the laboratories of Samar Basu, PhD in Uppsala, Sweden, as previously described.[49] In brief, an antibody was raised in rabbits by immunization with 8-iso-$PGF_{2\alpha}$ coupled to BSA at the carboxylic acid by 1, 1'-carbonyldiimmidazole method.[50]

Exercise Intervention

Following baseline testing, all participants began the exercise training phase. This consisted of monitored exercise sessions, 3 times per week for 24 weeks.

Initially, training sessions consisted of 20 minutes of exercise at 50 % VO_{2max}. Training duration was increased by 5 minutes per week for the first 5 weeks until 40 minutes was achieved and then training intensity was increased by 5 % per week until 70 % of VO_{2max} was achieved. After 10 weeks of training, a fourth unsupervised exercise session was added. Participants were required to record their exercise heart rate, exercise duration and mode of exercise in printed logs for all supervised sessions. They were also required to complete food frequency checklists every two months to monitor dietary intake and to ensure continued compliance with the AHA Step 1 diet.

Final Testing

Upon completion of the exercise training intervention, all tests performed at baseline were repeated. Participants continued exercise training until all final tests were completed. Blood samples for analysis were drawn 24-36 hours after an exercise session. Only subjects who completed the exercise intervention with > 70 % adherence were included in the final statistical analyses.

Genotyping

Genotyping for the AT1R polymorphisms was performed at the Functional Genomics Laboratory in the Department of Kinesiology, University of Maryland. Genotyping for the AT1R A1166C polymorphism was carried out by amplification of target sequences using polymerase chain reaction (PCR). The primers that were used are: AGTR1 (A1166C) forward primer 5'-AGA AGC CTG CAC CAT GTT TTG AG-3' and AGTR1 (A1166C) reverse primer 5'-CCT GTT GCT CCT CTA ACG

ATT TA-3'. The PCR product was digested with the *Dde I* enzyme, amplified using gel electrophoresis and read under ultraviolet light. Since the -825 T/A polymorphism does not create or destroy any known enzyme restriction site,[37] a genotype assay was developed using the 5'-nuclease allelic discrimination assay (TaqMan Assay) which was performed using a TaqMan PCR Core Reagent Kit (Applied Biosystems, Foster City, CA). First, the PCR was optimized using the AGTR1 (-825 T/A) forward primer 5'-AGC AGC GAG TGA CAG GAC TT-3' and AGTR1 (-825 T/A) reverse primer 5'-CCA TGG TCA AGT GAG GTT GA-3'. For the TaqMan assay, each PCR (12.5 µL) contained 1.5 µL of genomic DNA, 0.625µL of 20X diluted SNP mix and 6.25µL of 2X TaqMan universal PCR mix (Perkin Elmer, Applied Biosystems Division, rs# 275651). The cycling protocol for the PCR was as follows: 50°C for 2 minutes, 95°C for 10 minutes, 50 cycles of 95°C for 15 minutes and 60°C for 1 minute. Fluorescence in each well was measured using an ABI 7300 Real Time PCR system machine (Perkin Elmer, Applied Biosystems Division). Genotypes were determined by the ABI 7300 sequencer detection system software. For quality control purposes, 46 samples were directly sequenced. A large number of sequencing controls were necessary because of the low frequency (3%) of the AA genotype, however, only 8 samples were used as sequencing controls for each assay.

Genotyping for the ACE I/D and the AGT M235T polymorphisms was done at the University of Pittsburgh in the laboratories of Dr. Robert Ferrell. The primers that were used are: ACE (I/D)1 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3' and ACE (I/D)2 5'-GAT GTG GCC ATC ACA TTC GTC AGA T-3'; AGT(235) forward primer 5'-GAT GCG CAC AAG GTC CTG T-3' and AGT(235) reverse

primer 5'-TGC TGT CCA CAC TGG CTC gC-3' (lower case letter identifies a forced cut site for BstUI restriction enzyme).

Statistical Analysis

Primary statistical analyses were performed on the major outcome variables; plasma AngII, total SOD activity, urinary 8-iso-$PGF_{2\alpha}$ and urinary NOx, while secondary statistical analyses were performed on body composition, lipoprotein lipid profile, VO_{2max}, and BP. Data distribution was examined using the Shapiro-Wilk test of normality and homogeneity of variances (HOV) was determined using Levene's test. Plasma AngII levels were skewed and therefore were log transformed before analysis. Because of the low frequency of the AT1R 1166C homozygotes (4 %) and the AT1R -825A homozygotes (3 %), comparisons were made between carriers and non-carriers of the C allele for the A1166C polymorphism (AA vs. AC/CC), and carriers and non-carriers of the A allele for the -825 T/A polymorphism (TT vs. TA/AA). The interaction between these two polymorphisms was determined using risk allele combinations. Because of the low sample size in the group with no risk alleles (n = 3), participants with 1 risk allele were combined with this group for comparison. Therefore, in a separate analysis, participants were placed into one of 3 risk groups; group 1 = no risk alleles or 1 risk allele present, group 2 = 2 risk alleles present, and group 3 = 3 or more risk alleles present. Pearson correlation coefficients were used to examine relationships between outcome variables and to determine possible covariates for use with analysis of covariance (ANCOVA). Although previous data support the use of gender and HRT status as potential factors that may influence the outcome variables, a test of interactions between the AT1R

polymorphisms and these variables suggested no such interactions in this dataset. Chi-square tests were used to compare differences in categorical variables between genotype groups. This revealed a significant difference between genotype groups in the distribution of ethnicity for the AT1R A1166C polymorphism and for the AT1R -825 T/A polymorphism respectively ($p = 0.002$, $p = 0.002$) and as a result ethnicity was used as a covariate in all analyses. In addition, based on current literature, the potential influence of the angiotensinogen (AGT) M235T polymorphism and the angiotensin converting enzyme insertion/deletion (ACE I/D) polymorphism on AngII levels and the potential influence of age on oxidative stress levels, supports their use as covariates in the analyses. ANCOVA was used to compare differences between genotype groups at baseline and in response to aerobic exercise training. Baseline values were also used as covariates in analysis of training-induced changes. Paired t-tests were used to determine if there were significant changes in outcome variables with aerobic exercise training. Linear regression was used to assess the portion of variation in the outcome variables that was accounted for by the AT1R A1166C and -825 T/A polymorphisms. Chi-square tests were used to determine if the AT1R allele and genotype frequencies were in Hardy-Weinberg equilibrium. All statistical analyses were performed using SAS version 9.1. Statistical significance was set at $p \leq 0.05$ and all data are expressed as means ± SE.

RESULTS

General Characteristics for the Entire Group

Baseline characteristics of the 100 participants used in the analyses are presented in Table 1. Among the 100 participants, there were 45 men and 55 women and 20 % of the women were on HRT. Fifty-four percent of participants were Caucasian and 46 % were non-white. Genotype frequencies for the AT1R A1166C polymorphism were AA (62 %), AC (34 %), and CC (4 %) resulting in a rare allele frequency of 21 %. The frequency of the rare allele in the study population is slightly lower than previously reported [32, 51, 52] and is consistent with Hardy-Weinberg equilibrium ($p = 0.80$). Genotype frequencies for the AT1R -825 T/A polymorphism were TT (68 %), TA (29 %), and AA (3 %) resulting in a rare allele frequency of 17 %. The frequency of the rare allele for this polymorphism is similar to previous reports[53, 54] and is consistent with Hardy-Weinberg equilibrium ($p = 0.96$). Genotype and ethnicity frequencies for the AT1R A1166C polymorphism were AA + Caucasian (26 %), C allele carriers + Caucasian (28 %), AA + non-white (36 %), and C allele carriers + non-white (10 %). Genotype and ethnicity frequencies for the AT1R -825 T/A polymorphism were TT + Caucasian (44 %), A allele carriers + Caucasian (10 %), TT + non-white (24 %), and A allele carriers + non-white (22 %). There were no significant correlations among oxidative stress markers, urinary NOx and AngII levels at baseline in the entire group. In univariate analysis, ethnicity was the only independent predictor of AngII levels ($p = 0.02$), explaining 6.42 % of the observed variance however; after accounting for age, gender, A1166C, -825 T/A, M235T and ACE I/D polymorphisms, ethnicity was no longer an independent predictor <Table

2>. In multivariate analysis, the A1166C and -825 T/A polymorphisms explained ≤1 % of the observed variance in AngII levels, oxidative stress and urinary NOx. Altogether genetic factors investigated in the present study accounted for 4.46 % of the observed variance in plasma AngII, 0.36 % of the observed variance in urinary 8-iso-$PGF_{2\alpha}$, 0.82 % of the observed variance in urinary NOx, and 1.67 % of the observed variance in SOD activity.

Baseline Genotype Differences

AT1R A1166C Polymorphism

There were no significant differences at baseline between genotype groups for the AT1R A1166C polymorphism for any of the oxidative stress variables, urinary NOx or plasma AngII. Furthermore, baseline characteristics including systolic and diastolic BP were not genotype-dependent <Table 3>. In the AC + CC genotype group only, there was a borderline inverse correlation between urinary NOx excretion and plasma AngII ($p = 0.05$) <Table 4>.

AT1R -825 T/A polymorphism

There were no significant differences at baseline between AT1R -825 T/A polymorphism genotype groups for any of the oxidative stress variables, urinary NOx or plasma AngII <Table 5>. There was a tendency towards a positive correlation between urinary 8-iso-$PGF_{2\alpha}$ and plasma AngII ($p = 0.09$), and between urinary 8-iso-$PGF_{2\alpha}$ and SOD activity ($p = 0.07$) in the TT genotype group only <Table 6>.

Table 1. Characteristics of study participants at baseline

	N	Mean	Range
Ethnicity (w/nw)	54/46		
Gender (f/m)	55/45		
Age (yrs)	100	58.4 ± 0.5	50.0 – 75.0
Height (cm)	100	169 ± 1.0	149.9 – 192.0
Weight (kg)	95	84.2 ± 1.5	53.6 – 118.0
Total Fat (%)	89	37.8 ± 0.9	12.3 – 55.5
Total Cholesterol (mg/dL)	85	192.4 ± 4.0	113.0 – 258.0
BMI (Kg/m^2)	95	29.3 ± 0.4	19.8 – 37.0
TG (mg/dL)	85	118.9 ± 6.2	42.0 – 316.0
LDL-C (mg/dL)	85	115.6 ± 3.2	48.0 – 177.0
HDL-C (mg/dL)	85	50.5 ± 1.9	26.0 – 112.0
Systolic BP (mmHg)	85	132.6 ± 1.2	109.4 – 162.5
Diastolic BP (mmHg)	85	87.1 ± 0.7	72.4 – 102.0
GFR ($ml/min/1.73m^2$)	82	77.8 ± 1.4	59 – 132.9
VO_{2max} (ml/kg/min)	94	24.7 ± 0.5	17.0 – 35.4
Log_AngII (pg/ml)	87	2.1 ± 0.1	0.03 – 4.7
8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	87	0.32 ± 0.01	0.1 – 0.65
NOx ($\mu mol/L)x10^{-3}$	85	2.8 ± 0.1	1.0 – 6.0
SOD (U/ml)	91	1.02 ± 0.06	0.11 – 2.86

Data are expressed as mean ± SE. BMI, body mass index; TG, triglycerides; GFR, glomerular filtration rate; VO_{2max}, maximal oxygen consumption; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; BP, blood pressure; Log_AngII, log angiotensin II; NOx, urinary nitrates/nitrites; SOD, superoxide dismutase; 8-iso-$PGF_{2\alpha}$, 8-isoprostane prostaglandin F-2 alpha; f, female; m, male; w, white; nw, non-white; N, sample size. Due to the viability of some plasma/urine samples and the inability to obtain BP measurements for all participants, samples sizes are varied.

Table 2. Linear regression analysis with AngII as the dependent variable

	Univariate			**Multivariate**		
Independent Variables	Coefficient	t	p-value	Coefficient	t	p-value
Age	-0.001	-0.07	0.95	-0.015	-0.62	0.53
Gender	-0.042	-0.17	0.86	0.041	0.14	0.88
Ethnicity	-0.569	-2.41	0.02*	-0.595	-1.71	0.09
AT1R A1166C	0.064	0.26	0.79	0.072	0.25	0.79
AT1R -825 T/A	-0.095	0.37	0.71	0.053	0.17	0.86
ACE I/D	0.051	1.79	0.07	0.052	1.73	0.08
AGT M235T	-0.009	-0.35	0.72	0.019	0.62	0.54

* $p < 0.05$

Table 3. Baseline characteristics of study participants genotyped for the AT1R A1166C polymorphism

	AT1R A1166C Genotype		
	AA	AC + CC	p-value
Age (yrs)	58.4 ± 0.8 (62)	58.8 ± 0.9 (38)	0.80
BMI (Kg/m^2)	29.2 ± 0.6 (58)	29.2 ± 0.7 (37)	0.98
TG (mg/dL)	112.0 ± 8.3 (52)	117.9 ± 9.9 (33)	0.66
LDL-C (mg/dL)	112.1 ± 4.7 (52)	118.5 ± 5.6 (33)	0.39
HDL-C (mg/dL)	53.2 ± 2.7 (52)	48.0 ± 3.3 (33)	0.24
Systolic BP (mmHg)	132.1 ± 1.8 (53)	133.4 ± 2.2 (32)	0.65
Diastolic BP (mmHg)	87.2 ± 1.0 (53)	87.0 ± 1.2 (32)	0.81
GFR ($ml/min/1.73m^2$)	78.0 ± 2.1 (51)	79.3 ± 2.6 (31)	0.62
VO_{2max} (ml/kg/min)	25.0 ± 0.5 (57)	24.4 ± 0.6 (37)	0.55
Log_AngII (pg/ml)	2.06 ± 0.17 (55)	2.14 ± 0.21 (32)	0.78
8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	0.32 ± 0.02 (55)	0.32 ± 0.02 (32)	0.96
NOx ($\mu mol/L)x10^{-3}$	2.7 ± 0.2 (54)	2.8 ± 0.2 (31)	0.97
SOD (U/ml)	1.00 ± 0.09 (58)	1.17 ± 0.11 (33)	0.26

Data are expressed as mean ± SE. Values in parentheses represent sample size. BMI, body mass index; TG, triglycerides; GFR, glomerular filtration rate; VO_{2max}, maximal oxygen consumption; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; BP, blood pressure; Log_AngII, log angiotensin II; NOx, urinary nitrates/nitrites; SOD, superoxide dismutase; 8-iso-$PGF_{2\alpha}$, 8-isoprostane prostaglandin F-2 alpha.

Table 4. Baseline correlations between major outcome variables by the AT1R A1166C genotype group

			AA Genotype Group	
		8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	NOx (µmol/L)	Log_AngII (pg/ml)
SOD (U/ml)	r	0.20	-0.13	-0.09
	p-Value	0.15	0.35	0.50
	N	52	51	52
Log_AngII (pg/ml)	r	0.09	-0.02	
	P-Value	0.55	0.87	
	N	50	48	
NOx (µmol/L)	r	-0.03		
	P-Value	0.85		
	N	46		
			AC + CC Genotype Group	
SOD (U/ml)	r	0.25	0.09	-0.16
	p-Value	0.19	0.62	0.41
	N	29	28	30
Log_AngII (pg/ml)	r	0.27	-0.39ψ	
	p-Value	0.17	0.05	
	N	28	26	
NOx (µmol/L)	r	-0.17		
	p-Value	0.37		
	N	30		

SOD, superoxide dismutase; Log_AngII, log angiotensin II; NOx, urinary nitrates/nitrites; 8-iso-$PGF_{2\alpha}$, urinary 8-isoprostane prostaglandin F 2-alpha; r, correlation coefficient; N, sample size. ψ Denotes a borderline correlation within genotype group.

Table 5. Baseline characteristics of study participants genotyped for the AT1R -825 T/A polymorphism

	AT1R -825 T/A Genotype		
	TT	TA + AA	p-value
Age (yrs)	59.2 ± 0.7 (68)	57.2 ± 1.1 (32)	0.14
BMI (Kg/m^2)	29.0 ± 0.6 (65)	29.7 ± 0.9 (30)	0.54
TG (mg/dL)	109.0 ± 7.4 (60)	127.5 ± 12.0 (25)	0.21
LDL-C (mg/dL)	109.0± 4.1 (60)	129.0 ± 6.6 (25)	0.02*
HDL-C (mg/dL)	50.0 ± 2.5 (60)	53.4 ± 4.0 (25)	0.48
Systolic BP (mmHg)	132.0 ± 1.6 (60)	135.0 ± 2.6 (25)	0.32
Diastolic BP (mmHg)	87.0 ± 1.0 (60)	88.0 ± 1.5 (25)	0.51
GFR ($ml/min/1.73m^2$)	78.0 ± 1.9 (60)	79.0 ± 3.3 (22)	0.81
VO_{2max} (ml/kg/min)	25.5 ± 0.5 (64)	23.2 ± 0.7 (30)	0.02*
Log_AngII (pg/ml)	2.08 ± 0.17 (58)	2.13 ± 0.24 (29)	0.86
8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	0.31 ± 0.02 (59)	0.33 ± 0.03 (28)	0.64
NOx ($\mu mol/L)x10^{-3}$	2.9 ± 0.2 (58)	2.5 ± 0.2 (27)	0.33
SOD (U/ml)	1.09 ± 0.08 (62)	1.00 ± 0.13 (29)	0.55

Data are expressed as mean ± SE. Values in Parentheses represent sample size. BMI, body mass index; TG, triglycerides; GFR, glomerular filtration rate; VO_{2max}, maximal oxygen consumption; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; BP, blood pressure; Log_AngII, log angiotensin II; NOx, urinary nitrates/nitrites; SOD, superoxide dismutase; 8-iso-$PGF_{2\alpha}$, 8-isoprostane prostaglandin F-2 alpha.* Denotes statistically significant difference between genotype groups.

Table 6. Baseline correlations between major outcome variables by the AT1R -825 T/A genotype group

		TT Genotype Group		
		8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	NOx (μmol/L)	Log_AngII (pg/ml)
SOD (U/ml)	r	0.24ψ	-0.07	-0.11
	p-Value	0.07	0.57	0.41
	N	54	53	55
Log_AngII (pg/ml)	r	0.24ψ	-0.16	
	p-Value	0.09	0.28	
	N	52	49	
NOx (μmol/L)	r	0.08		
	p-Value	0.55		
	N	56		
		TA + AA Genotype Group		
SOD (U/ml)	r	0.13	-0.05	-0.15
	p-Value	0.52	0.81	0.45
	N	27	26	27
Log_AngII (pg/ml)	r	-0.003	-0.06	
	p-Value	0.99	0.74	
	N	26	25	
NOx (μmol/L)	r	-0.32		
	p-Value	0.10		
	N	26		

SOD, superoxide dismutase; Log_AngII, log angiotensin II; NOx, urinary nitrates/nitrites; 8-iso-$PGF_{2\alpha}$, urinary 8-isoprostane prostaglandin F 2-alpha; r, correlation coefficient; N, sample size. ψ Denotes borderline statistically significant correlation within genotype group.

AT1R Genotype Combinations

In light of previous reports of associations between the AT1R A1166C polymorphism and hypertension and oxidative stress [32, 36] and the suggestion of an association between the AT1R -825 T/A polymorphism and hypertension [54] and oxidative stress, predicted high-risk and low-risk AT1R genotype combinations were

determined and comparisons were made between the groups <Tables 7 & 7a >. There were no significant differences between risk allele groups at baseline for any of the oxidative stress variables, urinary NOx excretion or AngII levels <Table 8>. In group 2 only, there was a borderline inverse correlation between urinary NOx and AngII ($p = 0.06$) and between urinary 8-iso-$PGF_{2\alpha}$ and SOD activity ($p = 0.07$) <Table 9>.

Table 7. Distribution of AT1R genotype combinations and risk allele groups

Allele Combinations	-825T/1166A	-825T/1166C	-825A/1166A	-825A/1166C
-825T/1166A	TTAA (42)	TTAC (23)	TAAA (17)	-----
-825T/1166C	-----	TTCC (3)	TAAC (11)	TACC (1)
-825A/1166A	-----	-----	AAAA (3)	AAAC (0)
-825A/1166C	-----	-----	-----	AACC (0)

Numbers in parentheses represent sample sizes.

Table 7a. AT1R risk allele groups

Group	# of Risk Alleles	Genotype combination	N
1	0 or 1	TAAA, AAAA, AAAC	20
2	2	**TTAA, TAAC, AACC**	53
3	≥ 3	**TTAC, TACC, TTCC**	27

High risk combinations (i.e. 2 or 3 risk alleles) are shown in bold.

Table 8. Baseline characteristics of the AT1R risk allele groups

	AT1R Risk Allele Group			
	Group 1	Group 2	Group 3	p-Value
Age (yrs)	57.2 ± 1.4 (20)	58.6 ± 0.8 (53)	59.4 ± 1.2 (27)	0.56
BMI (Kg/m^2)	29.6 ± 1.1 (18)	29.2 ± 0.6 (51)	29.1 ± 0.9 (26)	0.94
TG (mg/dL)	125.2 ± 16.7 (14)	111.9 ± 8.4 (48)	113.2 ± 12.7 (23)	0.77
LDL-C (mg/dL)	128.3 ± 9.4 (14)	112.7 ± 4.7 (48)	111.2 ± 7.1 (23)	0.31
HDL-C (mg/dL)	57.0 ± 5.5 (14)	52.3 ± 2.8 (48)	45.5 ± 4.1 (23)	0.28
Systolic BP (mmHg)	130.0 ± 3.3 (16)	135.1 ± 1.8 (45)	130.3 ± 2.5 (24)	0.17
Diastolic BP (mmHg)	87.0 ± 1.9 (16)	88.0 ± 1.1 (45)	86.2 ± 1.5 (24)	0.73
GFR ($ml/min/1.73m^2$)	77.0 ± 4.2 (14)	78.0 ± 2.2 (44)	79.5 ± 3.1 (24)	0.88
VO_{2max} (ml/kg/min)	22.8 ±1.0 (18)	25.5 ± 0.6 (50)	24.7 ± 0.8 (26)	0.06
Log_AngII (pg/ml)	2.02 ± 0.30 (19)	2.15 ± 0.18 (46)	2.06 ± 0.27 (22)	0.92
8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	0.34 ± 0.03 (18)	0.31 ± 0.02 (46)	0.32 ± 0.03 (23)	0.72
NOx ($\mu mol/L)x10^{-3}$	2.4 ± 0.3 (18)	2.9 ± 0.2 (44)	2.8 ± 0.3 (23)	0.50
SOD (U/ml)	0.96 ± 0.15 (20)	1.02 ± 0.10 (46)	1.20 ± 0.13 (25)	0.45

Data are expressed as mean ± SE. Values in parentheses represent sample size. BMI, body mass index; TG, triglycerides; GFR, glomerular filtration rate; VO_{2max}, maximal oxygen consumption; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; BP, blood pressure; AngII, angiotensin II; NOx, urinary nitrates/nitrites; SOD, superoxide dismutase; 8-iso-$PGF_{2\alpha}$, 8-isoprostane prostaglandin F-2 alpha; Group 1, 0-1 risk allele; Group 2, 2 risk alleles; Group 3, ≥ 3 risk alleles. N, sample size.

Table 9. Baseline correlations between major outcome variables by the AT1R risk allele

			Group 1	
		8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	NOx (μmol/L)	Log_AngII (pg/ml)
SOD (U/ml)	r	0.07	-0.19	-0.10
	p-Value	0.78	0.44	0.68
	N	18	18	19
Log_AngII (pg/ml)	r	-0.11	0.24	
	p-Value	0.67	0.35	
	N	17	17	
NOx (μmol/L)	r	-0.22		
	p-Value	0.38		
	N	17		
			Group 2	
SOD (U/ml)	r	0.28ψ	-0.03	-0.13
	p-Value	0.07	0.81	0.39
	N	42	40	41
Log_AngII (pg/ml)	r	0.25	-0.31ψ	
	p-Value	0.11	0.06	
	N	42	39	
NOx (μmol/L)	r	-0.07		
	p-Value	0.636		
	N	43		
			Group 3	
SOD (U/ml)	r	0.22	0.06	-0.11
	p-Value	0.34	0.78	0.64
	N	21	21	22
Log_AngII (pg/ml)	r	0.26	-0.09	
	p-Value	0.29	0.71	
	N	19	18	
NOx (μmol/L)	r	0.05		
	p-Value	0.81		
	N	22		

SOD, superoxide dismutase; Log_AngII, log angiotensin II; NOx, urinary nitrates/nitrites; 8-iso-$PGF_{2\alpha}$, urinary 8-isoprostane prostaglandin F 2-alpha; r, correlation coefficient; N, sample size. ψ Denotes borderline statistically significant correlation within risk group.

Aerobic Exercise Training Changes

Changes with Training for the Entire group

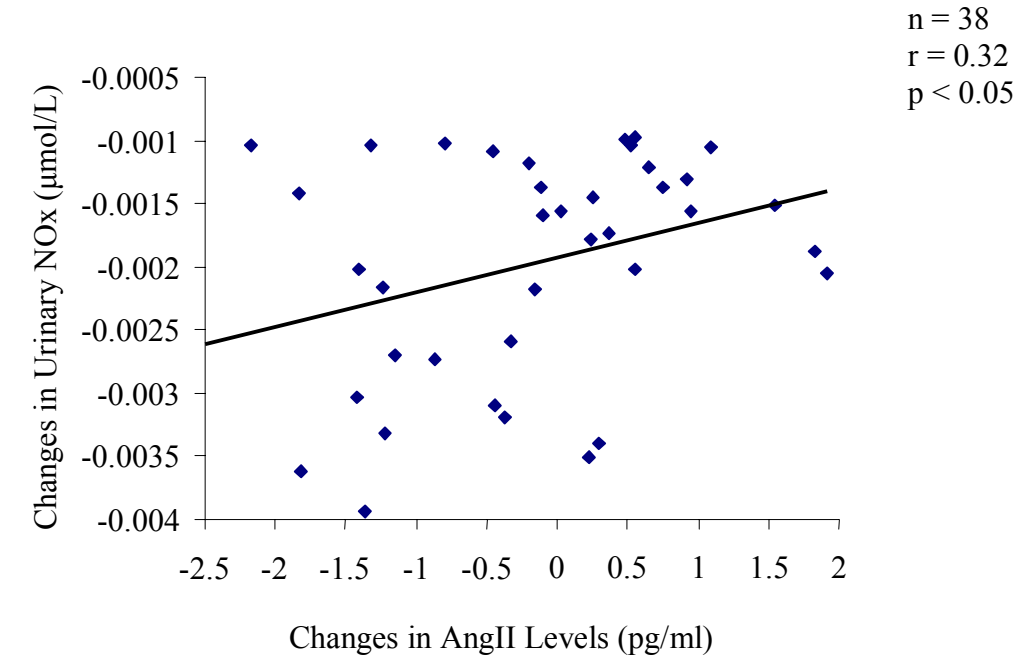
There was a significant increase in VO_{2max} in the entire group after six months of aerobic exercise training from 24.8 ± 0.5 to 28.8 ± 0.7 ml/Kg/min ($p < 0.0001$) suggesting that the training protocol was sufficiently effective to elicit CV adaptations <Table 10>. With an average body weight change of -1.14 %, participants remained within the required range (± 5 %) of their baseline body weight. Lean body mass significantly increased ($p = 0.04$) and percent body fat significantly decreased from 37.9 ± 1.0 to 35.0 ± 1.3 % ($p < 0.0001$) in the entire group. However, there were no significant changes in systolic or diastolic BP with exercise training. Urinary NOx excretion decreased significantly ($p < 0.0001$) and urinary 8-iso-$PGF_{2\alpha}$ increased significantly ($p = 0.002$) but there were no significant changes in SOD activity or AngII levels with six months of aerobic exercise training. The change in urinary NOx excretion was positively correlated with the change in AngII levels ($r = 0.32$, $p < 0.05$) and inversely correlated with the change in SOD activity ($r = -0.33$, $p = 0.02$). In addition, there was a significant correlation between the change in urinary 8-iso-$PGF_{2\alpha}$ and the change in SOD activity ($r = 0.32$, $p = 0.02$) <Figure 1>.

Table 10. Overall changes with training in the entire group

	N	Change	p-value
Total Fat (%)	58	-1.43 ± 0.28	< 0.0001
TG (mg/dL)	54	-8.87 ± 5.69	0.12
LDL-C (mg/dL)	54	-0.57 ± 2.24	0.79
HDL-C (mg/dL)	54	2.94 ± 0.71	0.0001
Systolic BP (mmHg)	57	-0.67 ± 0.95	0.48
Diastolic BP (mmHg)	57	-0.62 ± 0.67	0.36
VO_{2max} (ml/kg/min)	67	3.44 ± 0.36	< 0.0001
Log_AngII (pg/ml)	46	-0.20 ± 0.15	0.19
8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	56	0.09 ± 0.03	0.001
NOx (μmol/L)x10^{-3}	53	-2.0 ± 0.1	< 0.0001
SOD (U/ml)	54	0.02 ± 0.07	0.73

Data are expressed as mean ± SE. VO_{2max}, maximal oxygen consumption; TG, triglycerides; BP, blood pressure; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; Log_AngII, log angiotensin II; NOx, urinary nitrates/nitrites; SOD, superoxide dismutase; 8-iso-$PGF_{2\alpha}$, 8-isoprostane prostaglandin F-2 alpha; N, sample size. Not all participants completed exercise training and not all samples were viable for analysis; as a result sample sizes are varied for some variables.

Figure 1
A.


n = 38
r = 0.32
p < 0.05
Changes in Urinary NOx (µmol/L)
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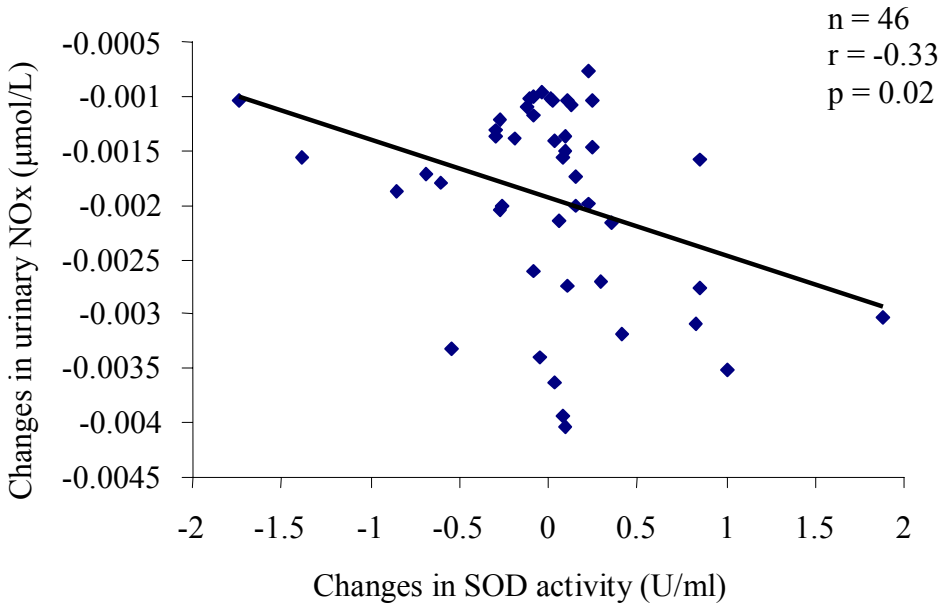
-0.001
-0.0015
-0.002
-0.0025
-0.003
-0.0035
-0.004
-2.5
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
Changes in AngII Levels (pg/ml)

B.


n = 46
r = -0.33
p = 0.02
Changes in urinary NOx (µmol/L)
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-0.001
-0.0015
-0.002
-0.0025
-0.003
-0.0035
-0.004
-0.0045
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
Changes in SOD activity (U/ml)

C.

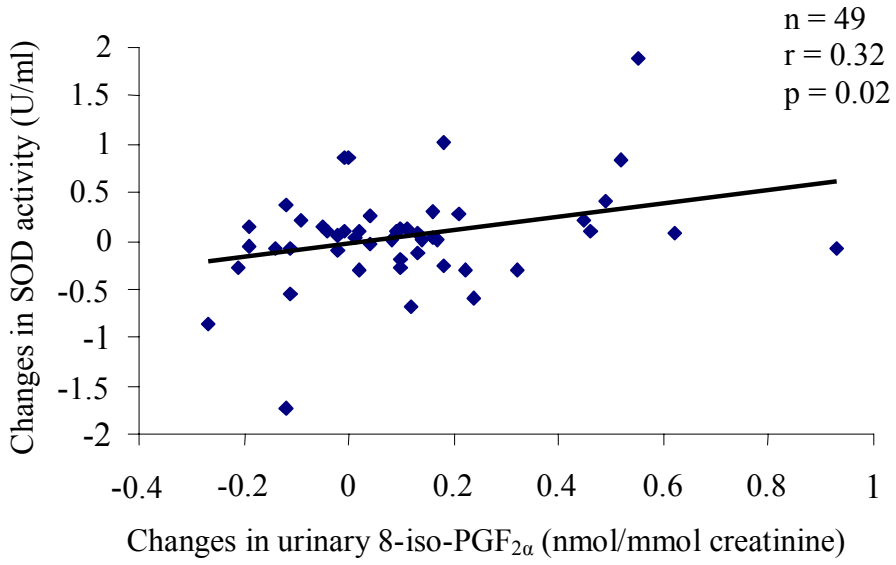


Figure 1. Correlation of changes with training values in the entire group between (A) AngII and urinary NOx excretion, (B) SOD activity and urinary NOx excretion, and (C) urinary 8-iso-$PGF_{2\alpha}$ and SOD activity.

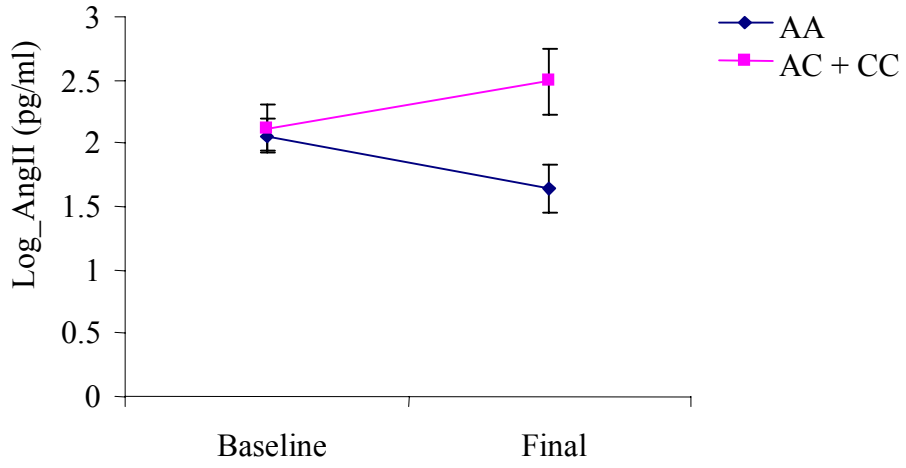
Changes with Training for the AT1R A1166C Polymorphism

There was a significant interactive effect of aerobic exercise training and the A1166C polymorphism on AngII levels ($p < 0.05$) <Figure 2>, resulting in a significant difference between genotype groups in the change in plasma AngII with exercise training. There were no significant differences between genotype groups in changes in oxidative stress markers or urinary NOx excretion after aerobic exercise training. Furthermore, the A1166C polymorphism and exercise training did not interactively affect urinary 8-iso-$PGF_{2\alpha}$, urinary NOx or SOD activity. Within both the AA genotype group and the AC + CC genotype group, there was a significant increase in urinary 8-iso-$PGF_{2\alpha}$ ($p = 0.03, 0.03$) and a significant decrease in urinary NOx excretion ($p < 0.0001, < 0.0001$) <Table 11>. The change in SOD activity was

inversely correlated with the change in urinary NOx excretion (r = -0.64, p = 0.008) and positively correlated with the change in urinary 8-iso-$PGF_{2\alpha}$ (r = 0.53, p = 0.03) in the AC + CC genotype group only <Table 12>. In addition, the change in urinary NOx was positively correlated with the change in plasma AngII in the AA genotype group only (p = 0.03).

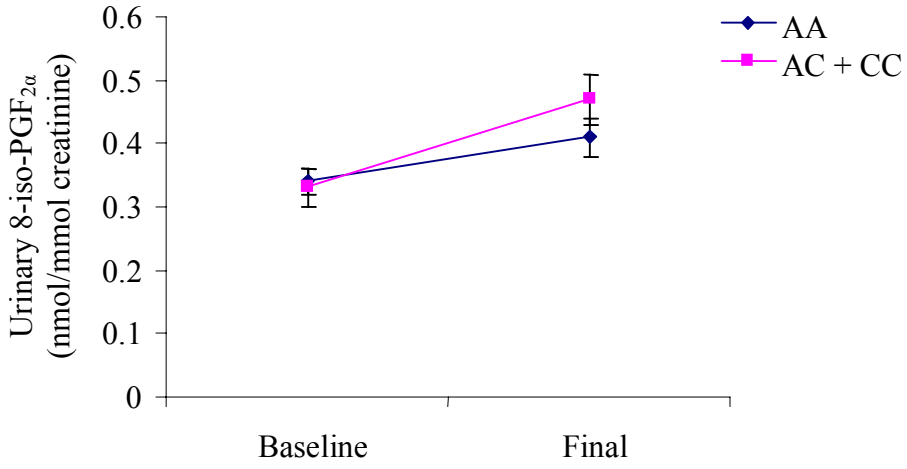
Figure 2.

A.



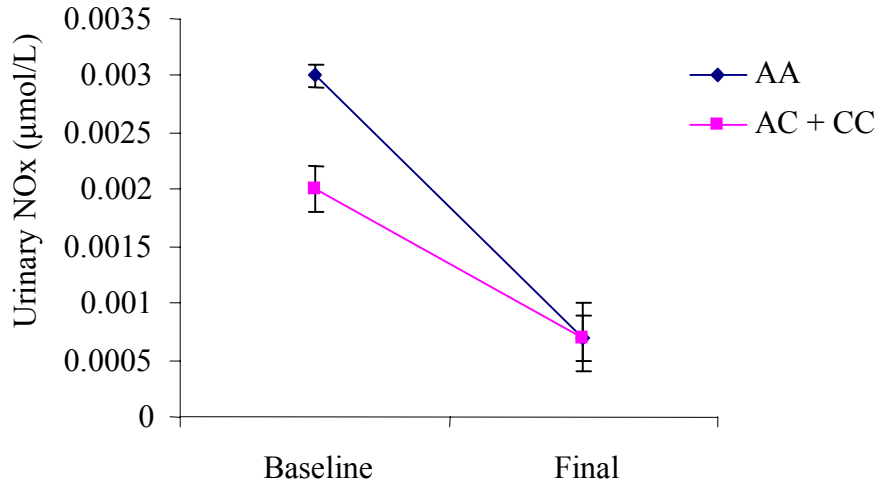
p < 0.05 for exercise training x A1166C interaction

B.



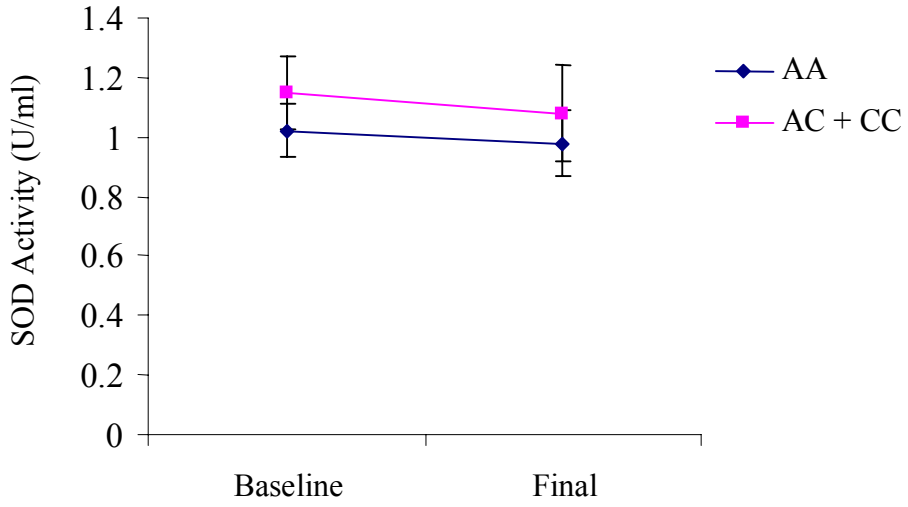
p = 0.37 for exercise training x A1166C interaction

C.



p = 0.36 for exercise training x A1166C interaction

D.



p = 0.91 for exercise training x A1166C interaction

Figure 2. Interaction between exercise training and the AT1R A1166C polymorphism on major outcome variables. (A) Log_AngII, (B) Urinary 8-iso-$PGF_{2\alpha}$, (C) Urinary NOx, and (D) SOD activity

Table 11. Changes with training by genotype group for the AT1R A1166C polymorphism

	AT1R A1166C Genotype		
	AA	AC + CC	p-value
Log_AngII (pg/ml)	-0.48 ± 0.18 (30)	0.24 ± 0.24 (16)	0.04*
8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	0.07 ± 0.05† (36)	0.15 ± 0.06† (20)	0.38
NOx (µmol/L)x10^{-3}	-1.9 ± 0.05† (34)	-2.1 ± 0.06† (19)	0.32
SOD (U/ml)	-0.08 ± 0.10 (36)	0.22 ± 0.14 (18)	0.12
Systolic BP (mmHg)	-0.05 ± 1.46 (36)	-0.84 ± 1.81 (21)	0.75
Diastolic BP (mmHg)	-0.97 ± 1.03 (36)	0.80 ± 1.28 (21)	0.32

Data are expressed as mean ± SE. AngII, angiotensin II; NOx, urinary nitrates/nitrites; SOD, superoxide dismutase; 8-iso-$PGF_{2\alpha}$, 8-isoprostane prostaglandin F-2 alpha. BP, blood pressure. † Denotes a statistically significant change with training within genotype group.

Table 12. Correlations between major outcome variables with aerobic exercise training by the AT1R A1166C genotype group

		AA Genotype Group		
		8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	NOx (μmol/L)	Log_AngII (pg/ml)
SOD (U/ml)	r	0.11	-0.12	-0.04
	p-Value	0.54	0.52	0.82
	N	32	30	28
Log_AngII (pg/ml)	r	-0.26	0.44†	
	p-Value	0.21	0.03	
	N	25	23	
NOx (μmol/L)	r	-0.02		
	p-Value	0.87		
	N	33		
		AC + CC Genotype Group		
SOD (U/ml)	r	0.53†	-0.636†	-0.14
	p-Value	0.03	0.008	0.61
	N	17	16	15
Log_AngII (pg/ml)	r	-0.18	0.11	
	p-Value	0.49	0.68	
	N	16	15	
NOx (μmol/L)	r	-0.16		
	p-Value	0.51		
	N	19		

SOD, superoxide dismutase; Log_AngII, log angiotensin II; NOx, urinary nitrates/nitrites; 8-iso-$PGF_{2\alpha}$, urinary 8-isoprostane prostaglandin F 2-alpha; r, correlation coefficient; N, sample size. † Denotes a statistically significant correlation within genotype group after aerobic exercise training.

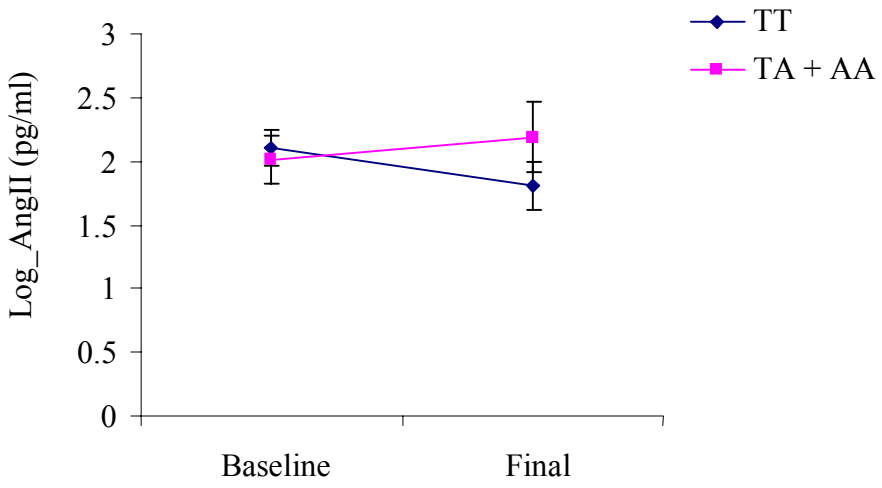
Changes with Training for the AT1R -825 T/A Polymorphism

No interaction was found between exercise training and the -825 T/A polymorphism for the changes in AngII levels, urinary 8-iso-$PGF_{2\alpha}$, urinary NOx or SOD activity <Figure 3>. In addition, changes in oxidative stress markers and urinary NOx excretion were not significantly different between genotype groups. However,

changes in plasma AngII levels were significantly different between genotype groups ($p = 0.02$). The TT genotype group had a significant reduction in plasma AngII ($p = 0.03$) while the TA + AA genotype group had a non-significant increase in plasma AngII <Table 13>. In the TT genotype group there was a significant increase in urinary 8-iso-$PGF_{2\alpha}$ ($p = 0.004$) and a significant decrease in urinary NOx excretion ($p < 0.05$). Similarly, in the TA + AA genotype group there was a significant decrease in urinary NOx excretion ($p < 0.0001$). In addition, in the TT genotype group only, there was an inverse correlation between the change in SOD activity and the change urinary NOx excretion ($r = -0.41$, $p = 0.02$), and a trend towards a positive correlation between the change in SOD activity and the change in 8-iso-$PGF_{2\alpha}$ ($r = 0.33$, $p = 0.06$) <Table 14>.

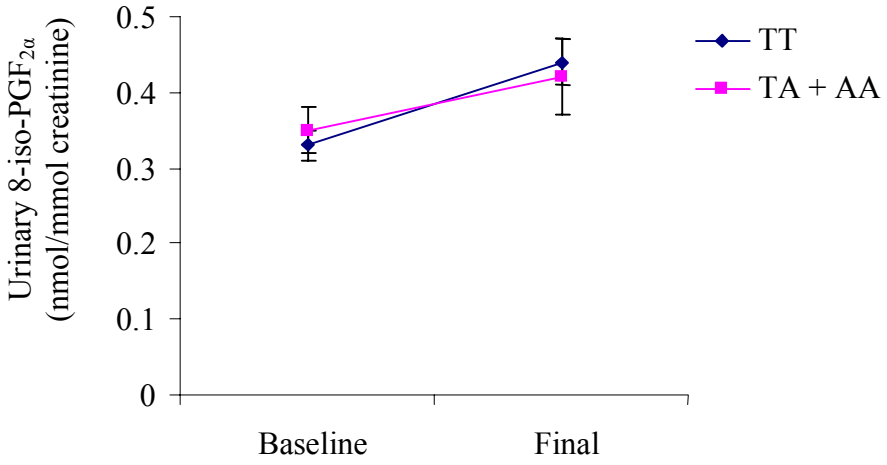
Figure 3.

A.



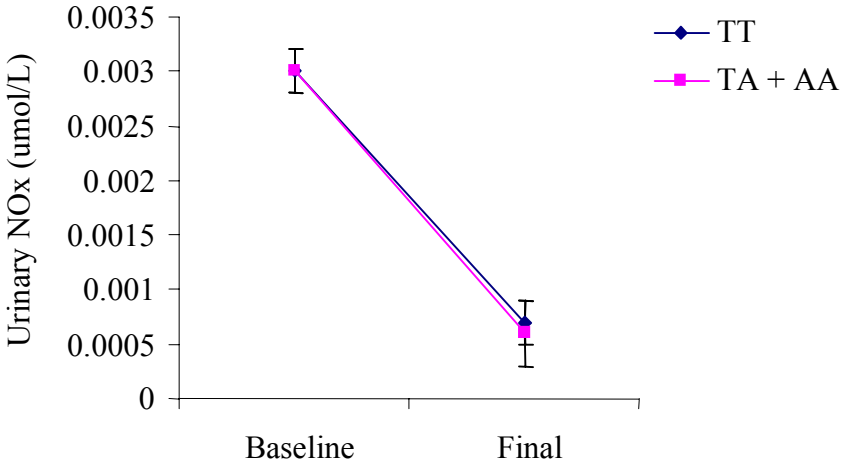
$p = 0.24$ for exercise training x -825 T/A interaction

B.



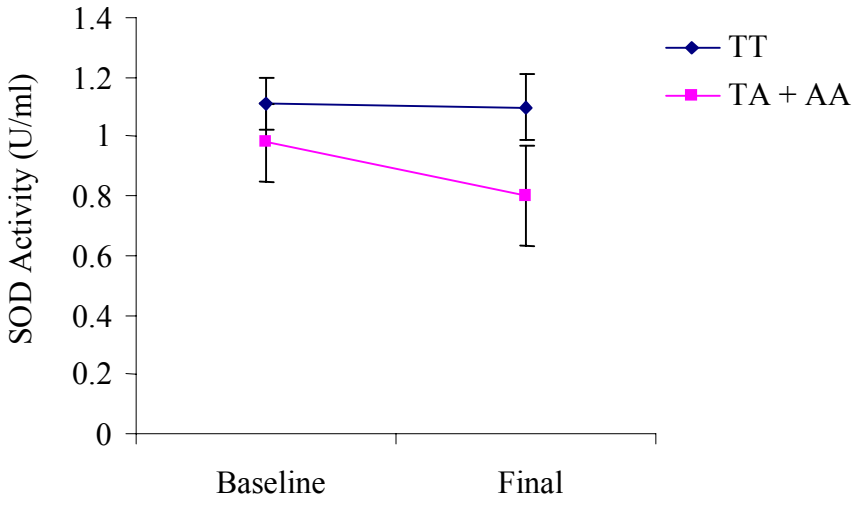
p = 0.68 for exercise training x -825 T/A interaction

C.



p = 0.81 for exercise training x -825 T/A interaction

D.



p = 0.50 for exercise training x -825 T/A interaction

Figure 3. Interaction between exercise training and the AT1R -825 T/A polymorphism on major outcome variables. (A) Log_AngII, (B) Urinary 8-iso-$PGF_{2\alpha}$, (C) Urinary NOx, and (D) SOD activity

Table 13. Changes with training by genotype group for the AT1R -825 T/A polymorphism

	AT1R -825 T/A Genotype		
	TT	TA + AA	p-value
Log_AngII (pg/ml)	-0.43 ± 0.16† (32)	0.36 ± 0.26 (14)	0.02*
8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	0.11 ± 0.04† (41)	0.08 ± 0.07 (15)	0.79
NOx (µmol/L)x10^{-3}	-2.0 ± 0.05† (37)	-1.9 ± 0.07† (16)	0.52
SOD (U/ml)	0.11 ± 0.09 (38)	-0.17 ± 0.14 (16)	0.11
Systolic BP (mmHg)	-0.48 ± 1.25 (42)	-0.08 ± 2.12 (15)	0.88
Diastolic BP (mmHg)	0.66 ± 0.85 (42)	-2.76 ± 1.48 (15)	0.06

Data are expressed as mean ± SE. Values in parentheses represent sample size. AngII, angiotensin II; NOx, urinary nitrates/nitrites; SOD, superoxide dismutase; 8-iso-$PGF_{2\alpha}$, 8-isoprostane prostaglandin F-2 alpha; BP, blood pressure. † Denotes a statistically significant change with training within genotype group.

Table 14. Correlations between major outcome variables with aerobic exercise training by the AT1R -825 T/A genotype group

		TT Genotype Group		
		8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	NOx (μmol/L)	Log_AngII (pg/ml)
SOD (U/ml)	r	0.33ψ	-0.41†	-0.007
	p-Value	0.06	0.02	0.97
	N	35	31	30
Log_AngII (pg/ml)	r	-0.28	0.24	
	p-Value	0.13	0.24	
	N	29	25	
NOx (μmol/L)	r	-0.20		
	p-Value	0.23		
	N	37		
		TA + AA Genotype Group		
SOD (U/ml)	r	0.30	-0.02	-0.14
	p-Value	0.29	0.93	0.65
	N	14	15	13
Log_AngII (pg/ml)	r	-0.10	0.42	
	p-Value	0.75	0.15	
	N	12	13	
NOx (μmol/L)	r	0.34		
	p-Value	0.21		
	N	15		

SOD, superoxide dismutase; Log_AngII, log angiotensin II; NOx, urinary nitrates/nitrites; 8-iso-$PGF_{2\alpha}$, urinary 8-isoprostane prostaglandin F 2-alpha; r, correlation coefficient; N, sample size. ψ Denotes a borderline significance within genotype group after aerobic exercise training; † Denotes a statistically significant correlation within genotype group after aerobic exercise training.

Changes with Training for AT1R Genotype Combinations

No significant interaction was found between aerobic exercise training and the genotype combinations for changes in oxidative stress, plasma AngII or urinary NOx. There were no significant differences between risk allele groups in urinary NOx levels, plasma AngII or oxidative stress with training, but there was a significant

reduction in plasma AngII in Group 2 only ($p = 0.04$) <Figure 4>. In group 2 only there was a significant increase in urinary 8-iso-$PGF_{2\alpha}$ ($p = 0.01$) and there was a significant decrease in urinary NOx excretion across all groups (Group 1, $p < 0.0001$; Group 2, $p < 0.0001$; Group3 $p < 0.0001$) with aerobic exercise training <Table 15>. In group 3 only, there was a significant positive correlation between the change in SOD activity and the change in urinary 8-iso-$PGF_{2\alpha}$ ($r = 0.60$, $p = 0.02$) and an inverse correlation between the change in SOD activity and the change in urinary NOx excretion ($r = -0.64$, $p = 0.02$) <Table 16>.

Figure 4.

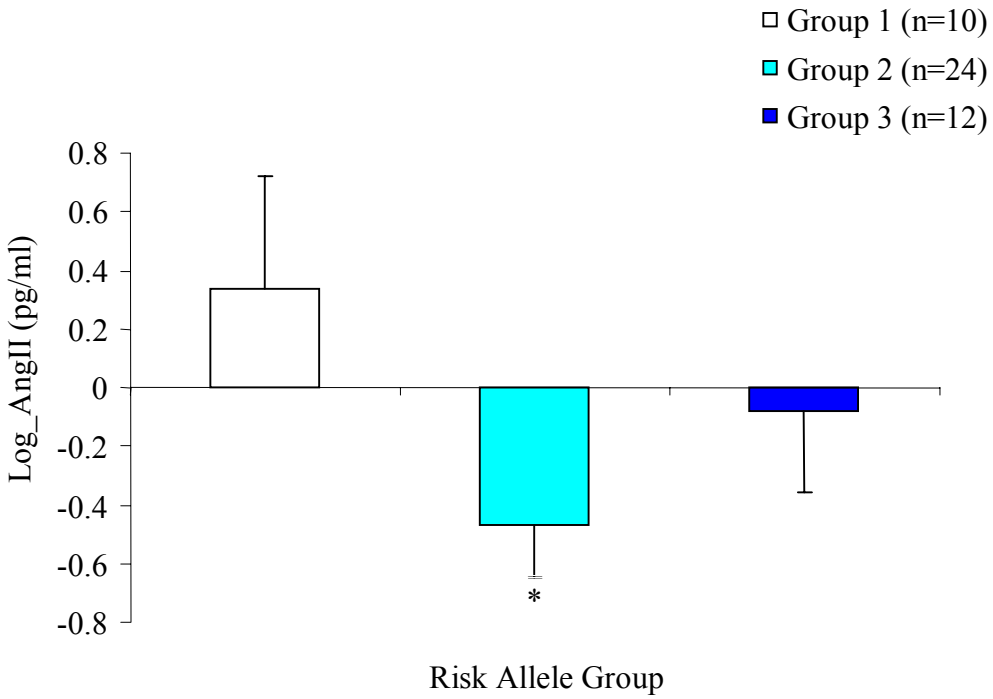


Figure 4. Changes in AngII levels with training by AT1R risk allele group.
* Denotes a significant change within genotype group.

Table 15. Changes with training for the AT1R risk allele groups

	AT1R Risk Allele Groups			
	Group 1	Group 2	Group 3	p-Value
Log_AngII (pg/ml)	0.34 ± 0.32 (10)	-0.47 ± 0.18† (24)	-0.08 ± 0.28 (12)	0.11
8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	0.03 ± 0.09 (11)	0.10 ± 0.05† (29)	0.14 ± 0.07 (16)	0.69
NOx (μmol/L)x10^{-3}	-1.9 ± 0.09† (12)	-2.0 ± 0.05† (26)	-2.1 ± 0.07† (15)	0.51
SOD (U/ml)	-0.28 ± 0.17 (13)	0.01 ± 0.10 (26)	0.28 ± 0.15 (15)	0.08
Systolic BP (mmHg)	0.91 ± 3.00 (10)	-0.12 ± 1.60 (31)	-1.09 ± 2.29 (16)	0.88
Diastolic BP (mmHg)	-4.18 ± 2.20† (10)	0.31 ± 1.17 (31)	1.49 ± 1.68 (16)	0.15

Data are expressed as mean ± SE. Values in Parentheses represent sample size. Log_AngII, log angiotensin II; NOx, urinary nitrates/nitrites; SOD, superoxide dismutase; 8-iso-$PGF_{2\alpha}$, 8-isoprostane prostaglandin F-2 alpha; BP, blood pressure. Group 1, 0-1 risk allele; Group 2, 2 risk alleles; Group 3, ≥ 3 risk alleles. † Denotes a significant change within risk allele group.

Table 16. Correlations between major outcome variables with aerobic exercise training by the AT1R risk allele

		Group 1		
		8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	NOx (μmol/L)	Log_AngII (pg/ml)
SOD (U/ml)	r	0.32	-0.03	-0.12
	p-Value	0.34	0.91	0.73
	N	11	12	10
Log_AngII (pg/ml)	r	-0.31	0.637	
	p-Value	0.45	0.06	
	N	8	9	
NOx (μmol/L)	r	0.32		
	p-Value	0.34		
	N	11		
		Group 2		
SOD (U/ml)	r	0.005	-0.18	0.13
	p-Value	0.98	0.43	0.58
	N	24	21	21
Log_AngII (pg/ml)	r	0.05	0.38	
	p-Value	0.81	0.12	
	N	21	18	
NOx (μmol/L)	r	-0.04		
	p-Value	0.85		
	N	26		
		Group 3		
SOD (U/ml)	r	0.60†	-0.64†	-0.15
	p-Value	0.02	0.02	0.64
	N	14	13	12
Log_AngII (pg/ml)	r	-0.46	0.12	
	p-Value	0.13	0.72	
	N	12	11	
NOx (μmol/L)	r	-0.32		
	p-Value	0.24		
	N	15		

SOD, superoxide dismutase; Log_AngII, log angiotensin II; NOx, urinary nitrates/nitrites; 8-iso-$PGF_{2\alpha}$, urinary 8-isoprostane prostaglandin F 2-alpha; r, correlation coefficient; N, sample size; † Denotes a statistically significant correlation within risk allele group after aerobic exercise training.

DISCUSSION

AngII participates in the pathogenesis of end-organ injury through the regulation of two main receptors: the AT1R and the AT2R; however, it is widely shown that the AT1R mediates the majority of the pathological actions of AngII including vasoconstriction, cell proliferation and vascular hypertrophy.[55, 56] Furthermore, it has become apparent that an important consequence of AT1R activation in the CV system is the production and release of ROS.[57, 58] Genetic variation in the AT1R has been associated with hypertension, aortic stiffness, increased response to AngII, increased sensitivity to AngII and more recently oxidative stress.[32, 36, 59-62] There is a plethora of information providing evidence that oxidative stress plays a critical role in the development of hypertension. Therefore, given the apparent association between AngII, AT1R, oxidative stress and hypertension, this study investigated the influence of two AT1R polymorphisms: the A1166C and the -825 T/A variants, on AngII levels, urinary NOx excretion and oxidative stress in hypertensive subjects.

The major findings of the present study were that after 6 months of aerobic exercise training 1) there was a significant increase in oxidative stress in the entire group, 2) there was a significant interactive effect of the AT1R A1166C polymorphism and aerobic exercise training on AngII levels, 3) the TT genotype group of the AT1R -825 T/A polymorphism had significantly greater reductions in plasma AngII with training compared to A allele carriers, and 4) the AT1R A1166C and -825 T/A polymorphisms were not associated with differential changes in oxidative stress or urinary NOx excretion.

Influence of AT1R polymorphisms at baseline

Genetic factors have been shown to play a significant role in the regulation of BP, with heritability estimates ranging from 30 % to more than 50 % for both systolic and diastolic BP depending on the study design. The RAS plays a major role in the regulation of BP and the genes encoding the components of the RAS, specifically AGT and ACE, have received the most attention with regard to hypertension. The M235T polymorphism of the AGT gene has been associated with higher AGT levels and a corresponding increased risk of hypertension.[63] An I/D polymorphism in intron 16 of the ACE gene has also been implicated in the regulation of BP and studies show a strong association of this polymorphism on plasma ACE levels.[64] In addition, it has been reported that increasing ACE activity is associated with the D allele of this polymorphism and thus, it is possible that those carrying the D allele may have higher AngII levels. In light of the potential influence of these polymorphisms on AngII levels and therefore, on oxidative stress and hypertension, for the first time, to our knowledge, the M235T and ACE I/D polymorphisms were accounted for in all statistical analyses.

Our data show that there were no associations at baseline between the AT1R polymorphisms and AngII levels, markers of oxidative stress or urinary NOx excretion. Contrary to our findings, Cameron et al. reported that heart failure patients with the CC genotype of the A1166C polymorphism had significantly higher plasma levels of MPO and PCs (markers of oxidative stress) compared to other genotype groups.[36] The A1166C polymorphism is located in the 3'UTR of the AT1R gene and therefore, it is widely speculated that the C allele may be in linkage disequilibrium

(LD) with a functional variant that may alter mRNA stability in response to increasing levels of AngII. [32, 53] The use of different markers of oxidative stress and the difference in populations between these two studies could account for these inconsistent findings. In addition, there are several markers of oxidative stress and some markers may be more sensitive in certain disease populations than others. Therefore, without the standardization of markers of oxidative stress relating to specific diseases, comparisons between studies is difficult. We found an almost significant inverse correlation between urinary NOx and plasma AngII at baseline in C allele carriers, but not AA homozygotes, for the AT1R A1166C polymorphism. The C allele has been shown to be associated with increased sensitivity[60] and an increased responsiveness to AngII.[61] In addition, ROS formation via AngII stimulation, which results in the inactivation of NO and the production and release of more harmful oxidants contributing to eNOS uncoupling, could account for the inverse relationship observed in this genotype group.

To our knowledge, this is the first study to investigate the influence of the AT1R -825 T/A polymorphism on AngII levels, urinary NOx excretion and oxidative stress. This promoter polymorphism is in almost complete concordance with the AT1R -153 A/G polymorphism.[53] The -153 A/G polymorphism was reported to be associated with urinary 8-iso-$PGF_{2\alpha}$ in a mixed group of normotensives and hypertensives, with the G allele having lower urinary 8-iso-$PGF_{2\alpha}$ excretion compared to AA homozygotes.[62] Given the almost complete LD between these two promoter polymorphisms, it is reasonable to hypothesize that the -825 T/A polymorphism would also be associated with oxidative stress. Poirier et al. reported that the A allele

of the -825 T/A polymorphism was associated with a lower risk of myocardial infarction,[53] while Jin et al. reported that the frequency of the A allele was higher in Chinese patients with essential hypertension combined with coronary heart disease compared to controls.[54] A likely explanation for the inconsistency in these results could be the differences in population characteristics and the phenotype being studied. AngII, via the AT1R is reported to stimulate proliferation and growth in breast cancer cell lines[65] thus, it is hypothesized that variation in the AT1R gene could be associated with breast cancer risk. Consistent with this hypothesis, Koh et al. reported that the A allele of the -825 T/A polymorphism, was associated with a reduced risk of breast cancer compared to TT homozygotes in Chinese women, further supporting the possibility of a protective effect of the A allele.[66] This polymorphism is thought to destroy a binding site for GATA transcription factors and, thus, it is plausible that the presence of the A allele may be associated with lower receptor activity and lower oxidative stress. We sought to determine the association between the -825 T/A polymorphism and AngII levels, urinary NOx and oxidative stress. We found no significant differences between genotype groups for any of the above variables, however, there was a trend towards a positive correlation between urinary 8-iso-$PGF_{2\alpha}$ and AngII levels and urinary 8-iso-$PGF_{2\alpha}$ and SOD activity in the TT genotype group only and this is consistent with the theory that the A allele may offer a protective effect and possibly promote lower oxidative stress. Urinary 8-iso-$PGF_{2\alpha}$, an end-product of oxidized arachadonate, is the most reliable biological marker of lipid peroxidation *in vivo*, moreover, it is not modified by the lipid content of the diet and its chemical stability in urine makes it suitable for large-scale

studies.[67-69] Elevation of isoprostanes have been found in disease states such as diabetes,[70] renal disease,[71] hypercholesterolemia,[72] and models of AngII-induced hypertension.[29-31] AngII-induced superoxide formation leads to the formation of 8-iso-$PGF_{2\alpha}$ and this increase in oxidative stress may cause a compensatory increase in SOD activity which is known to be augmented by oxidative stress.[73]

In the present study, risk allele analysis revealed no significant differences at baseline in AngII levels, urinary NOx and oxidative stress between risk allele groups. Using this type of analysis to determine the interaction between two polymorphisms is reasonable especially when the two polymorphisms are not in LD.[37, 74] To the best of our knowledge there is no evidence to suggest a significant LD between the AT1R A1166C and -825 T/A polymorphisms however, the combination of the two polymorphisms, rather than each alone, may be more informative when determining the genetic contribution to certain phenotypes. In the present study, risk allele analysis did not reveal any significant differences at baseline between groups for any of the variables tested.

Influence of aerobic exercise training

The presence of increased systemic oxidative stress plays a critical role in the progression of CVD. Risk factors for CVD, including hypertension, diabetes, atherosclerosis and obesity, are linked and further complicated by the presence of oxidative stress; however, modulation of oxidative stress and the endogenous antioxidant system is regarded as a beneficial approach to reducing the risk of CV morbidity and mortality.[75, 76]

Several studies report that aerobic exercise training reduces oxidative stress and enhances antioxidant enzyme capacity, however, the precise mechanism by which this occurs still need to be fully elucidated. Although an acute bout of exercise is considered to be a form of oxidative stress due to the generation of ROS that exceeds antioxidant capacity, it has been observed that exercise training results in a higher antioxidant reserve, resulting in resistance to exercise-induced oxidative stress.[77] It is widely accepted that aerobic exercise training decreases oxidative stress, thereby increasing NO availability and improving endothelial function, but studies investigating the effect of long-term aerobic exercise training on oxidative stress are lacking.

The most unexpected, but important finding of the present study was that there was a significant increase in oxidative stress with 6 months of aerobic exercise training in healthy, middle-aged to older, pre- and stage 1 hypertensive men and women. This was accompanied by a significant decrease in urinary NOx excretion. The reduction in urinary NOx after training could be an indication of reduced NO availability due to scavenging by superoxide and may lead to impairment of endothelial function. Our results are consistent with those from Goto et al. who reported that 12 weeks of high intensity aerobic exercise training (75 % VO_{2max}) in previously sedentary healthy young men, resulted in a significant increase in plasma 8-hydroxy-2'-deoxyguanosine (8-OhdG), a marker of oxidative stress to DNA, and MDA levels when compared to moderate intensity exercise (50 % VO_{2max}).[78] In addition, Bergholm et al. reported that 12 weeks of high intensity (70 – 80 % VO_{2max}) exercise training significantly decreased endothelial function and all circulating

antioxidants in healthy men, indicating an increase in oxidative stress.[79] The results of our study, which required participants to exercise at 70 ± 5 % $VO_{2max,}$ are consistent with these previous reports. It must be noted however, that our findings are inconsistent with those reported by Park et al.[74] who found that with 6 months of aerobic exercise training there was a significant decrease in TBARS levels in participants of the Gene Exercise Research Study (GERS). Although the participants used in the present study were also members of the GERS, we selected those participants that were pre- and stage-1 hypertensives only and this could account, in part, for the inconsistencies observed in the two studies. In addition, TBARS has been criticized for inadequate specificity to oxidant-driven peroxidation,[80] because not only does it reflect lipid peroxidation, but it is also a by-product of cyclooxygenase activity in platelets.[81] On the other hand, isoprostanes, specifically 8-iso-$PGF_{2\alpha}$, represent the stable end-products of lipid peroxidation formed *in situ* in cell membranes following free radical interaction with arachadonic acid independent of the cyclooxygenase pathway.[67, 81, 82] Thus, the clinical use of TBARS as a measure of lipid peroxidation is questionable and results should be interpreted with caution. We found no changes in SOD activity after exercise training, therefore, our findings suggest that long-term high intensity aerobic exercise training may reduce NO availability because of the inability of antioxidant enzymes to cope with the increase in ROS.

In the entire group the change in urinary NOx and the change in AngII levels with aerobic exercise training were positively correlated. The influence of the AT1R on NADPH oxidase activation has been well established, thus, changes in receptor expression could influence NADPH oxidase activation and oxidative stress. AngII-

induced activation of NADPH oxidase and subsequent generation of superoxide are offset by AngII binding to the AT2R and depends on the ratio of the AT1R/AT2R. In patients with stable coronary artery disease, aerobic exercise training resulted in a significant decrease in mRNA and protein expression of the AT1R and a significant increase in the expression of the AT2R resulting in a lower AT1R/AT2R ratio.[42] In the same study, it was reported that the shift in the ratio of the receptors after training lead to a reduction in NADPH oxidase-mediated superoxide production. Furthermore, it has been clearly demonstrated that NADPH oxidase-mediated superoxide production leads to a reduction in NO availability.[83, 84] Therefore, the downregulation of NADPH oxidase expression and activity after exercise training observed by Adams et al.[42] may account for an increase in NO availability and the positive correlation observed between AngII levels and urinary NOx with aerobic exercise training in the present study.

Gene-exercise training interaction

We observed a significant interactive effect of the A1166C polymorphism and aerobic exercise training on AngII levels, where C allele carriers exhibited a greater increase in plasma AngII levels after exercise training compared to AA homozygotes who decreased AngII levels after training though both genotype groups were similar at baseline. Although there was not a significant interactive effect of the -825 T/A polymorphism and exercise training on AngII levels, there was a significant difference in the change in AngII between genotype groups, with the TT genotype group having a significant reduction in AngII levels with exercise training. AngII, the AT1R and NADPH oxidase are highly linked,[57, 85] suggesting that a reduction in

plasma AngII should be accompanied by a reduction in oxidative stress. Furthermore, several investigators have reported that aerobic exercise training increases antioxidant capacity,[38, 86] improves NO bioavailability,[76, 87] and contributes to an overall reduction in oxidative stress.[88] We hypothesized that two AT1R polymorphisms would influence exercise-induced changes in oxidative stress. We observed a significant increase in urinary 8-iso-$PGF_{2\alpha}$, a significant decrease in urinary NOx and no change in SOD activity in AA homozygotes and C allele carriers of the A1166C polymorphism and in TT homozygotes of the -825 T/A polymorphism with aerobic exercise training. Although the A allele carriers of the -825 T/A polymorphism showed a small but non-significant increase in urinary 8-iso-$PGF_{2\alpha}$, they also showed a significant decrease in urinary NOx.

Despite a reduction in plasma AngII after training in the AA genotype group of the A1166C polymorphism and the TT genotype group of the -825 T/A polymorphisms, we observed an increase in oxidative stress accompanied by a reduction in NO bioavailability across all genotype groups. Furthermore, risk allele analysis revealed that those with 2 risk alleles exhibited a significant decrease in plasma AngII but this was accompanied by an increase in oxidative stress. Recent evidence suggests that locally produced AngII may be more important in determining CV risk. Therefore, it must be noted that the measurement of circulating (plasma) levels of AngII may not accurately represent long-term adaptations that occur in the vasculature in response to exercise training. Thus, we cannot deny the possibility that the increase in oxidative stress after training may be a result of 1) changes in tissue-based production of AngII and 2) other vasoactive factors released from the

endothelium, including endothelin-1 (ET-1). Preliminary results from our lab suggest that plasma ET-1 levels are significantly increased with aerobic exercise training in the same cohort used in the present study. Evidence presented by Duerrschmidt et al. show that ET-1 induces superoxide production via NADPH oxidase in cultured human endothelial cells.[89] In addition, Wedgewood et al. reported that ET-1 via the type A receptor, can stimulate superoxide production in cultured pulmonary artery smooth muscle cells.[90]

The change in SOD activity was inversely correlated with the change in urinary NOx and positively correlated with the change in urinary 8-iso-$PGF_{2\alpha}$ in the entire group. This relationship was also observed in only the C allele carriers of the AT1R A1166C polymorphism, in only the TT homozygotes of the AT1R -825 T/A polymorphism and subjects with 3 or more risk alleles. Our findings suggest that the higher lipid peroxidation that may be present in these high risk genotype groups and the possible compensatory increase in SOD activity, coupled with high intensity exercise and the apparent lack of upregulation of antioxidants in response to aerobic exercise training, may contribute to oxidative stress.

Summary

We found no main genotype effects for differences in oxidative stress variables at baseline or in response to exercise training, however, our results suggest that the AT1R A1166C and -825 T/A polymorphisms may synergistically interact with high intensity aerobic exercise training resulting in enhanced oxidative stress in this hypertensive population. Data suggest that it is clinically important to select the appropriate exercise intensity, as high intensity exercise can be hazardous to human

vessels.[91] Our results indicate that when prescribing exercise intensity, consideration should be placed on the patient population. Furthermore, it is possible that any improvements in oxidative stress or endothelial function observed with moderate intensity exercise may be abolished during high intensity aerobic exercise through reduced ability of antioxidants to cope with increasing ROS. Therefore, two major questions arise based on our findings 1) is there a threshold for the beneficial effects of exercise on oxidative stress? and 2) is the AT1R gene important for the modulation of oxidative stress in response to an optimal/intense volume of exercise?

Conclusion

These data suggest that in previously sedentary hypertensive adults the AT1R A1166C and -825 T/A polymorphisms may modulate the exercise-induced response to oxidative stress. Reduced plasma AngII in AA homozygotes and TT homozygotes of the A1166C and -825 T/A polymorphisms respectively were not associated with beneficial changes in oxidative stress in these genotype groups as was expected however, this does not exclude the possibility that tissue AngII may have played an important role in the increase in oxidative stress observed after training. Our findings may have clinical implications for the influence of exercise training and genetics on oxidative stress involved in the development and progression of CVD, as an exercise intensity of 70 % VO_{2max} may exceed the threshold for the beneficial effects of exercise in this hypertensive population. Future studies need to 1) address the mechanisms involved in the increase in oxidative stress in response to high intensity aerobic exercise training, 2) clarify whether the increase in oxidative stress in response to high intensity exercise is AT1R genotype-dependent, and 3) verify the

influence of the -825 T/A polymorphism on oxidative stress, endothelial function and CVD.

REVIEW OF LITERATURE

Cardiovascular disease (CVD) is a class of diseases that involves several traditional risk factors that tend to aggregate together possibly because of a common underlying cause. [9, 92, 93] Essential hypertension is one of the major risk factors for CVD and is considered to be a large public health issue due to the fact that it affects ~25-35% of the adult population.[22, 93] Several mechanisms have been suggested to explain how hypertension might give rise to an increased risk for CVD. These include structural and functional changes in the arterial wall, accelerated atherogenesis and more recently oxidative stress.[94, 95] There has been increasing evidence to support the role of ROS in the development of hypertension and hypertension-induced organ damage.[96-98] Furthermore, evidence supports the role of AngII in the production and release of ROS.[9, 22, 58] Increased production of ROS may be responsible for the increase in endothelium-dependent vasoconstriction and loss of endothelium-dependent vasodilation which may lead to an increase in vascular smooth muscle tone and subsequently hypertension.[22]

It is well established that sodium, fluid balance and vasomotor tone are integral mechanisms involved in BP regulation.[93] However, by promoting ROS-mediated inactivation of NO and CV remodeling, oxidative stress can cause endothelial dysfunction and ultimately hypertentsion.[97] This review will focus on the roles of oxidative stress, NO availability and AngII on the development of hypertension, the involvement of the AT1R gene and the impact of exercise on oxidative stress and hypertension.

Hypertension

Hypertension is a well established risk factor for the development and acceleration of atherosclerosis.[99] The prevalence of hypertension worldwide is estimated to be 1 billion, and more than 7.1 million deaths per year may be attributed to this disease.[47] Accordingly, for every 20 mmHg rise in systolic BP or every 10 mmHg rise in diastolic BP, mortality rates double for both ischemic heart disease and stroke.[47] AngII is the primary effector of the renin-angiotensin system (RAS) and has been implicated in the pathogenesis of hypertension.[8, 9, 23, 29, 100, 101] The RAS is comprised of renin, angiotensinogen, angiotensin I, AngII and aldosterone. Angiotensinogen is released from the liver and renin is released from the kidney. In the circulation, renin converts angiotensinogen to angiotensin I which is then converted to AngII by the angiotensin converting enzyme (ACE). In addition, tissue RAS is also of importance in the pathogenesis of hypertension as all components needed for the generation of AngII are found in isolated blood vessels.[21] Furthermore, AngII can also be formed by the endopeptidase chymase which is produced abundantly in the vessel wall.[20]

Oxidative Stress in Hypertension

Compelling evidence is available to support a role of oxidative stress in the pathogenesis of genetic and acquired hypertension,[6] and evidence suggests that these effects are mediated by inactivation of NO and a compromised antioxidant defense system in both the vasculature and the kidney. [102-105] There are several lines of evidence to support a causal role of oxidative stress in hypertension in both animal models and in humans. First, it has been shown that markers of oxidative stress are

increased in models of hypertension. For example, plasma levels of uric acid, a major metabolite of xanthine oxidase, were higher in spontaneously hypertensive rats (SHR) compared to normal rats.[106] Chrysohoou et al. found that individuals with pre-hypertension had lower total antioxidant capacity and higher oxidized low density lipoprotein (LDL) levels compared to normortensives.[94] It has also been reported that patients with essential hypertension have significantly lower levels of the antioxidants SOD and glutathione peroxidase (GPX), and higher levels of MDA, a marker of lipid peroxidation, compared to their age-matched normotensive counterparts.[107, 108] Furthermore, it was reported that there is a linear correlation between BP and plasma hydrogen peroxide levels.[109] Although this correlation does not indicate cause and effect, it highlights the potential link between hypertension and oxidative stress.

Second, interventions designed to promote oxidative stress have been shown to lead to the gradual development of hypertension.[110] Glutathione depletion in otherwise intact genetically normotensive animals was shown to lead to a significant increase in nitrotyrosine, a reduction in urinary NOx excretion and marked elevation in arterial pressure.[111] The increase in nitrotyrosine, which is a footprint of NO interaction with superoxide, coupled with a reduction in NOx excretion suggests that diminished NO bioavailability via oxidative stress contributes to the development of hypertension. Lead-induced hypertension was shown to lead to oxidative stress and subsequent elevation in arterial pressure.[26] Furthermore, elevation of BP in lead-exposed rats was coupled with a reduction in urinary NOx excretion and increased activation of the NADPH oxidase complex.[26] Infusing a subpressor dose of AngII does not cause an acute increase in BP however, after several days of AngII infusion

BP gradually rises.[23, 30, 101] This effect has been shown to result primarily from the induction of oxidative stress which precedes the elevation in BP.[30]

Third, there are observations in animals that oxidative stress can precede the development of hypertension. In 4 week old SHR, there is enhanced renal protein and mRNA expression of the p47phox subunit of NADPH oxidase.[112] This observation suggests that oxidative stress is important in the development of hypertension especially because in this model the rise in BP does not occur until after 4 weeks of age.[112, 113]

Fourth, gene deletion studies in mice point to the role of oxidative stress in the development of hypertension. Genetic deletion of p47phox leads to inhibition of superoxide production and a failure to develop hypertension after treatment with AngII.[114] In addition, extracellular SOD (ec-SOD) deletion has been shown to result in marked elevations in oxidative stress and BP.[110, 115]

Finally, there are a number of studies which report that the correction of oxidative stress in models of hypertension also corrects hypertension.[110] The consistent association with oxidative stress and a favorable response to antioxidant therapy in models of hypertension are extremely suggestive of a causal relationship.[97] Gene transfer of ec-SOD into hypertensive rats results in a significant reduction in their BP.[116] Various types of antioxidant therapy are shown to reduce oxidative stress and ameliorate hypertension.[24, 27, 28, 110, 117, 118] Zhan et al. reported that lifelong consumption of an antioxidant-rich diet lead to amelioration of oxidative stress and hypertension in prenatal SHR.[119] In this study, one week pregnant SHR were given either a diet rich in antioxidants or a normal diet. It was reported that plasma

hydrogen peroxide, MDA and nitrotyrosine concentrations were lower and upregulation of kidney NADPH oxidase subunits was attenuated in the offspring of treated SHR when compared to the untreated (normal diet) SHR.[119] This is indicative of a reduction in superoxide production and therefore oxidative stress. Furthermore, the increase in arterial pressure was lower and began later in the offspring of treated SHR when compared to the offspring of untreated SHR.[119]

Genetics of Hypertension

Regulation of BP is an important process that involves many physiological pathways, each of which might be influenced by multiple gene products.[120] Failure to control BP may lead to clinical end points such as end-stage renal disease and stroke due to long term chronic hypertension. Thus, it is reasonable to expect that several genetic and physiological safety mechanisms have evolved to maintain BP within an acceptable biological range, and that many gene variants have the potential to confer resistance or susceptibility to hypertension.[120] Consequently, hypertension is a common disorder that is thought to result from the complex interaction of several genetic and environmental factors including age, obesity, diet, stress and physical inactivity.[121, 122] There are multiple forms of hypertension, ranging from monogenic disorders to multifactorial forms that more than likely involve multiple susceptibility genes.[123] According to Agarwal et al. the threshold model of multifactorial inheritance predicts that those with an inherited genetic liability above a certain threshold will develop hypertension, especially when exposed to unhealthy environmental stimuli.[124] This model further indicates that the risk of developing hypertension increases with the number of affected relatives.

Heritability estimates within a population are reported to be ~30 -50 % for both diastolic and systolic BP.[121, 125] Given that hypertension is a complex disorder, it is likely that a number of genes rather than a single gene account for heritability. Three strategies to identifying genes for hypertension have been used: genome wide scans, candidate gene evaluation and investigation of mendelian disorders.[126] A genome wide scan for hypertension in severely affected sibling pairs who had early onset hypertension, revealed a principle locus on chromosome 6 that attained genome-wide significance with a LOD score of 3.21.[127] In the same study, three additional loci on chromosomes 2, 5 and 9 also showed genome-wide significance. In addition, Levy et al. reported strong evidence for a BP quantitative trait locus on chromosome 17 in selected participants from the Framingham Heart Study.[126] The candidate gene approach assumes that a gene or set of genes involving a specific physiological or cellular function contribute to BP variation.[128] As a result, there is a growing list of candidate genes hypothesized to influence BP and for many, evidence of linkage or association with hypertension has been reported.[32, 129, 130] Finally, the investigation of mendelian disorders influencing BP has established the identification of several genes, however the contribution of these rare disorders to overall BP variation is very small.[126, 128]

Oxidative Stress

During normal cellular metabolism oxygen undergoes a series of univalent reductions, sequentially leading to the production of superoxide, hydrogen peroxide and water.[98, 102] Oxygen is ubiquitous in the environment, and the oxidant status of an organism depends in part on the state of oxygenation of the organism or cell.[102]

Because oxygen is ubiquitous, most organisms have developed signaling mechanisms that utilize ROS.[131] As such, ROS are produced in a controlled manner and are likely involved in critical signaling functions.[132, 133] Furthermore, ROS produced by activated macrophages and leukocytes are essential for defense against invading microorganisms.[97] However, in order for ROS to act as second messengers both their production and inactivation must be tightly regulated and as a result, antioxidant defenses are integral in modulating the steady-state levels of ROS.[132-134]

Oxidative stress is commonly used to describe a disturbance in the balance between oxidant production and antioxidant reserve.[98, 102, 110, 135, 136] Under normal conditions, organisms contain specific antioxidant enzyme systems that can sense and inactivate ROS.[131, 132] When these protection mechanisms are overwhelmed by excessive ROS production, oxidative stress occurs leading to processes that oxidize macromolecules such as DNA, protein, carbohydrates and lipids resulting in tissue injury.[102, 131, 136]

ROS include superoxide, hydrogen peroxide, hydroxyl radical, nitric oxide, and peroxynitrite.[104, 133] However, superoxide and hydrogen peroxide appear to mediate vascular smooth muscle cell growth, differentiation and apoptosis and the lipid peroxidation and protein nitration induced by peroxynitrite are early events in the disease process.[137] As a result, oxidative stress is a major cause of vascular injury in hypertension.[6]

Dismutation of superoxide by SOD produces the more stable, non-radical ROS, hydrogen peroxide which, in the presence of free transition metals such as iron or copper, can give rise to hydroxyl radical, an extremely reactive species that

induces local damage at the site where it is formed. [6, 97, 131, 137] In addition, hydrogen peroxide can also serve as a substrate for the generation of hypochlorous acid by the phagocyte enzyme myeloperoxidase (MPO).[97, 138, 139]

Sources of ROS

Normally oxidative processes occur predominately in the mitochondria but mitochondrial oxygen can sometimes leak through the electron transport chain and result in the formation of free radicals.[102] These free radicals can diffuse out of the mitochondria becoming a source of oxidant stress.[102] Additional important sources of excess oxidants involved in the disease process include NADPH oxidase, xanthine oxidase, MPO, and NO synthase.[10, 102, 136, 137, 140-142] Vascular ROS are produced in endothelial, adventitial and vascular smooth muscle cells and are derived primarily from NADPH oxidase which is a multi-subunit enzyme.[6, 10, 140, 143] This enzyme catalyzes the production of superoxide by the one electron reduction of oxygen using NADPH as the electron donor. Endothelial, vascular smooth muscle and fibroblast NADPH oxidase enzymes are not identical, but have unique subunit structures and mechanisms of regulation. For example, ROS production by vascular smooth muscle cell NADPH oxidase occurs intracellularly and is therefore ideally suited to modify signaling pathways and gene expression, profoundly influencing both normal physiology and the course of vascular disease.[137] In addition, macrophage ROS production occurs extracellularly and can activate metalloproteinases which may degrade the extracellular matrix, weaken the fibrous cap and lead to plaque rupture.[137]

NADPH oxidase has been identified as the most important source of superoxide[85] and NADPH oxidase activity has been shown to play and important role

in AngII-mediated hypertension.[142] Administration of AngII to rats raises BP and increases vascular superoxide production and this effect was found to be dependent on membrane-bound NADPH oxidase.[101] An increased superoxide production via NADPH oxidase was observed in adult SHR and this was associated with impaired endothelium-dependent vasodilation.[144] In addition, it was reported that there was an association between NADPH oxidase-mediated superoxide production and reduced NO-dependent vasodilation in hypertensive rats and humans.[83, 84] Increased NADPH oxidase activity may also be important in other CV diseases. For example, human saphenous veins from patients with atherosclerosis were shown to generate superoxide predominately by NADPH oxidase.[84] In addition, Warnholtz et al. reported that superoxide production was increased 2-fold in hypercholesterolemic rabbits compared to controls, and this increase was associated with activation of NADPH oxidase.[145]

Xanthine oxidase has been implicated as one of the many factors that are involved in the pathogenesis of hypertension.[146, 147] It can exist in two interconvertible forms: xanthine dehydrogenase and xanthine oxidase.[136] Xanthine dehydrogenase reduces NAD^+ whereas xanthine oxidase reduces molecular oxygen, leading to the production of both superoxide and hydrogen peroxide.[136] Xanthine oxidase is expressed on the luminal surface of the endothelium and catalyzes the conversion of hypoxanthine to uric acid, producing superoxide in the process.[148] Houston et al. have demonstrated that increased xanthine oxidase binding to endothelial cells *in vitro* causes a marked reduction in NO bioactivity.[149] Furthermore, free radical production is increased in the microcirculation of a rat

model of hypertension (SHR) and this can be prevented by a xanthine oxidase inhibitor.[150] In patients with chronic heart failure, xanthine oxidase activity and serum levels of uric acid were markedly increased, SOD activity was decreased and endothelium-dependent relaxation was found to be impaired.[151]

MPO is a heme-containing enzyme secreted by activated neutrophils, monocytes and tissue-associated macrophages.[152] The main biological function of MPO is the defense against microbial invasion.[153] However, the reactive oxidizing and chlorinating species produced by MPO may also damage normal tissue[153] and therefore, are possible contributors to oxidative damage associated with a variety of diseases in which inflammatory cells participate.[152] As such, MPO release and the resultant superoxide and hydrogen peroxide formation, results in lipid peroxidation and the consumption of NO, thereby contributing to endothelial dysfunction.[153, 154]

Vaziri et al. reported a marked increase in endothelial nitric oxide synthase (eNOS) expression in pre-hypertensive and hypertensive rats compared to normotensive rats.[155] As a matter of fact, the expression of eNOS has been shown to be increased rather than decreased in situations where endothelial dysfunction is caused by oxidative stress.[14] Endothelial dysfunction in the presence of increased eNOS expression suggests that the ability to produce NO may be limited.[14] Evidence pointing to superoxide generation by eNOS in the absence of cofactor tetrahydrobiopterin (BH_4) or substrate (L-arginine) deficiency, has led researchers to the concept of eNOS uncoupling.[6, 14] Landmesser et al. reported that in hypertensive rats NADPH oxidase-derived superoxide production results in oxidation of BH_4 leading to eNOS uncoupling and further contribution to ROS production.[156]

Uncoupled eNOS may lead to oxidative stress and endothelium dysfunction by at least three mechanisms: 1) the production of NO is reduced so that the radicals it normally reacts with and contains are free to attack other cellular targets, 2) eNOS produces superoxide rather than NO, further contributing to oxidative stress, and 3) eNOS may become partially uncoupled such that it produces superoxide and NO simultanseously.[136]

8-Isoprostane Prostaglandin $F_{2\alpha}$

As stated before, there are several targets of free radical oxidation including lipids, protein and DNA. Of these, lipid peroxidation is a central feature of oxidative stress.[157] A number of prostaglandin-like compounds are formed from the non-enzymatic peroxidation of arachadonic acid. These include, F_2 isoprostanes which are derived from the free radical lipid peroxidation of arachadonic acid and are presumed to be involved in oxidative injury-derived diseases.[49] They are formed *in situ* esterified to phospholipids, released into the plasma by phospholipase, removed from the plasma via the kidney and are excreted in the urine.[68, 158] Evidence suggests that the measurement of F_2 isoprostanes in urine or plasma provides a reliable measure of oxidative stress status *in vivo* in both animal models and in humans.[157] The ability to quantify F_2 isoprostanes allows the exploration of the role of oxidative stress in the pathophysiology of a wide range of human diseases.[157] As such, elevated F_2 isoprostane concentration in both plasma and in urine have been found in patients and animals with a number of disease conditions in which injury caused by oxidative stress is apparent.[68, 157]

There are 64 different isoprostanes that can be generated from arachadonic acid [67] however, 8-Isoprostane Prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) has been identified as the most abundant F_2 isoprostane and is considered the major urinary marker of systemic oxidative stress *in vivo*.[67, 159] Furthermore, urine contains negligible quantities of arachadonic acid/lipids thereby limiting *ex vivo* oxidation and isoprostane production after urine collection. Therefore, urinary measurement of 8-iso-$PGF_{2\alpha}$ is presumed to be an integrated assessment of oxidative stress with time.[28, 158] It has been reported that analysis of a 24-hr urine sample would not give a more reliable value of 8-iso-$PGF_{2\alpha}$ than a morning urine sample or a sample collected at any other time of the day.[50]

8-iso-$PGF_{2\alpha}$ was reported to have potent vasoconstrictive properties *in vivo* and *in vitro*.[160, 161] These effects have been shown to be abolished with the addition of a thromboxane A2 receptor agonist suggesting that 8-iso-$PGF_{2\alpha}$ may target this receptor.[162] There is evidence to suggest however, that 8-iso-$PGF_{2\alpha}$ exerts it's biological actions on vascular smooth muscle through activation of target receptors that are similar to but distinct from the thromboxane A2 receptor.[162, 163]

Antioxidants

Under a variety of abnormal conditions, the rate of ROS production may exceed the natural antioxidant capacity leading to oxidative stress, which uncontained can attack the functional or structural molecules resulting in tissue injury and dysfunction.[1] The natural antioxidant system consists of a series of antioxidant enzymes, and numerous endogenous and dietary antioxidant compounds that react with and inactivate ROS. Accordingly, SOD, catalase and glutathione peroxidase

(GPX) constitute the principle components of the endogenous antioxidant system and their deficiencies can cause oxidative stress.[164] Catalase is a ubiquitous enzyme found in all known organisms and is most abundant in the liver, kidney and erythrocytes.[165] It has a very high turnover number, decomposing hydrogen peroxide to molecular oxygen at an exceedingly high rate. Catalase has been suggested to play a central role in the protection against severe oxidative stress and low catalase activity may be considered a risk factor for diseases influenced by ROS.[166] GPX is a selenium-containing tetrameric enzyme which reduces hydrogen peroxide and lipoperoxides to their corresponding hydroxyl compounds using glutathione as the hydrogen donor.[164] GPX aids in the removal of hydrogen peroxide by using reduced glutathione to convert it to water, molecular oxygen and the oxidized form of glutathione (glutathione disulfide). Reduced glutathione can then be regenerated by using glutathione reductase to catalyze the reduction of glutathione disulfide using NADPH as the hydrogen donor. Although GPX and catalase both share the substrate hydrogen peroxide, GPX alone can react effectively with hydrogen peroxide and lipids, and is considered the major source of protection against low levels of oxidative stress.[164]

Dietary antioxidants such as vitamin C and vitamin E are essential components of the cellular defense against both endogenous and exogenous oxidants.[167] Vitamin E is a ubiquitous antioxidant which is lipid-soluble, whereas vitamin C is a water-soluble antioxidant.[168] Both vitamins have been reported to increase BH4, stimulate activation of NOS activity and increase NO synthesis in endothelial cells, contributing to improved endothelial-dependent relaxation in hypertension.[169] Furthermore, both vitamins are associated with decreased activation

of NADPH oxidase and increased activity of SOD.[169] The principle role of vitamin E as an antioxidant is thought to be scavenging of lipid peroxyl radicals which propagate lipid peroxidation.[167] These mechanisms would result in increased NO synthesis and bioavailability, thereby improving endothelial function, decreasing oxidative stress and ameliorating hypertension.

Superoxide Dismutase

SOD is the frontline defense against ROS-mediated injury.[73] It converts superoxide to hydrogen peroxide which is then dissociated to water and oxygen in the presence of catalase and GPX.[73, 164, 165] Three isoforms of SOD have been identified to date; copper/zinc SOD (Cu/Zn SOD) which is present in the cytoplasm, manganese SOD (Mn-SOD) present in the mitochondria and ec-SOD present in interstitial fluid.[1] ec-SOD accounts for the majority of SOD activity in the plasma, lymph and synovial fluid.[170] It binds to glycosaminoglycans such as heparin in the vascular extracellular matrix,[171] and in mammals 90-99% of the ec-SOD is found in the interstitial spaces of tissues.[170] In addition, ec-SOD activity accounts for almost 50% of the total SOD activity and given the location of the enzyme it is likely critical for removal of superoxide from the plasma and vascular tissues.[105]

Vascular SOD levels are important for the ability of NO to modulate vascular tone.[151] In African American hypertensives it was reported that there was significant reduction in ec-SOD activity coupled with a significant increase in plasma 8-iso-$PGF_{2\alpha}$ and nitrotyrosine compared to normotensives.[105] The ec-SOD Arg213Gly mutation which leads to accelerated release of ec-SOD from the interstitial matrix such that the arterial wall has insufficient antioxidant capacity,[170] was found to be

associated with an increased risk for ischemic heart disease.[172] Once ec-SOD has been secreted from the smooth muscle cells into the interstitial space, the extracellular localization of this enzyme prevents any effect on intracellular superoxide.[171] Evidence suggests however, that it still plays a critical role in the maintenance of vascular function in hypertension by increasing NO bioavailability via scavenging of superoxide.[171] As such, it has been reported that in oxidative stress-associated hypertension there is impairment of ec-SOD activity and application of ec-SOD or a SOD mimetic leads to improvements in endothelial dysfunction in hypertension.[173]

Genetics of Oxidative Stress

As mentioned earlier, there is increasing evidence that ROS may play an important role in the development of organ damage associated with CVD in general and hypertension in particular. It was recently reported that hypertensive patients exhibited a significantly higher production of plasma hydrogen peroxide than normotensive subjects.[109] In this study, Lacy et al. reported that normotensives with a positive family history of hypertension had significantly higher plasma hydrogen peroxide levels and a higher systolic BP than normotensives without a positive family history of hypertension. Furthermore, a family history of hypertension was more important than BP status in determining a patient's plasma hydrogen peroxide level.

In a follow up study, Lacy et al. assessed hydrogen peroxide formation in a family-based cohort of people with essential hypertension in order to directly examine the role of heredity in the overall determination of this trait.[174] It was reported that values for parent-offspring correlations and sib-sib correlations were consistently greater than those for spousal correlations. As a matter of fact, spousal

correlations were near zero which is a pattern consistent with a genetic component to the overall determination of this trait. Accordingly, approximately 20-35 % of the observed variance in hydrogen peroxide can be attributed to genetic factors. These results might suggest that as a heritable trait, hydrogen peroxide may have earlier penetrance than elevated BP and may predict subsequent development of hypertension.[174]

Total antioxidant status (TAS) reflects the balance between oxidants and antioxidants. ROS producing enzymes determine how many free radicals are produced while the antioxidant system determines whether these ROS are in excess, which classes of molecules they will oxidize and what pathological changes they will leave behind.[175] TAS represents the oxidative stress in plasma relative to oxidation-induced pathological processes and as a result, it is possible that genetic factors may contribute to this bioregulation. As such, Wang et al. assessed the genetic contributions of plasma total antioxidant activity and reported that the additive effect of genes accounted for more than 50 % of the total phenotypic variation in TAS.[175]

ec-SOD is the major scavenger of superoxide and thus, may play an important role in vascular function and CV health. A single base-pair substitution (Arg213Gly) at the heparin binding domain in the ec-SOD gene contributes to the known genetic effects on plasma ec-SOD levels. Accordingly, this mutation was found to be associated with a 10-fold increase in plasma SOD content.[170] In an Australian family based cohort, after elimination of individuals possessing this mutation, it was reported that the additive effect of genes contributed to more than 35 % of the total phenotypic variation in ec-SOD levels.[176]

Since NO plays an important role in the modulation of vascular tone, determination of the genetic contribution to basal NO production is important for studying the potential role of NO in hypertension.[177] Wang et al. studied 428 members from 108 nuclear families of the Heart Health Education Program for family-based primary coronary prevention in Australia. They reported that the additive effect of genes accounts for more than 25 % of the basal plasma NO production.[177]

Nitric Oxide

NO is a small lipophilic molecule with a half-life of several seconds and can freely diffuse across plasma membranes and lipoproteins, and react with multiple intracellular targets to elicit its effects.[11, 178] It is synthesized by NO synthase (NOS) isoenzymes that oxidize L-arginine to L-citrulline. [11, 17] In addition, cofactors such as BH_4 are also involved in NO production.[17] NOS consists of a family of three main isoenzymes that are either constitutive or inducible: neuronal NOS (nNOS), eNOS, and inducible NOS (iNOS).[11, 178, 179] These isoforms are present in a variety of cell types including vascular endothelial cells, smooth muscle cells, platelets, neuronal cells, macrophages, and neutrophils.[180] In addition to regulating vascular tone and BP, NO has been reported to prevent platelet aggregation and inhibit proliferation of vascular smooth muscle cells.[181] These actions are the result of activation of soluble guanylyl cyclase, the major target for the physiological effects of NO, and subsequent generation of cGMP.[178, 181]

The major biological end products of NO metabolism are NOx, which are excreted in the urine.[11, 182, 183] As a free radical, NO can react very quickly with

species containing unpaired electrons such as molecular oxygen, superoxide and metals, resulting in deleterious effects.[11, 178] An additional target of NO is cytochrome c oxidase which is the final enzyme in the electron transport chain.[181, 184] NO has been shown to reversibly inhibit cytochrome c oxidase in a manner that is competitive with oxygen,[181, 184] and this inhibitory effect may occur at physiological levels of NO resulting in the generation and release of superoxide anion.[5, 181] The influence of NO on oxidative stress depends on the relative concentration of NO, superoxide and antioxidant enzymes. As such, studies show that endothelial cells from hypertensive patients have greater NOS activity but yet produce less bioactive NO presumably because of inactivation of NO by superoxide.[5, 12] Thus, in disease states when NO production increases, the chemical interaction between NO and superoxide leads to the formation of peroxynitrite, resulting in lipid peroxidation, DNA damage and protein nitration.[11] This suggests that NO is not only a homeostatic regulator in the vasculature but may also contribute to the generation of oxidative stress in pathologic conditions such as hypertension.

Endothelial Dysfunction

The vascular endothelium is a single cell lining covering the internal surface of blood vessels.[9, 185] As the major regulator of local vascular homeostasis, the endothelium senses mechanical and hormonal stimuli and in response to these stimuli, produces numerous vasoactive factors that regulate the balance between; vasodilation and vasoconstriction, and thrombogenesis and fibrinolysis.[185] In addition, the vasoactive factors released from the endothelium, also regulate the balance between inhibition and promotion of smooth muscle cell proliferation and migration, and are

involved in the prevention and stimulation of platelet adhesion and aggregation.[185] When the delicate balance between these processes is disturbed, endothelial dysfunction occurs causing damage to the arterial wall.[17, 185] At least three vasodilators are synthesized and released by the endothelium in response to various hormonal and mechanical stimuli. These include prostacyclin, endothelium-derived hyperpolarizing factor and NO.[9, 185] Inhibition of NO production is reported to stimulate endothelial ACE activity and increase AngII and superoxide generation, leading to vasoconstriction followed by sustained and pronounced hypertension.[22] Furthermore, mice lacking the eNOS gene were reported to have higher systolic BP than wild type animals,[186] while mice overexpressing the eNOS gene were reported to be hypotensive.[187]

The earliest detectible changes in vascular disease states are abnormalities of the endothelium resulting in loss of the endothelium's normal homeostatic functions.[140] Thus, endothelial dysfunction refers to several pathological conditions, including impaired anticoagulant and inflammatory properties, increased platelet aggregation and leukocyte adhesion, and dysregulation of vascular remodeling.[17, 136] However, the widely accepted definition of endothelial dysfunction is a reduction in NO bioavailability, detected by a decrease in endothelium-dependent vasodilation.[136, 179] Taddei et al. found that subjects with a familial history of hypertension exhibited a blunted vasodilatory response to acetylcholine compared to subjects without a family history of hypertension.[15] In addition, patients with risk factors for coronary artery disease, including hypertension, exhibited impaired endothelial-dependent vascular reactivity prior to any other evidence of CVD.[16] Therefore, a disruption of

endothelial function via the loss of NO is considered a key event in the development of atherosclerosis.[188]

Mechanisms Underlying Endothelial Dysfunction

Although endothelial dysfunction occurs in many disease processes and underlying mechanisms may be multifactorial,[9] oxidative stress can be identified as the common factor.[17, 136, 140] It is widely known that in the presence of CV risk factors endothelial dysfunction is often present.[14] Accordingly, several potential abnormalities have been proposed which could account for the prevalence of impaired endothelial-dependent relaxation: 1) changes in the activity or the expression of eNOS, 2) decreased sensitivity of vascular smooth muscle cells to NO, and 3) degradation of NO via interaction with ROS.[14] The degradation of NO by ROS appears to be the most appealing because in the presence of CV risk factors, endothelial dysfunction can be abolished by administration of antioxidants.[14, 96, 169, 189] Furthermore, ROS has been shown to regulate several classes of genes, including adhesion molecules, chemotatic factors, antioxidant enzymes and vasoactive substances.[137] As such, ROS play an important role in both normal physiology and pathophysiology of the vasculature.[185, 190]

NO is normally produced by eNOS, but in inflammatory states iNOS, contributes significantly to NO production.[190] Under these conditions, the expression of iNOS is increased *de novo* producing 1000 times more NO than eNOS, and this cellular production of NO goes on for hours.[191] Therefore, excessive production of superoxide coupled with an increase in NO production results in these two radicals reacting very rapidly to form the harmful pro-oxidant species, peroxynitrite.[135, 185, 190]

The reaction between NO and superoxide is reported to be three times faster than the dismutation of superoxide by SOD, and ultimately may inhibit the physiological functions of NO.[14] Not only does superoxide reduce NO bioavailability but it also directly inhibits soluble guanylyl cyclase.[14] Peroxynitrite oxidizes eNOS and it's cofactor BH_4, inhibits guanylyl cyclase and enhances oxidative stress by inhibiting SOD, further leading to a reduction in NO availability and perpetuating the generation of superoxide in endothelial cells.[7, 14, 185 135, 136] In high concentrations, peroxynitrite is highly toxic and is reported to cause oxidative damage to DNA, proteins and lipids.[14] Superoxide also contributes to the oxidative modification of polyunsaturated fatty acids by producing bioactive isoprostanes that target thromboxane receptors on vascular smooth muscle cells leading to vasoconstriction.[68, 185] In addition, ROS faciltates the mobilization of calcium and activates cyclooxygenases, thereby promoting vasconstriction of vascular smooth muscle cells and production of endothelium derived contracting factors.[185, 192] Therefore, increased levels of oxygen radicals and decreased antioxidant defenses are considered critical factors in the development of endothelial dysfunction.[19] This information provides compelling evidence that the impact of overproduction of ROS on NO bioavailability is of importance in the pathogenesis of endothelial dysfunction and ultimately hypertension.

Measurement of Urinary NOx Excretion

The *in vivo* concentration of NO cannot be measured directly because of its extremely short half life and the low levels at which it is produced.[193] Therefore, the stable end products of NO metabolism or NOx, offer a useful index of NO generation

when dietary NOx intake has been accounted for.[182, 193] The measurement of urinary NOx allows the repeated and non-invasive estimation of systemic NO production *in vivo*.[18] Urinary NOx levels have been shown to be lower in patients with various diseases compared to healthy subjects.[18, 110] In addition, urinary NOx excretion was shown to be independent of age and sex, suggesting that it could be a useful index for systemic NO production in humans.[18]

Lyamina et al. reported that urinary NOx excretion was higher in patients with high normal BP compared to those with optimal BP.[19] The authors suggested that at the early hypertensive stage, increased NO production may play a compensatory role to limit the increase in BP. This idea is consistent with studies in rats which have shown that established hypertension is preceded by NO overproduction via iNOS.[141, 155] Lyamina et al. further reported that after one year, there was a trend towards a decrease in urinary NOx values in the high normal BP group. They also reported that urinary NOx excretion was significantly lower in stage I and stage II hypertensives compared to controls with normal BP. Therefore, it appears that eventually NO production falls in patients with advanced hypertension probably due to decreased activation of NO via oxidative stress mechanisms.

Biological Activities of Angiotensin II

AngII is known to cause potent increases in systemic and local BP via its vasoconstrictive effects.[92] Additionally, AngII may increase receptor density and sensitivity for other factors that modulate growth of vascular smooth muscle, regulate the gene expression of several bioactive substances (vasoactive hormones, growth factors, extracellular matrix components and cytokines) and activate multiple

intracellular signaling cascades (mitogen-activated protein kinase cascades, tyrosine kinases and transcription factors).[194, 195] Furthermore, evidence suggests that AngII may cause CV and end-organ injury independent of its BP elevating effects, through mechanisms that include increases in oxidative stress.[101, 110] For example, subpressor doses of AngII have been shown to increase free isoprostane and urinary 8-iso-$PGF_{2\alpha}$ production.[23, 29, 31] AngII-stimulated superoxide production results in impairment of the balance between relaxing and contracting factors released from the endothelium, leading to endothelial damage and hypertension.[22] AngII has also been shown to increase superoxide formation in models of AngII-induced hypertension and in human vascular smooth muscle cells.[29, 85, 100, 196] There is also evidence to suggest a role of the NADPH oxidase enzymatic complex in AngII-induced oxidative stress. Exposure of human vascular endothelial cells to AngII significantly increases NADPH oxidase activity and superoxide production.[143] In addition, Mollnau et al. reported that AngII infusion in rats increases the expression and activity of NADPH oxidase leading to superoxide production and endothelial dysfunction.[83] It was also reported that in AngII-induced hypertension, increased vascular superoxide production was associated with activation of NADPH oxidase.[101]

AngII has prolonged effects on vascular smooth muscle through Phosolipase D (PLD), phosphorylation of p47phox and activation of NADPH oxidase.[114, 197] AngII-mediated activation of NADPH oxidase, is somewhat delayed but can be detected after one hour and is sustained for up to 24 hours after infusion of AngII.[197] Once AngII binds to the AT1R, PLD is activated and this results in the production of phosphatidic acid and diacylglycerol, leading to activation of protein kinase C (PKC).

It is not clear if PKC is the major way in which phosphorylation of p47phox (a NADPH oxidase subunit) occurs, however Touyz et al. reported that when PKC was inhibited in vascular smooth muscle cells, the AngII-induced hydrogen peroxide formation was significantly reduced.[196] This suggests that PKC in part regulates AngII-induced formation of oxidative stress.[197]

AngII-stimulated superoxide production is dependent on p47phox in both endothelial cells and vascular smooth muscle cells.[114, 196, 198] Once p47phox is phosphorylated, this subunit which resides in the cytosol, is translocated to the cell membrane, resulting in the assembly and activation of the NADPH oxidase enzymatic complex.[114] Landmesser et al. reported that the increase in vascular superoxide production in response to a subpressor dose of AngII was diminished in p47phox knockout mice.[114] In the same study, BP was markedly blunted in p47phox knockout mice, suggesting a role of NADPH oxidase activation and superoxide production in the increase in BP response to AngII.

Enzyme activity, protein levels and mRNA expression of the antioxidant enzyme ec-SOD were found to increase 2-3 fold in response to AngII infusion in a dose-dependent manner in both a rat model of AngII-induced hypertension and human smooth muscle cells.[199] Furthermore, this effect of AngII was reportedly due to increased transcriptional rate and mRNA stability of ec-SOD. In the same study, it was reported that AngII but not epinephrine, increased ec-SOD activity and mRNA expression, suggesting that hypertension alone is not the cause of increased SOD activity. Evidence also suggests that AngII directly upregulates ec-SOD independent of superoxide production or activation of NADPH oxidase via activation of a MAP

kinase pathway.[199] Thus, the increased vascular levels of ec-SOD (and subsequent increased scavenging of superoxide) in response to AngII may represent an important compensatory mechanism that blunts the BP response in conditions where AngII is elevated.[199]

AngII Receptors

AngII exerts its effects through G-protein coupled receptors that can be separated into two main classes: the type 1 and type 2 receptors. Most of the known actions of AngII are mediated by the AT1R. However, in addition to the type 2 receptors, there are two other angiotensin receptor subtypes: the type 3 and type 4 receptors. The type 3 receptor has only been described in cell lines, is peptide specific, mainly recognizes AngII and does not bind non-peptide ligands.[55] The type 4 receptor is distributed in the heart, lungs, kidney, brain and liver, and only binds AngIV.[55]

AngII Type 2 Receptor

The AngII type 2 receptor (AT2R) is ubiquitously expressed in the developing fetus but decreases rapidly after birth suggesting a role for this receptor in fetal development.[55, 194, 200] The functional role and the exact signaling pathways of AT2R still need to be clarified but evidence suggests that it may antagonize AT1R-mediated effects by inhibiting cell growth and inducing vasodilation.[55] The AT2R has been shown to be coupled to a different G-protein and therefore has opposite actions compared with the AT1R when stimulated.[201] Accordingly, the AT1R receptor activates a kinase that tends to phosphorylate while the AT2R activates a phosphatase

that will dephosphorylate.[195] The AT2R is thought to signal through cGMP/NO and/or bradykinin, and it has recently been shown that short term stimulation of AT2R *in vivo* in SHR will lower BP.[200] Thus, it is possible that the AT2R opposes the actions of the AT1R leading to inhibition of proliferation, vasodilation, natriuresis and apoptosis.[201] In addition, activation of the AT2R by AngII may also induce activation of the antioxidant GPX.[202]

Evidence suggests that AngII-induced superoxide production may be mediated by both receptors in human vascular endothelial cells.[143] In contrast, Chabrashvili et al. investigated the effect of blockade of the AT1R and AT2R on the excretion of 8-iso-$PGF_{2\alpha}$ and MDA during a slow pressor dose of AngII.[57] They reported that blockade of the AT1R prevented an increase in excretion of 8-iso-$PGF_{2\alpha}$ and MDA but blockade of AT2R resulted in further increases in both 8-iso-$PGF_{2\alpha}$ and MDA, supporting the hypothesis that the AT1R and AT2R have opposing effects.

AngII Type 1 Receptor

The AT1R is a member of the seven transmembrane-spanning G-protein coupled receptor family, binds to a heterotrimeric G-protein and typically activates phospholipase C (PLC).[55, 194] It is ubiquitously and abundantly distributed in adult tissues including blood vessels, heart, kidney, adrenal gland, liver, brain and lungs.[194]AngII promotes its effects by directly acting through this receptor, indirectly through the release of other factors, and possibly via cross talk with intracellular signaling pathways of other vasoactive agents and growth factors.[55] In addition to the classic responses of AT1R activation, it has been reported that AngII also increases

oxidative stress via this receptor, in part, because the AT1R is linked to activation of NADPH oxidase in vascular walls.[58]

The type 1 receptors are coupled to multiple, distinct signal transduction processes, leading to various biological actions.[55] The signaling processes are mulitphasic and as such, early, immediate and late signaling events occur within seconds, minutes and hours, respectively. AngII-induced PLC phosphorylation occurs within seconds and therefore, constitutes immediate signaling events. Activation of phospholipase D, tyrosine kinases and mitogen-activated protein kinases (MAPKs) occurs within minutes and are classified as early signaling events, while generation of oxidative stress and protein synthesis occurs within hours and constitute late signaling events.[55]

The AT1R mediates several critical CV responses, including vasoconstriction, vascular and cardiac remodeling and cell survival/cell death,[203] and has been implicated in the pathogenesis of various CV diseases.[204, 205] As such, the atherosclerotic process characterized by increased oxidation of LDL, increased uptake of oxidized LDL by macrophages, and foam cell formation is promoted by activation of the AT1R.[58] Furthermore, the expression of the receptor for oxidized LDL, LOX-1, is remarkably increased by activation of the AT1R.[58] Activation of the AT1R increases oxidative stress by increasing NADPH oxidase activity, decreasing ec-SOD concentration and decreasing the bioavailability of NO. Taken together, this information suggests that the AT1R plays a major role in initiating atherosclerosis and hypertension via oxidative stress, impaired NO bioavailability and endothelial dysfunction.

AT1R Gene

The AT1R gene maps to the long arm of chromosome 3, is more than 55Kb long and contains 5 exons and 4 introns. The coding region of the gene is located in the 5th exon and the first 3 exons encode 5' untranslated region (UTR) sequences.[206] A low salt diet has been shown to decrease AT1R expression, while a high salt diet has been shown to upregulate AT1R expression.[204] It has been reported that gene expression of the AT1R is increased in young SHR and thus, may be responsible in part for the hypertensive phenotype in this model.[207] Several polymorphisms have been identified in the 3'UTR and in the promoter region of the gene that may be related to hypertension and thus, oxidative stress.

A1166C polymorphism

This polymorphism involves an A → C substitution at position 1166 in the 3' UTR of the AT1R gene. The allele frequency ranges from 22-36 % in white populations with a lower frequency (~5 %) reported in African Americans.[208, 209] The A1166C has been found to be associated with hypertension in various populations however, the results are inconsistent with several studies failing to confirm an association with hypertension.[209-212] In contrast, Bonnardeaux et al. reported that the C allele was more frequent in white hypertensives with a positive family history compared to normotensives.[32] In the same study it was reported that the more severe and the earlier the onset of the hypertension, the greater the frequency of this variant. Further, association of this polymorphism with hypertension in a Chinese population was confirmed by Jiang et al.[35] In a Japanese population, the C allele was found to be more frequent in hypertensives compared to normotensives.[213] van Geel et al.

reported that this polymorphism was associated with different responses to AngII in isolated human arteries from patients with coronary artery disease.[61] They showed that the response to AngII was significantly higher, and the contractile response to increasing doses of AngII was significantly greater in patients with the CC genotype compared to A allele carriers. In the same study, it was reported that ACE inhibition increased the responsiveness to AngII in the CC genotype group possibly due to receptor upregulation. Furthermore, since the increased responsiveness to AngII in the CC genotype group was not masked by ACE inhibition, the authors suggested that this polymorphism may be in LD with a mutation that increases the responsiveness to AngII. The A1166C polymorphism was also found to be associated with increased sensitivity to AngII [60] and increased aortic stiffness.[59] The CC genotype was also found to be associated with relative CV risk independent of BP in a prospective study of middle-aged white males.[52] Recently, it was reported that this polymorphism was associated with oxidative stress in patients with heart failure. Cameron et al. reported that in patients homozygous for the 1166C allele, plasma levels of protein carbonyls (PCs) (an index of protein oxidation) and MPO were significantly higher compared to A allele carriers and controls. They also reported that the AT1R genotype was an independent predictor of PCs and MPO levels.[36]

The AT1R A1166C polymorphism is located at the 5' end of the 3'UTR and does not appear to alter an mRNA polyadenylation or destabilization signal although this has not been investigated. It is possible that this polymorphism may be in LD with a functional variant located elsewhere in the AT1R gene or within a nearby gene that could explain the observed associations of this variant with CV phenotypes.[51]

Studies have identified several polymorphisms located in the coding, 3' and 5' flanking regions of the AT1R gene, however LD was only found between a SNP located in the coding region (T573C) and the A1166C.[32] Although the T573C is located in the coding region it does not alter the amino acid sequence for the encoded protein. It has been suggested that the lack of LD between SNPs in the promoter region and A1166C represent a functional role for the 3' block. As such, studies in Chinese hamster ovary cells have revealed that at least one cellular protein binds to the 3'UTR of the AT1R, and influences the receptor function in response to AngII.[214] Therefore, it is possible that the 3'UTR may influence cell signaling, proliferation and translation.

-825T/A Polymorphism

It is interesting to note that only two other identified SNPs in the AT1R gene were found to be associated with hypertension. Takahashi et al. reported a higher frequency of the -535T allele of the C-535T polymorphism in Japanese hypertensives,[215] and Jin et al. reported a possible association of the AT1R -825 T/A promoter polymorphism with essential hypertension in Chinese subjects.[54] In this study, it was reported that hypertensive patients carrying the A allele had an increased risk for coronary heart disease morbidity compared to T homozygotes. In contrast, the frequency of the A allele was reported to be lower in patients with myocardial infarction and was associated with a lower risk for MI.[53] The -825 T/A polymorphism is of interest because it is reported to destroy a binding site for GATA transcription factors.[37] Furthermore, it has been shown that the AT1R promoter is active in rat cardiac muscle in response to pressure overload, and that this response is mediated by

AP-1 and GATA transcription factors.[216] Thus, it is possible that this variant may alter AT1R expression and the influence of AngII on hypertension via oxidative stress mechanisms.

Aerobic Exercise Training

A sedentary lifestyle has long been established as an independent risk factor for CVD. However, regular physical activity is one factor that can cause dramatic improvements in health by managing and preventing lipid abnormalities, diabetes and hypertension with the greatest benefits observed in sedentary individuals who begin to exercise.[217] Epidemiological studies indicate that greater fitness is associated with lower BP and a reduction in CV morbidity and mortality.[218] Although mechanisms surrounding the reduction in BP with aerobic exercise training are generally unclear, they have been reported to include a reduction in vascular resistance in which the sympathetic nervous system and the RAS appear to be involved. In addition, a down-regulation of the oxidative stress system may also be involved with a reduction in BP in response to exercise training.[91]

Aerobic Exercise, Endothelial Function & BP

According to Blair et al., subjects with low levels of physical fitness have a high relative risk for the development of hypertension when compared with subjects having high levels of physical fitness.[219] In fact, a lifestyle of physical activity can reduce the risk of developing hypertension by 30-50 %.[39] As such, regular moderate physical activity has been shown to decrease systolic BP by 6-10 mmHg and diastolic BP by 4-8 mmHg in patients with essential hypertension.[91] It is possible that the

reduction in BP with exercise training may be due, in part, to a reduction in oxidative stress and improved endothelium-dependent vasodilation via enhanced NO bioavailability.

Activation of eNOS to produce NO in the presence of L-arginine activates guanylyl cyclase and increases cGMP content in vascular smooth muscles cells, resulting in relaxation of vascular tone.[91, 178, 181] Thus, it seems reasonable that in hypertension there is some disruption in this pathway, and it is possible that aerobic exercise training may improve this disturbance. Higashi et al. reported that the acetylcholine-induced vasodilation in forearm arteries was significantly blunted in hypertensive subjects.[220] In the same study, 12 weeks of aerobic exercise training resulted in the reduction of both systolic and diastolic BP and improvements in endothelium-dependent relaxation, presumably due to increased production, release and availability of NO. Animal models of hypertension have also confirmed this observation. Six weeks of moderate intensity exercise training completely restored maximal endothelium-dependent relaxation in adult SHR to levels of that found in age-matched normotensive rats (WKY).[221] Endothelium-dependent vasodilation was shown to be preserved in older endurance trained athletes.[222] Furthermore, it was reported that endothelium-dependent relaxation was not significantly different in older athletes compared to younger athletes suggesting that there is a correlation between physical activity and vessel health. It has also been shown that basal urinary excretion of NOx increases with increasing levels of physical activity and urinary NOx levels are significantly increased after training in patients with coronary artery disease.[223]

Shear Stress

Fluid shear stress is the frictional force generated by blood flow over the vascular endothelium. Endothelial function, shape, physiology and pathophysiology are regulated by the types and magnitude of shear stress exerted on the endothelial cells.[224] As such, laminar shear stress occurs in linear vascular segments and is thought to exert atheroprotective effects, including improved vascular tone and prevention of apoptosis and monocyte adhesion. This protective effect is thought to be mediated by the expression of atheroprotective genes and antioxidant enzymes.[224] On the other hand, oscillatory shear stress or disturbed blood flow, occurs in branched or curved arteries and has been shown to induce various inflammatory and pro-atherogenic responses.[224, 225] Exercise training may exert its beneficial effects on endothelial function and BP via induction of shear stress. It is thought that mechanosensors on the membranes of endothelial cells sense shear stress leading to activation of various protein kinases which increase eNOS and SOD activity.[91]

Aerobic Exercise & Oxidative Stress

It is well established that intense aerobic exercise induces oxidative stress due to generation of ROS that exceeds the normal antioxidant defense capacity.[77, 226] Bergholm et al. reported that in healthy men, intense physical training for 3 months resulted in impaired endothelium-dependent relaxation and a significant reduction in all circulating antioxidants.[79] However, in a number of other studies antioxidant capacity is higher in individuals undergoing endurance training.[76, 86, 227] Modulation of oxidative stress and antioxidant enzyme capacity is thought to result from the cumulative effect of repeated bouts of exercise on the gene expression of antioxidant

enzymes.[77] As a result, evidence suggests that the exercise-induced improvement in antioxidant enzyme capacity is important in the reduction of CVD risk.[76]

Moderate physical activity has been shown to result in the decrease of markers of oxidative stress including plasma isoprostanes and TBARS.[41] It was recently reported that after six months of aerobic exercise training, there was a 16 % decrease in TBARS in previously sedentary individuals.[74] In another study, exercise training was reported to lead to an increase in antioxidant capacity and reduce oxidative stress in previously sedentary men and women.[38] In aortic endothelial cells of miniature pigs, 16-19 weeks of aerobic exercise training resulted in a significant increase in SOD activity, and a significant decrease in MDA when compared to controls.[43] In addition, total antioxidant capacity and GPX activity were shown to be increased in response to training in older men.[88] Thus, regular physical activity appears to be associated with ameliorated antioxidant capacity and decreased oxidative stress which may be possible mechanisms for the training-induced improvements in BP.

Ookawara et al. demonstrated that three months of endurance training in healthy men did not significantly change plasma Cu/ZnSOD levels. [86] In contrast, Rush et al. reported that endurance training resulted in a significant increase in protein levels and activity of Cu/ZnSOD in pig aortic endothelial cells.[43] It was also reported that endurance training did not affect MnSOD levels at rest but it significantly increased MnSOD after an acute bout of excersie.[86] The authors suggested that if the main source of MnSOD was leakage from skeletal muscle, the increased level after an acute bout of exercise after training could be due to a higher level of muscle MnSOD in the trained subjects. Interestingly, training caused a

significant decrease in ec-SOD levels at rest, and an acute bout of exercise after training significantly increased levels of ec-SOD. The authors hypothesized that the reduction in plasma ec-SOD at rest after training suggests that it may more readily accumulate in tissues due to easier incorporation into the cells as a result of exercise training. This hypothesis was supported when IV administration of heparin induced a three-fold increase in plasma ec-SOD.[86]

ec-SOD exists in secretory form and one of its unique properties is its affinity for heparin analogues. It is thought that after being released in the extracellular space, it is distributed to specific regions of the extracellular matrix or cell surface allowing ec-SOD to efficiently scavenge superoxide in the extracellular space.[86, 171] The conversion of superoxide to hydrogen peroxide by SOD prevents not only the formation of toxic metabolites such as peroxynitrite but also prevents the degradation of NO. It is thought that SOD may have an important role in the modulation of NO bioactivity because as superoxide is scavenged by SOD, NO is readily available to synthesize cGMP and subsequently cause vasodilation. In addition, the increase in ec-SOD expression in response to training could enhance the formation of hydrogen peroxide which may increase eNOS expression.[228] Lauer et al. reported that hydrogen peroxide plays an important role in the endothelial adaptation to exercise by stimulating upregulation of eNOS since eNOS expression was increased after training in normal mice but not in mice over expressing catalase.[229]

Gene-Exercise Interactions

Regulation of NO bioavailability and homeostatic maintenance of harmful vasoconstrictors is a complex process with important clinical ramifications. In several

disease states, exercise training or physical activity has been reported to reduce oxidative stress, increase NO availability and improve endothelial function (endothelium-dependent relaxation) presumably leading to an enhanced quality of life. Although the molecular basis for these improvements still need to be fully elucidated, two main hypotheses are clear; the first is that training leads to improved endothelial function via an increase in NO bioavailability by increasing the expression of eNOS, and the second is that exercise training decreases free radicals which would otherwise inactivate NO.[230] It was reported that in patients with chronic heart failure, twelve weeks of aerobic exercise training lead to increases in mRNA expression of both Cu/ZnSOD and GPX but not eNOS expression.[230] The authors concluded that their data does not support a model of training-induced increases in steady-state levels of NO but that the data is entirely consistent with regard to the alternate hypothesis that reduced oxidative stress in response to training may account for increased NO-mediated vasodilation.

Since shear stress is known to modulate endothelial function, it stands to reason that the expression of the eNOS gene and various antioxidant enzymes could be regulated by intermittent increases in blood flow that occur with training. It has been reported that the promoter region of the eNOS gene contains a cis-acting shear stress response element and that Cu/ZnSOD is a shear stress-sensitive gene product.[43, 230] To determine whether chronic shear stress increased expression of Cu/ZnSOD, Inoue et al. exposed human aortic endothelial cells to varying levels of shear stress.[231] They reported that increasing levels of shear stress increased Cu/ZnSOD mRNA in a dose dependent manner. In addition, at a shear level of 15 dyne/cm^2 they showed that

the enzyme's transcriptional rate, protein levels and enzymatic activity were significantly increased. As shear stress did not alter the expression of Cu/ZnSOD in vascular smooth muscle cells, the authors proposed that the effect of shear stress is specific to the endothelium. Takeshita et al. reported that physiological levels of laminar shear stress upregulate GPX mRNA levels and enzymatic activity in a time dependent manner in endothelial cells contributing to the antioxidant state of the vessel wall.[232] In addition, exercise training-induced elevations in eNOS mRNA expression in coronary vessels from dogs suggests that chronic exercise increases NO production probably by increasing endothelial shear stress. [233]

As mentioned earlier, SOD appears to modulate NO activity via scavenging of superoxide. However, it is possible that NO itself might modulate ec-SOD expression in response to endurance training. Vascular ec-SOD is localized in high concentrations between the endothelium and the smooth muscle where endothelium-derived NO must pass to stimulate smooth muscle relaxation.[171] Thus, regulation of ec-SOD by NO represents an important feed-forward mechanism whereby NO enhances it's own biological effects by reducing superoxide in the critical extracellular site.[228] As such, Fukai et al. reported that NO increased ec-SOD expression in human aortic endothelial cells.[228] Furthermore, they reported that while aortas from wild type mice exhibited increased eNOS and ec-SOD expression after three weeks of exercise training, training had no effect on mice lacking eNOS suggesting that the effect of exercise was mediated by endothelium derived NO.

Regular physical activity is also reported to reduce mRNA and protein expression of the AT1R, increase mRNA expression of the AT2R, reduce mRNA

expression of subunits of NADPH oxidase and reduce NADPH oxidase activity with a concomitant reduction in ROS production.[42] The change in ratio of these two receptors in response to training may be important for the net vasoactive effect of AngII, potentially leading to a reduction in AngII-mediated vasoconstriction and superoxide production, subsequently improving oxidative stress and endothelial function.

Conclusion

Hypertension is one of the major risk factors for CVD and has a strong underlying genetic component. Several mechanisms have been suggested to contribute to the development and maintenance of hypertension and hypertension-induced organ injury. Among them, oxidative stress and the resulting endothelial dysfunction appear to have defining roles in the etiology of this multifaceted disorder. Subsequently, the availability of NO and the homeostatic maintenance of the ratio between vasodilators and vasoconstrictors in the endothelium, contribute to overall vascular health by regulating vascular tone, BP, platelet aggregation and cell proliferation. As such, the loss of the endothelium's normal homeostatic functions is considered to be the earliest detectible changes in the development of vascular disease.[140] Accordingly, endothelial dysfunction, defined as a reduction in NO bioavailability, is prevalent in several disease states.[8, 16, 17]

The ROS produced during normal cell metabolism are contained by various antioxidant enzymes including SOD, GPX and catalase. However, in pathologic states a reduction in antioxidant capacity coupled with the overproduction of oxidants, or oxidative stress, has been identified as the critical mechanism underlying

endothelial dysfunction and subsequent development of hypertension. Accordingly, gene-deletion studies of some antioxidants have shown significant increases in BP and increased oxidative stress. The overproduction of superoxide anion has been shown to reduce NO bioavailability, uncouple eNOS, induce peroxynitrite formation, and increase the generation of vasoconstrictive isoprostanes and other contracting factors. As a result, oxidative stress has been identified as a critical factor in the pathogenesis of hypertension and heritability studies demonstrate that oxidative stress also has an underlying genetic component.

There are several sources of ROS, among them NADPH oxidase has been identified as the most important source of superoxide.[85] As such, increased NADPH oxidase activity has been reported in several disease states.[84, 144, 145] AngII via the AT1R, has been identified as one of the main contributors to the activation of NADPH oxidase and therefore, is a determining factor in oxidative stress-induced hypertension and end-organ injury. Variation within the AT1R gene may modulate the influence of AngII on BP and oxidative stress. The A1166C polymorphism has been extensively studied and has been found to be associated with hypertension and oxidative stress while the -825 T/A polymorphism, although not as extensively studied, may modulate the influence of AngII on BP and oxidative stress.

Aerobic exercise training has been shown to be beneficial in reducing BP and reducing CV morbidity and mortality. Accordingly, several studies have reported that exercise training leads to amelioration of oxidative stress and improved endothelial function and it is probable that shear stress mechanisms are in part responsible for this response to aerobic exercise training. Furthermore, it is postulated that the

intermittent increases in blood flow in response to training may modulate the adaptive response of the vasculature by increasing the expression of atheroprotective genes.

APPENDIX A – Limitations of the Study

Delimitations

1. All participants were members of the larger Gene Exercise Research Study which investigated the influence of genetics on changes in BP in response to aerobic exercise training.
2. All participants were recruited from the Washington DC metropolitan area through advertisements and public service announcements.
3. All participants were sedentary (physical activity < 20 minutes, < 2 days per week), non-smokers, non-diabetic and free from CV disease.
4. Participants were classified as pre- or stage 1 hypertensive (SBP: 120-159; DBP: 80-99) without medication.
5. All women were post menopausal for > 2 years and were required to stay on their HRT regime, either on or not on, for the duration of the study.

Limitations

1. A mixed ethnic population was used to improve the statistical power of the present study.
2. The current study lacked a control group. Study participants included stage 1 hypertensives, and the current JNC VII guidelines recommend at least 30 mins of physical activity most days of the week for this group. Our training intervention included 6 months of aerobic exercise training and because no other intervention was available in this study, it would be unethical to include hypertensives in a control group and not treat them.
3. Participants were asked to avoid exercise that was not consistent with the training protocol however, we could not control their compliance outside of our training facility.
4. The viability of some plasma/urine samples and the inability to obtain BP measurements for all participants resulted in samples sizes that were not similar for all variables. As a result the reduced sample size may have reduced the statistical power.
5. The results of this study may be applicable only to the study population and similar populations and may not be representative of the general population.

APPENDIX B – Definition of Terms

Aerobic exercise – refers to any type of exercise performed at moderate intensity for extended periods of time in order to maintain an increased heart rate.

Allele – an alternate form of a gene.

Antioxidants – molecules (both endogenous and exogenous) that slow or prevent the rate of oxidation of other biological compounds and structures.

Cardiovascular disease – a term that encompasses a broad range of diseases and refers to any disorder that affects the heart and/or blood vessels.

Cyclooxygenase – an enzyme responsible for the formation of important biological mediators called prostanoids.

Endothelial dysfunction – impairment of endothelium-dependent relaxation caused by a loss of nitric oxide bioactivity in the vessel wall.

Endothelium – the endothelium is a single cell lining covering the internal surface of blood vessels. It senses mechanical and hormonal stimuli and produces numerous vasoactive factors that regulate vascular tone, local homeostasis, recruitment and activity of inflammatory cells and vascular wall proliferation processes.

Genome – the total genetic information, in particular the DNA that carriers this information, belonging to a cell or organism

Hypertension – commonly referred to as "high blood pressure." It is a medical condition where blood pressure is chronically elevated.

Intron – a non-coding region of a gene that is transcribed into an RNA molecule but is then spliced during production of the mRNA.

Linkage disequilibrium – this is also referred to as allelic association. It is the occurrence of alleles at two loci more frequently than expected given the known allele frequencies and the recombination fraction between the two loci.

Oxidative stress – a disturbance in the balance between oxidant production and antioxidant defense.

Polymerase chain reaction – a 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 complimentary strands.

Polymorphism – a variation in the sequence of DNA among individuals that must occur in a least 1 % of the population to be considered common.

Promoter – a nucleotide sequence in DNA to which RNA polymerase and transcription factors bind to initiate transcription.

Reactive oxygen species – a family of molecules including molecular oxygen and its derivatives produced in all aerobic cells.

Single nucleotide polymorphism – a DNA sequence variation that occurs when a single nucleotide (A, C, T or G) in the genome is changed.

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