ABSTRACT

Title of Dissertation: **NFKB1 Gene Promoter Polymorphism and Unidirectional Laminar Shear Stress: Implications for NF-κB activation, eNOS Protein Expression and Endothelial Function**

Joon Young Park, Doctor of Philosophy, 2006

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Exercise stimulus can be defined as endothelial wall shear stress. In the endothelial cells, the nuclear factor–kappa B (NF-κB) is an important intracellular signaling molecule by which changes in wall shear stress, sensed by mechanosensors, are transduced into the nucleus to initiate downstream eNOS gene expression. Recently, a polymorphism in the promoter region of the gene encoding a p50/p105 NF-κB subunit, NFKB1, has been identified. The NFKB1 ATTG insertion (I) / deletion (D) (NFKB1 I/D) promoter polymorphism transcriptionally regulates NFKB1 gene expression. However, the functional significance of this polymorphism has not been elucidated in endothelial cells under LSS and in endothelial function in humans.

Therefore, the purpose of this study was to investigate whether the NFKB1 I/D promoter polymorphism had functional genetic properties in human umbilical vein endothelial cells (HUVECs) under physiological levels of unidirectional laminar shear stress (LSS), and further, whether the polymorphism was associated with changes in endothelial function after endurance exercise training in pre- and stage I hypertensive individuals.

The major findings of the present study were that 1) a protein present in HUVECs preferentially and specifically binds to the I allele promoter compare to the D allele; 2) the I allele had significantly higher promoter activity than the D allele; and accordingly, the II homozygote cells had higher p50/p105 NFKB1 protein levels than the DD homozygote cells; 3) the II homozygote cells showed a greater increase in eNOS protein levels than the DD homozygote cells under unidirectional LSS; and 4) the I-allele carrier group had a greater reactive hyperemic forearm blood flow response, a measure of endothelial function, before exercise training; however, the NFKB1 I/D polymorphism was not significantly associated with the differential changes in endothelial function following exercise training.

These results have potential clinical implications for endothelial dysfunction that are related to the development and progression of atherosclerosis and cardiovascular disease. In addition, our findings provide insight into the molecular mechanisms involved in the intracellular signaling transduction process of eNOS gene expression and function of the NFKB1 gene promoter region.

NFKB1 GENE PROMOTER POLYMORPHISM AND UNIDIRECTIONAL LAMINAR SHEAR STRESS: IMPLICATIONS FOR NF-κB ACTIVATION, eNOS PROTEIN EXPRESSION AND ENDOTHELIAL FUNCTION

By

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May, 2006

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LIST OF ABBREVIATIONS

AHA: American Heart Association
AUC: area under the forearm blood flow curve
bp: base pairs
BP: blood pressure
CP1: CCAAT-binding Protein 1
CV: cardiovascular
D: deletion
DNA: Deoxyribonucleic acid
eNOS: endothelial nitric oxide synthase
FBF: forearm blood flow
FBS: fetal bovine serum
FVR: forearm vascular resistance
GRR: glycine-rich region
HUVEC: human umbilical vein endothelial cell
I: insertaion
IKKα: inhibitor kappa B kinase alpha subunit
IκB: inhibitor kappa B
kDa: kilodalton
L-NMMA: N-monomethyl-L-arginine
LDL: low density lipoprotein
LPS: lipopolysaccharide
LSS: laminar shear stress
Luc: luciferase
MBP: mean blood pressure
NF-κB: nuclear factor of k-light polypeptide gene enhancer in B cells
NLD: nuclear localization domain
NO: nitric oxide
OSS: oscillatory shear stress
PCR: polymerase chain reaction
PLB: passive lysis buffer
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SSRE: shear stress responsive element
VO_{2max}: maximal oxygen consumption

INTRODUCTION

The endothelium is a monolayer of epithelial cells lining the innersurface of all of the blood vessels in the body. At the interface between circulating blood and tissues, these cells function as a protective biological barrier and also as a fine sieve to regulate selective passage of macro- and gaseous molecules in and out of the circulating blood. In addition, the endothelial cells release a number of autocrine and paracrine substances[50, 126]. Because of its strategic location, the endothelial cells readily sense changes in hemodynamic forces[5, 131] and blood borne signals[53], and respond by releasing a number of vasoactive substances (i.e. nitric oxide (NO)[50, 126]) and by initiating expression of the downstream genes such as endothelial nitric oxide synthase (eNOS) gene [5, 79, 131, 191].

Over the last two decades, function of the vascular endothelium has emerged as a strong marker for monitoring cardiovascular (CV) health on the basis that impairment of endothelial function is the earliest event in the process of developing atherosclerosis[110]. Endothelial dysfunction has been frequently associated with all common CV risk factors predisposing to atherosclerosis, such as age[19, 37, 174, 190], obesity[170], post-menopause[173], hypertension[108, 143-145, 176], hypercholesterolemia[16, 28, 69], diabetes[119, 120, 185], smoking[18, 69], and physical inactivity[48].

In the presence of endothelial dysfunction, improvement in the endothelial function has been observed with various pharmacological treatments[9, 75, 77, 86, 158], and also was evident with behavioral modifications[43, 159] including increased physical activity[48, 56, 102, 172].

Therefore, investigation of the mechanisms underlying the salutary effect of physical activity and its related stimulation on endothelial function has an important clinical significance.

Endothelial nitric oxide synthase

NO is a well-known endothelium-derived, atheroprotective, vasodilatory, and anti-proliferative molecule which maintains vascular integrity and regulates endothelial function[50, 141]. The classical endothelium-derived relaxing factor, NO, is synthesized by a reaction converting L-arginine to L-citruline, a reaction catalyzed by eNOS[50, 140, 141].

L-arginine-based inhibitors of eNOS increase blood or perfusion pressure and vascular resistance and reduce blood flow in vivo[76, 152] and in vitro[155]. eNOS knockout mice (-/-) are mildly hypertensive relative to wild-type littermate control mice (+/+). Moreover, the suppressor effect of an eNOS inhibitor is attenuated in the -/- mice and endothelium-dependent relaxation in response to acetylcholine is abrogated in the isolated vessels[83, 166].

eNOS is constitutively expressed in endothelial cells[131], but it is also subjected to a modest degree of regulation in certain conditions. In vitro, eNOS expression is increased by shear stress[131], cyclic strain[5], exposure to lyshosphatidylcholine[191], low concentrations of oxidized low density lipoprotein (LDL)[79], and cyclic GMP analogues[151]. In vivo, the most important physiological agonist for NO release is fluid shear stress.

eNOS posttranslational modification/activation occurs via calcium/calmodulin-dependent and independent mechanisms[49, 163, 164]. Flow shear stress induces eNOS activation and NO release by recruitment of upstream kinases[11, 35, 45, 123]

Under basal conditions, systemic NO production and bioavailability is diminished in patients with cardiovascular diseases, and the impaired NO release may play a role in pathogenesis of hypertension and atherosclerosis[47, 111].

Thus, the shear-mediated regulation of eNOS gene expression is an important issue related to NO bioavailability to maintain endothelial function in pathophysiologic conditions.

Hemodynamic shear stress and mechanotransduction: Regulation of eNOS gene expression

Vascular endothelial cells are constantly exposed to hemodynamic shear stress. The magnitude and pattern of the shear stress vary depending mainly on the level of local blood flow and vascular geometry, which varies by location in the vascular tree.

The regions of arterial narrowing or extreme curvature, where low-velocity and oscillatory flow is frequently observed, have a predilection for atherosclerotic lesions. In contrast, the straight regions of the vascular tree where unidirectional laminar flow is dominant, are generally atheroprotective[113]. Based on in vitro studies, unidirectional laminar shear stress (LSS) greater than 15 dyne /cm^2 is associated with upregulation of vasoprotective endothelial genes (i.e. eNOS gene) which have anti-inflammatory, antioxidant, anti-apoptotic, and anti-proliferative properties.

LSS activates the inhibitor kappa B kinase α subunit (IKKα) via the c-Src and MEK 1/2 dependent signaling pathway, increases proteosome-dependent degradation of inhibitor kappa B alpha subunit, and induces nuclear translocation of the p50/p65 NF-κB complex[10, 31-33, 60, 67]. The NF-κB complex binds to the shear stress response element at

position -982 in the human eNOS gene promoter resulting in increased gene expression[33]. Moreover, inhibition of the NF-κB -mediated signaling completely blocks the increase in eNOS transcription in endothelial cells exposed to the shear stress[60]. Accordingly, the p50/p65 NF-κB complex is an essential transcription factor in the response of eNOS gene expression to LSS in endothelial cells.

Exercise, endothelial function, and heritability

At the cellular level, exercise acts as a physiological stressor that disturbs resting homeostasis. In the vasculature, the exercise stimulus can be defined as endothelial wall shear stress. During exercise, flow velocities are approximately double those observed during rest, and flow reversal and regions of the low velocity/oscillatory shear stress that existed under resting condition are eliminated[24, 127, 178]. Independent of reducing CV risk factors, the increased flow-mediated shear stress during exercise plays a significant role in the improvement of endothelial function with physical activity[50, 101, 130, 165, 189].

Exercise intervention studies have shown that exercise training improves endothelial function in both healthy[94, 100, 169] and diseased[76, 102] individuals. Animal studies have shown that endurance aerobic exercise training increased eNOS protein content in coronary and small resistance arteries[34, 101]. In addition, the application of physiological levels of unidirectional LSS increased eNOS mRNA and protein levels in endothelial cells *in vitro*[150, 189]. Taken together, enhancement of the eNOS gene expression and activation seems to play a significant role in the beneficial effect of exercise on the endothelium.

A number of studies have proposed a modest contribution of genetic factors to the variability in blood pressure and endothelial function[3, 8, 184]. Also several studies have shown that a positive family history of CV diseases is an important predictor of impaired endothelial cell function[25, 51, 160]. In addition, it has been proposed that genetic factors may be involved in determining the direction and magnitude of the beneficial cardiovascular adaptations that occur with exercise training in individuals with CV risk factors[63, 109, 186].

Nuclear factor kappa B and the p50/p105 NFKB1 gene

NF-κB is a ubiquitous dimeric transcription factor that regulates expression of various genes related to inflammation, cell adhesion, cell division, apoptosis, and stress responses[52]. There are five members of the NF-κB family in mammals: p50/p105, p65/RelA, c-Rel, RelB, and p52/p100. Although many dimeric forms of NF-κB have been detected, the major inducible form of NF-κB is a heterodimer of the p50 and p65 subunits[21].

In a quiescent cell, the NF-κB complex is retained in the cytoplasm by binding to a protein inhibitor, inhibitor kappa B (IκB)[7]. The formation of the NF-κB/IκB complex masks a critical nuclear localization domain (NLD) on a conserved N-terminal region of NF-κB proteins, called the Rel homology domain, and this prevents the translocation of NF-κB dimers into the nucleus. Once the cell is stimulated by appropriate stimuli, IκB kinase (IKK) phosphorylates the IκB complex at Ser32 and Ser36 on the IκBα subunit followed by ubiquitination and proteosome-dependent degradation of the IκB complex[2, 6]. Dissociation of IκB from NF-κB exposes the NLD of the NF-κB complex, so that the

NF-κB complex is translocated into the nucleus and binds to a κB-binding site on a target gene to initiate gene transcription[7].

The human NFKB1 gene has been localized to 4q23-q24. It encodes two proteins, a 105 kilodalton (kDa) non DNA-binding cytoplasmic molecule (p105) and a 50 kDa DNA-binding protein (p50) that corresponds to the N terminus of p105[71]. Recently, a functional promoter polymorphism, NFKB1 ATTG insertion (I) / deletion (D) (NFKB1 I/D) promoter polymorphism, has been described[91]. The polymorphism is located between transcription factor binding sites for AP-1 and κB in the promoter region. The allele frequencies of NFKB1 I and NFKB1 D allele in the normal population are approximately 60% and 40%, respectively. The NFKB1 I/D promoter polymorphism has been shown to transcriptionally regulate NFKB1 gene expression in HT-29 and HeLa cells[91]. However, the functional significance of the polymorphism has not been elucidated in endothelial cells under flow shear stress.

PURPOSE OF THE STUDY

Vascular endothelial function has emerged as a strong marker for monitoring cardiovascular health on the basis that the impairment of the endothelial function is the earliest event in the process of atherosclerosis. Nitric oxide is a key endothelium-derived relaxing factor and a strong atheroprotective substance, and it is generated by endothelial nitric oxide synthase (eNOS).

As a systemic stressor, a bout of endurance exercise is translated into unidirectional laminar shear stress (LSS) in endothelial cells due to increased blood flow during exercise. There are several lines of evidence showing that LSS underlies the adaptations of the endothelium to chronic endurance exercise training. In addition, nuclear factor kappa B (NF-κB) is an important intracellular signaling molecule in endothelial cells by which changes in wall shear stress, sensed by mechanosensors, are transduced into the nucleus for downstream eNOS gene expression. Recently, a functional insertion (I) / deletion (D) polymorphism in the promoter region of the gene encoding a p50/p105 NF-κB subunit, NFKB1, has been identified. However, the functional significance of the polymorphism has not been elucidated in endothelial cells under LSS and in endothelial function in humans.

Therefore, the purpose of this study was to investigate whether the NFKB1 I/D promoter polymorphism has functional genetic properties in eNOS gene expression under physiological levels of unidirectional LSS in human endothelial cells, and further, whether the polymorphism is associated with endurance exercise training-induced changes in endothelial function in Pre- and Stage I hypertensive individuals.

HYPOTHESES

The central hypothesis of this study is that the NFKB1 -94 ATTG insertion (I) / deletion (D) promoter polymorphism has functional properties in eNOS gene expression that respond to unidirectional laminar shear stress (LSS) in Human Umbilical Vein Endothelial Cells (HUVECs), and that the polymorphism is associated with differential changes in endothelial function after exercise training. The specific aims of this study are shown below.

Hypothesis #1 (In vitro study)

The NFKB1 I/D promoter polymorphism has functional genetic properties that respond to *in vitro* unidirectional LSS in HUVECs.

Specific Aims

1.1 Determine whether NF-κB-mediated signaling is induced by unidirectional LSS in HUVECs.

1.2 Determine whether the NFKB1 I/D promoter polymorphism affects DNA-protein binding activity between the polymorphic promoter region and nuclear proteins extracted from HUVECs.

1.3 Determine whether the NFKB1 I/D promoter polymorphism affects transcriptional activity under unidirectional LSS in HUVECs.

1.4 Determine whether the NFKB1 I/D promoter polymorphism affects intracellular levels of eNOS protein under unidirectional LSS in HUVECs.

Hypothesis #2 (*In vivo* study)

The NFKB1 I/D polymorphism is associated with differential changes in endothelial function following exercise training.

Specific Aims

2.1 Determine whether the NFKB1 I/D polymorphism is associated with changes in peak forearm blood flow with endurance exercise training.

2.2 Determine whether the NFKB1 I/D polymorphism is associated with changes in total reactive hyperemic forearm blood flow with endurance exercise training.

METHODS

Study Design Overview

To address the hypotheses, a complementary study design including *in vitro* unidirectional laminar shear stress (LSS) experiments and an *in vivo* human exercise intervention study were conducted. <Figure 1>

In the *in vitro* shear stress experiments, primary-cultured Human Umbilical Vein Endothelial Cells (HUVECs) from different donors allowed us to obtain cells with different genotypes (II, ID, and DD genotypes). In some experiments, heterozygote HUVECs were subjected to transient transfection with I-type or D-type promoter-reporter gene constructs to establish genetic models. The HUVECs growing on a gelatinized culture dish were exposed to physiological levels of unidirectional LSS generated by a cone-and-plate apparatus. The NFKB1 gene promoter activities and the protein expression levels were measured in the cells. Therefore, the *in vitro* shear stress experiments provided molecular genetic data which elucidated the function of NFKB1 I/D promoter polymorphism on eNOS gene expression in HUVECs.

In the *in vivo* human exercise intervention study, sedentary middle-aged to older Pre- and Stage I hypertensive men and women were recruited and screened for diseases or clinical conditions that may confound the data interpretation. The qualified subjects underwent a 6 week dietary stabilization period and those who were using antihypertensive medications were tapered from their medication. Subjects were genotyped for the NFKB1 I/D polymorphism and underwent 6 months of exercise

training. To assess the endothelial function, reactive hyperemic forearm blood flow were measured before and after the exercise training.

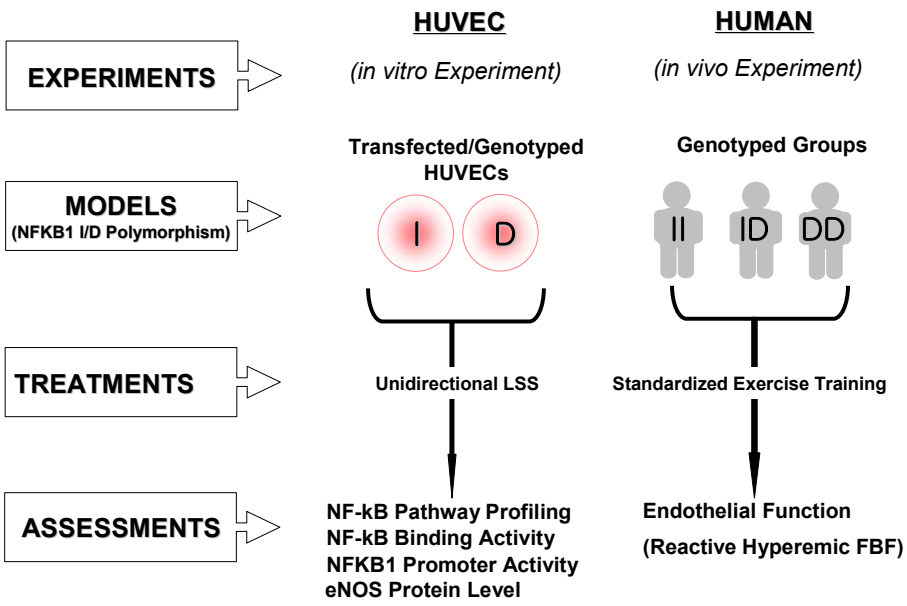


Figure 1. Complementary *in vivo* and *in vitro* Experimental Study Design

Human hypertensive subjects and Screening

Human Pre and Stage I hypertensive subjects were recruited to the project investigating the interaction between genetic factors and an exercise intervention on the regulation of blood pressure (BP). Men and women responding to media advertisements and fliers were contacted by telephone to assess their eligibility. All the protocols involved human subjects were approved by the Institutional Review Board of the University of Maryland, College Park (IRB no. 01075 and no. 00736; IRB HSR ID no. 04-0319).

Subjects were sedentary (aerobic exercise <2 times/wk, <20 min/session, sedentary job); 50 - 75 yrs of age; nondiabetic; not on lipid-lowering medications; suspected of having Prehypertension or Stage 1 Hypertension (systolic BP: 120-159; diastolic BP: 80-99); and have no evidence of chronic obstructive pulmonary disease. Subjects also would not have any other medical conditions precluding vigorous exercise. Subjects had to have a body mass index <37 kg/m^2. Women were postmenopausal (absence of menses for >2 yrs). Hypertensive subjects using more than one antihypertensive medication were excluded from participation. Women were excluded from further participation in the study if they changed their hormone replacement therapy regimen during the course of the study.

Orientation Visit: Subjects visited our laboratory and had their medical history forms reviewed by the study physician to determine if they met any criteria that would exclude them from the study. The written informed consent form was signed by the qualified subjects and co-signed by principal investigators.

Screening Visit #1: Subjects reported in the morning after an overnight fast of >12 hours and blood samples were drawn for basic blood chemistries and deoxyribonucleic acid (DNA) analysis. After the baseline blood draw, subjects were instructed to drink 10 fl oz of a glucose tolerance test beverage within two minutes. Another blood draw took place in 2 hours in order to measure 2 hour glucose level. Subjects were excluded if they had a hematocrit <35, serum creatinine >135 mmol/L, evidence of renal or liver disease, or fasting glucose >126 mg/dL.

Screening Visit #2: Qualified subjects then underwent a maximal treadmill exercise test to screen for coronary heart disease, preceded by a physical examination by a

physician to screen for CV disease. BP and ECG was recorded before the test, at the end of every stage of the exercise, and every other minute for 6 minutes after exercise. The test was terminated when the subject could no longer continue or CV signs/symptoms occurred. Subjects who had > 2 mV ST-segment depression or CV signs/symptoms were excluded from the study.

Diet Stabilization and Medication Withdrawal: Because differences in dietary intake between the subjects could independently affect some of the outcome variables, all subjects maintained the American Heart Association (AHA) Dietary Guidelines for Healthy American Adults, formerly called a "Step I" diet. Qualified subjects underwent 6 wks of instruction in the principles of an AHA low-fat (< 30% total calorie intake) and < 3 g/day sodium diet that maintained for the entire study period. This training consisted of 2 sessions / week and was instructed by a research dietitian.

Subjects on one antihypertensive medication began a tapering schedule recommended by their physician at the start of the Dietary Stabilization period. The study physician also supervised the medication tapering process. All subjects had their BP measured weekly. If a subject's systolic or diastolic BP was >159 or >99 mm Hg, respectively, for 3 consecutive weeks at any time during the study, they were excluded from further participation in the study and referred back to their personal physician.

Measurement of Casual Blood Pressure

Casual BP was measured according to the seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC VII) guidelines. In brief, subjects fasted for more than 12 hours before the

measurement. Subjects rested in a seated position for 20 minutes and BP was measured at least two times until both systolic and diastolic BP values were within 4 mm Hg. For each day the casual BP was measured, all values that deviated by less than 4 mm Hg were averaged for both systolic and diastolic BP. The casual BP was calculated by averaging the average BPs obtained across all three days.

Measurement of Maximal Oxygen Consumption

All subjects underwent a second maximal treadmill exercise test to assess maximal oxygen consumption (VO_{2max}) and to prescribe exercise training. This test was supervised by the study physician. This test started at 70% of the peak heart rate achieved on each subject during their screening exercise test of each subject and the treadmill grade was increased by 2% every 2 minutes. BP and ECG were monitored. The criteria for VO_{2max} included a respiratory exchange ratio (RER) $\geq$ 1.15, failure of increasing oxygen consumption $\geq$ 2 ml/kg/min as the exercise intensity increased, and the subject could no longer continue to exercise.

Measurement of Forearm Blood Flow During Reactive Hyperemia

Forearm blood flow (FBF, $ml \cdot min^{-1} \cdot 100ml^{-1}$ forearm tissue volume) was measured in the morning (7:00 ~ 9:00 am) after an overnight fast (12 hr) in the non-dominant arm using venous occlusion plethysmography method as described elsewhere.[76, 134, 145] Subjects were instructed to rest in the supine position for 20 minutes before the testing was performed. A strain gauge was placed around the widest part of the forearm, which was elevated slightly above the level of the right atrium. The strain gauge was connected

to a Hokanson EC-5R plethysmograph (Hokanson Inc, WA) calibrated to measure the change in forearm volume. For each measurement, a BP cuff placed around the upper arm was inflated to 55 mm Hg and connected to a rapid cuff inflator (E-20, Hokanson Inc) to occlude venous outflow from the extremity. A wrist cuff was inflated to suprasystolic pressure before each measurement to exclude the hand circulation from the measurement of FBF.

Baseline FBF were measured 3 times, each separated by 10-15 seconds. Reactive hyperemia was then induced by inflating the cuff on the upper arm to 50 mm Hg above the systolic BP for 5 minutes to occlude the FBF. Immediately after the cuff was released, FBF was measured for 3 minutes at 15 second intervals (7 sec measure + 8 sec recovery). BP was measured each minute during the reactive hyperemic period.

Three parameters were obtained from the FBF curve during the procedure: peak FBF; minimum forearm vascular resistance (FVR) (mm $Hg \cdot ml^{-1} \cdot min^{-1} \cdot 100ml^{-1}$), calculated from peak FBF and mean blood pressure (MBP) measured just after the release of the cuff; and duration of reactive hyperemia, defined as the interval (seconds) between the release of occlusion and the return to +5% of the baseline FBF. Furthermore, total hyperemic FBF was determined from the area under the FBF curve (AUC) during the period of recovery after release of occlusion. Reactive hyperemia variables were calculated as follows: flow debt (ml/100ml) = baseline FBF X duration of occlusion; excess flow (ml/100ml) = total hyperemia flow – (baseline FBF X duration of reactive hyperemia). Hyperemia repayment (%) was calculated a ratio of excess flow and debt flow. FBF was calculated by 2 independent observers unaware of the subjects' profiles.

Endurance Aerobic Exercise Training Program

After completing baseline testing, subjects underwent 3 supervised exercise sessions / wk for 6 months. The exercise session included warm-up and stretching exercises and whole body dynamic aerobic exercise. Heart rate was used to monitor their training intensity. Initial exercise training consisted of 20 min 50% VO_2max, and the training duration and intensity were gradually increased until 40 minutes of exercise at 70% of VO_2max was completed. After 10 weeks, one day of walking exercise for 40 minutes was added to the exercise program. Exercise modes included walk/jogging, stair stepping, and cycle, cross-country ski, and rowing ergometry.

Each subject's resting BP was monitored weekly throughout the exercise training intervention. The body weight was monitored throughout the study and subjects were instructed to maintain their body weight within ±5% of their initial body weight.

Genotyping the NFKB1 and NOS3 Gene Polymorphisms

A blood sample was drawn into 10mL EDTA tubes from an antecubital vein at the first screening visit. The anti-coagulated whole blood was centrifuged at 3,000 g for 20 minute to separate a white buffy coat region. Genomic DNA was isolated using the Genomic DNA Purification Kit (Gentra Systems, MN). For HUVECs' genomic DNA isolation, HUVECs grown in a 175cm^2 culture flasks were harvested with 0.25% trypsin/EDTA. Cell pellets underwent a standard procedure provided by the Gentra PUREGENE DNA Purification kit (Gentra Systems, MN) for cells and tissues.

Genotyping for the NFKB1 -94 insertion/deletion ATTG polymorphism was performed as described by Karban et al.[91] by a combined polymerase chain reaction

(PCR)-restriction digestion method. Briefly, a 289 bp PCR fragment was amplified from genomic DNA using specific forward and reverse primers, F:5'-TTCAGTTGTCACTCCACCCA-3' and R:5'-CTCTGGCTTCCTAGCAGGG-3'. Products were digested by the enzyme *Van 91I* (new England BioLabs, Inc., MA), which cleaves the -94 insertion ATTG containing product twice and the -94deletion ATTG containing product once, and the fragment length was analyze on a 2.5% agarose gel. <Figure 2> Genotyping accuracy was confirmed with a direct sequencing.

Based on recent finding of significant functional effect of the C allele in the T → C single nucleotide substitution at -786 of eNOS gene on shear stress insensibility[17], the eNOS gene polymorphism was also screened to exclude CC homozygote HUVECs in the present study. Briefly, the flanking regions of NOS3 gene was amplified using a pair of primers, F: 5'-CACCCAGGCCCACCCCAACCT-3' and R: 5'-GCCGCAGGTCGACAGAGAGACT-3'through PCR. The PCR amplicon was digested overnight at 37ºC using *Msp I* (new England BioLabs, MA) followed by electrophoresis for 4 hours in a gel composed of 2% agarose + 1% Nusieve (FMC, ME). The T allele (absence of the *Msp I* restriction site) yields a fragment of 415 bp, and the C allele (presence of the *Msp I* restriction site) yields fragment of 370 bp and 45 bp.

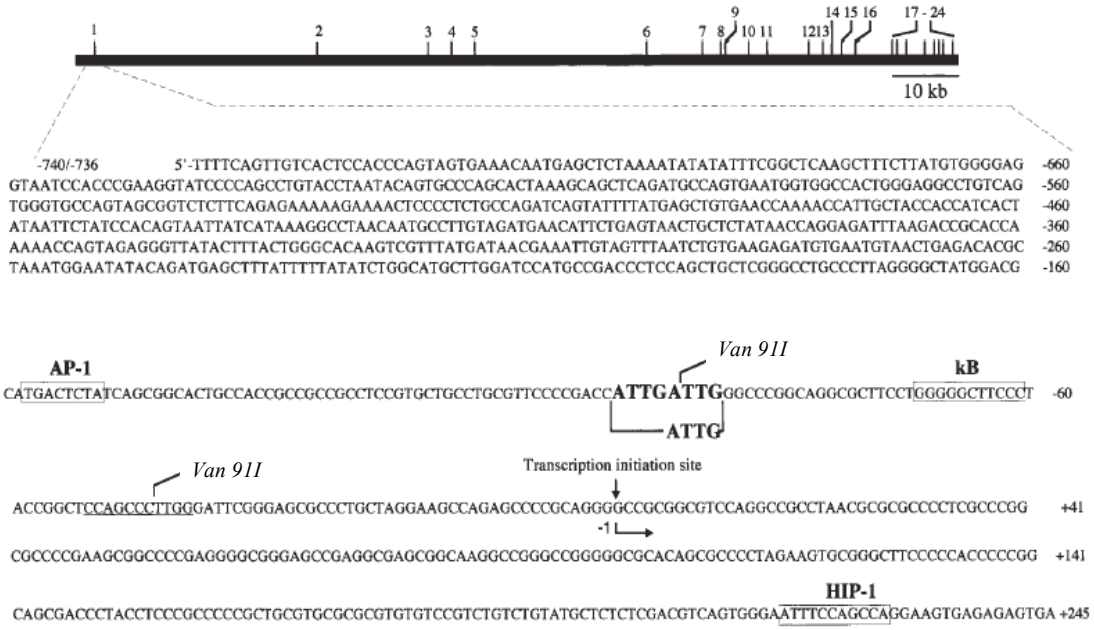


Figure 2. NFKB1 gene structure

Diagram for genomic structure with the location of the 24 exons (top) and sequence of the -740 bp 5' of exon 1 through +245 (bottom) are shown. The -94 insertion/deletion ATTG polymorphism is indicated. Van91I restriction sites used for genotyping and AP-1, κB and HIP-1 DNA binding motifs are designated. (Modified from Karban et al. 2004[91])

HUVECs and cell culture

HUVECs were purchased from the Emory Skin Diseases Research Center, Department of Dermatology, Emory University School of Medicine. These HUVECs were isolated from umbilical veins obtained from area hospitals. This tissue was viewed as discarded tissue. The protocol was approved by the Emory University IRB for the use of this randomly obtained, discarded tissue. They were collected in sterile specimen containers with no patient information included with the specimen. HUVECs were grown in special endothelial cell culture medium supplemented with 20% Fetal Bovine Serum (FBS), 16 U/mL heparin, 50 μg/mL endothelial growth supplement, 2 mmol/L glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and cultured with 5% CO_2 at 37 °C in dishes coated with 0.1% gelatin to assist cell adhesion.

Shear Stress Experiments

The unidirectional LSS and oscillatory shear stress (OSS) experiments were performed as described by Grumbach et al.[61] Confluent HUVECs grown in 100-mm tissue culture dishes were exposed to unidirectional LSS in the growth medium by rotating a Teflon cone (0.5° cone angle). To mimic unstable shear conditions *in vivo*, cells were exposed to OSS with directional changes of flow at 1 Hz cycle by rotating the cone back-and-forth using a stepping motor (Servo Motor, OH) controlled by a computer program (DC Motor Company, OH). The shear experiments were conducted in a humidified environment with 5% CO_2 at 37°C. Based on previous *in vitro* studies, unidirectional LSS greater than 15 dyne /cm^2 is associated with upregulation of atheroprotective genes (i.e. eNOS) and downregulation of proatherogenic genes[113, 118]; moreover, direct vascular models of the low shear stress or OSS regions have revealed shear values on the order of ± 5 dyne/cm^2 compared with greater than 10 dyne/cm^2 in the atheroprotective or LSS regions.[90] Therefore, we set the level of the unidirectional LSS of + 15 dyne/cm^2 (1.5 N/m^2; arterial or high shear stress level) and OSS of ± 5 dyne/cm^2 for the appropriate physiological level of fluid shear stress. The duration varied depending on the hypothesis that was being addressed.

Preparation of Cell Lysates

Cell lysate samples were prepared using a standard procedure using a total protein extraction method. Briefly, after the fluid shear stress or lipopolysaccharide (LPS, 100 or 500 ng/ml) stimulation, the culture dishes containing HUVECs with static and shear

conditions were immediately placed on ice and washed three times with 1 mL of ice-cold PBS buffer solution followed by 1 mL of total protein extraction solution (containing 1% 1X Triton X-100). After 3 minutes incubation on ice, the solution containing total protein extracts was harvested by scraping. The samples were homogenized using an ultrasound sonicator and stored at -80ºC until used.

Preparation of Nuclear and Cytoplasmic Extracts

For the nuclear protein analysis, cytoplasmic and nuclear proteins were fractionated with the NE-PER Extraction reagent (PIERCE, IL). Briefly, HUVECs were washed three times using 1 ml cold PBS buffer solution and harvested by gently scraping with 1 ml ice-cold PBS using a cell lifter. The suspended cells were centrifuged at 500 x g for 2-3 minutes at 4 ºC. Using a pipet, the supernatant was gently discarded, leaving the cell pellet as dry as possible. 200 µl of ice-cold CER I solution was added and the tube was vortexed vigorously for 15 seconds to fully resuspend the cell pellet and then the tube was incubated on ice for 10 minutes. Then, 11 µl of cold CER II solution was added to the tube and vortexed for 5 seconds on the highest setting and the tube was incubated on ice for 1 minute. After vortexing the tube again for 5 seconds on the highest setting, the tube was centrifuged for 5 minutes at maximum speed in a microcentrifuge (16,000 x g). The supernatant (cytoplasmic fraction) was immediately transferred into a clean pre-chilled tube and placed on ice until storage, and 100 µl of ice-cold nuclear extraction reagent was added. Then, the tube was vortexed for 15 seconds and returned to ice every 10 minutes for a total of 40 minutes. The nuclear extract was obtained by centrifuging the

tube at 16,000 x g for 10 minutes and the supernatant was immediately transferred to a pre-chilled tube and stored at -80 ºC until used.

Plasmid Construction / DNA Cloning

NFKB1 I or D promoter-luciferase gene constructs were generously donated by Dr. Steven Brant at Johns Hopkins University. The reporter constructs include the NFKB1 gene genomic sequence from -736 to +245 having either insertion or deletion allele of the DNA sequence variation. Restriction analysis and complete DNA sequencing confirmed the orientation and integrity of each construct's inserts. To monitor the NF-κB signaling pathway, a commercially available reporter gene system, The Mercury^{TM} Pathway Profiling Systems (Clontech, CA) was utilized. This profiling system allows for monitoring of translocation of conventional p50/p65 NF-κB complex. In the Mercury profiling vector, the specific *cis*-acting –κB binding sequence is located upstream from TATA box (P_{TA}), which is followed by a luciferase reporter gene (pNFkB-TA-Luc). The negative control vector used in this assay was pTA-Luc vector, which lacks the –κB binding sequence (pTA-Luc). For an internal control, the thymidine kinase promoter-*Renilla* luciferase control vector (phRL-TK) (Promega, WI), which contains the herpes simplex virus thymidine kinase promoter and *Renilla* luciferase gene, was used in all transfection assays.

Transient Transfection

HUVECs having ID genotype were transiently transfected with the plasmid constructs of either pGL3-NFKB1(I) / pGL3-NFKB1(D), or pTA-Luc / pNFκB-TA-Luc, respectively.

Equal number of HUVECs (1 X 10^6 cells per dish) were plated in each 10 cm^2 culture dish between 18-24 hours before the transient transfection. Subconfluent (approx. 70-80%) HUVEC cells cultured in 100-mm dishes were transiently co-transfected with 5 μg of either pTA-Luc, pNFκB-TA-Luc, pGL3-NFKB1(I), or pGL3-NFKB1(D) reporter vector and 250 ng of the phRL-TK using 3 μl of FuGENE6 according to the manufacturer's manuals (Roche Molecular Biochemicals, IN). The phRL-TK vector was used as an internal control for transfection efficiency. The concentrations of each vector were determined following a Qiagen purification procedure in parallel using a spectrophotometer. Before transfection assay was conducted, culture media was replaced by special transfection media, containing only minimum supplements that were previously optimized.

Preparation of cell lysate and luciferase assay

After exposure to static or shear conditions, the transfected HUVEC cells lysate was harvested using the Passive Lysis Buffer (PLB) (Promega, WI) as per manufacturer's instructions. Briefly, HUVEC cells were washed twice with ice-cold PBS buffer and 600 μl of PLB was added to each dish. Dishes were incubated at room temperature on a motorized orbital shaker for 20 minutes and cell lysates were harvested by scraping and stored at -80 ºC until used. A dual luciferase system (Promega, WI) was used for firefly and *Renilla* luciferase assays. 50 μl of LAR II reagent was predispensed into glass test

tube. 20 μl of cell lysate was carefully transferred into the LARII in the test tube then place the tube in the Tropix luminometer after mixed by pipetting 2 or 3 times. After the reading was completed 50 μl of Stop&Glo reagent was added and briefly mixed, then the tube was placed again in the luminometer. A luminometer was programmed to perform a 2-second premeasurement delay, followed by a 20-second measurement period for each promoter assay. Firefly luciferase activities were normalized to *Renilla* luciferase activity as 'relative luciferase activity'.

Electrophoretic Mobility Shift Assay

Nuclear protein extracts were obtained from HUVECs grown at 37 ºC with 5% CO_2 in M199 supplemented with 20% FBS, L-glutamine, heparin and penicillin/streptomycin. Each of the nuclear protein extracts was obtained using the NE-PER kit from Pierce (MI) as described above. Complementary single-stranded oligonucleotide probes were synthesized based on the NFKB1 promoter as below.

Insertion (I)	:	TCCCCGACCATTGATTGGGCCCGGC
Deletion (D)	:	TCCCCGACCATTGGGCCCGGC
Deletion variant (D1)	:	*gcgt*TCCCCGACCATTGGGCCCGGC
M1	:	TCCCCGACC*cagt*ATTGGGCCCGGC
M2	:	TCCCCGACCATTG*cagt*GGCCCGGC
M3	:	TCCCCGACCATTGA*a*TGGGCCCGGC

The canonical NF-κB p50/p65 protein binding consensus sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3') was used as control[162]. An additional 4-base overhang (gatc) was added at the 5' ends of each oligonucleotide to optimize end-labeling with ^{32}P. Complimentary oligomers were allowed to anneal, and then

radioactively labeled with dATP [α-^{32}P] and dCTP [α-^{32}P] according to the method of Feinberg and Vogelstein.[42] Following purification by Qiagen the labeled, double-stranded DNA oligomers were then incubated for 30 minutes with individual nuclear extracts at room temperature. Electrophoretic mobility shift assay (EMSA) was performed as previously described.[149]

Western Blotting

Aliquots of cell lysates (20 μg of protein each) were resolved on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with a primary antibody overnight at 4 ºC, and then with a secondary antibody conjugated with alkaline phosphotase (for eNOS protein, 1 hour at room temperature) or horseradish peroxidase (for p50/p105 and p65, 1 hour at room temperature), which was detected by chemiluminescence. The intensities of immunoreactive bands in Western blots were analyzed using National Institutes of Health IMAGE Program (Scion Image Beta 4.02). The primary antibody included eNOS (BD Bioscience, NJ), p50/p105 (Cell Signaling Technology, MA), p65 (BD, NJ) and Actin antibody (Santa Cruz Biotechnology, CA) will be used for internal control.

Statistical Analysis

Each variable was plotted and analyzed for variances to identify missing values, outliers, normality and patterns of skewness in the distributions.

Analysis of covariance (ANCOVA) with repeated measures was used to test for differences in reactive hyperemic FBF responses among genotype groups. ANCOVA was

also used to test for differences in reactive hyperemic parameters between resting and reactive hyperemic conditions, and also for difference in percent changes in peak FBF, minimum FVR and total AUC between the genotype groups (II and ID). Based on the results of previous studies, demographic (age, gender and ethnicity) and biological (body weight, MBP and body mass index) confounding factors were used as covariates in all FBF analyses. As no ethnicity*genotype or gender*genotype interactions were observed, these groups were combined for all the analyses. A total of 12 out of 996 ((47+36) X 12 time points) FBF values were not obtained during testing. In order to calculate total AUC, the missing values in the reactive hyperemic FBF measures were replaced by estimated values using a modified interpolation method, in which the mean % change value from the previous time point to the next time point among the total population was used to calculate the missing value. If the 15 second time point was missed, the sample was excluded from the analysis. In the analysis of genotype effect on the % change in reactive hyperemic FBF response, Student's *t*-tests were used to test if there were significant changes in subject characteristics between before and after exercise training.

In the reporter gene expression analyses, Student's *t*-tests were used to test for differences in luciferase activities between static and unidirectional LSS conditions for the I-type and D-type promoter constructs under LPS or fluid dynamic conditions, and for eNOS protein expression levels among the genotyped HUVECs during the different fluid dynamic conditions. In addition, a Student's *t*-test was used to test for difference in changes in eNOS expression level in response to unidirectional LSS between genotype groups.

In vivo study data are expressed as the adjusted LS mean ± SEM except for the demographic and the biological characteristics. The subject characteristics and *in vitro* study data are expressed as mean ± SEM. An α-level was set at 0.05 for all hypothesis tests.

RESULTS

Unidirectional laminar shear stress activates translocation of NF-κB p50/p65 in HUVECs

To determine whether the NF-κB complex was activated by unidirectional laminar shear stress (LSS) in HUVECs, NF-κB translocation under unidirectional LSS was monitored using the Mercury^{TM} Pathway Profiling System (Clontech). In this experiment, either pNFkB-TA-luciferase gene (Luc) reporter vector or pTA-Luc control vector was co-transfected along with phRL-TK *Renilla* luciferase control vector followed by 24 hours of unidirectional LSS stimulation. In the pNFkB-TA-Luc vector, a minimal promoter (TA-luc) is under the control of multimerized kB binding sites. Reporter and control vectors were introduced into HUVEC cells by lipofection. After 24 hours of LSS, cell extracts were assayed for luciferase activity. The NF-κB sites in the pNFkB-TA-Luc vector significantly increased activity of the promoter (> 100 fold increase over the control vector) under both static and unidirectional LSS conditions ($P<0.001$). <Figure 3> However, no significant difference in the level of reporter gene expression was observed between the two fluid dynamic conditions. This result indicated that the NF-κB complex is active in human endothelial cells and may be involved in the induction of downstream gene expression. However, it was unclear whether unidirectional LSS triggered the activation and translocation of NF-κB complex due to the high gene expression levels observed in the static condition. Similar high basal activity of the NFkB-TA-Luc vector has been observed in other cell types (unpublished results: I.K. Farrance and T.B. Rogers)

In order to clarify the involvement of unidirectional LSS in the activation of the NF-κB complex, we monitored the translocation of the NF-κB subunits, p50/p65, under unidirectional LSS by subsequent immunoblot assays. HUVECs were subjected to unidirectional LSS for up to 60 minutes and nuclear extracts were prepared for p50/p65 protein immunoblot assays at the 0, 15, 30, and 60 minute time periods. In this experiment, gradual translocation of both p50 and p65 subunits was observed under the unidirectional LSS. <Figure 4> This observation was consistent with what we observed using the conventional NF-κB activation method using lipopolysaccharide (LPS, 100 ng/ μl) stimulation. <Figure 5>

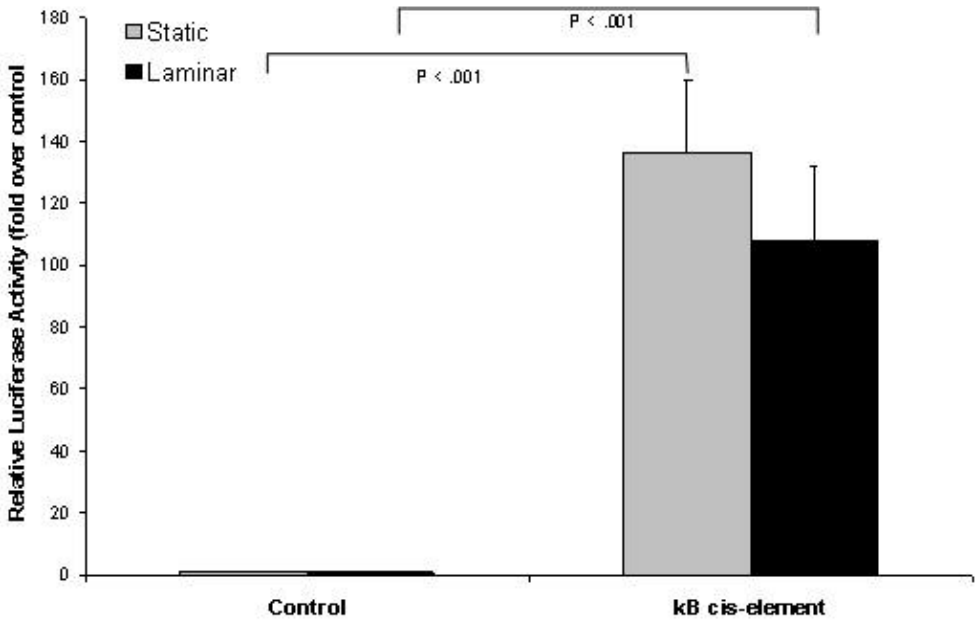


Figure 3. Addition of NF-κB binding sites in the pNFkB-TA-Luc vector significantly increases activity of the promoter under both static and unidirectional LSS conditions. Data represent the results of statistical analysis of fold increase in adjusted luciferase activity over the control observed in the 24 hour unidirectional LSS stimulations (mean values and SEM, n=3). HUVECs were transfected with MercuryTM Pathway Profiling System including plasmid constructs with or without multiple –kB binding sites in the enhancer region followed by luciferase reporter gene, expressed by TA-LUC and kB-TA-LUC, respectively, and the cells were subjected to 24 hour unidirectional LSS. *Renilla* luciferase control plasmid was co-transfected as a internal control.

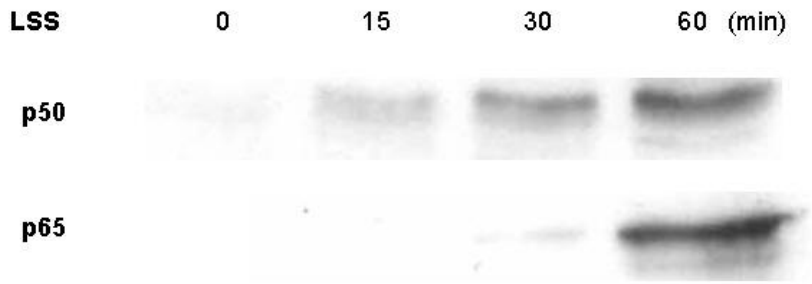


Figure 4. Both p50 and p65 NF-κB subunits are gradually translocated under unidirectional LSS in HUVECs.
Primary cultured HUVECs were subjected to unidirectional LSS and the levels of p50 and p65 proteins were measured in the nuclear extracts. LSS: Laminar shear stress

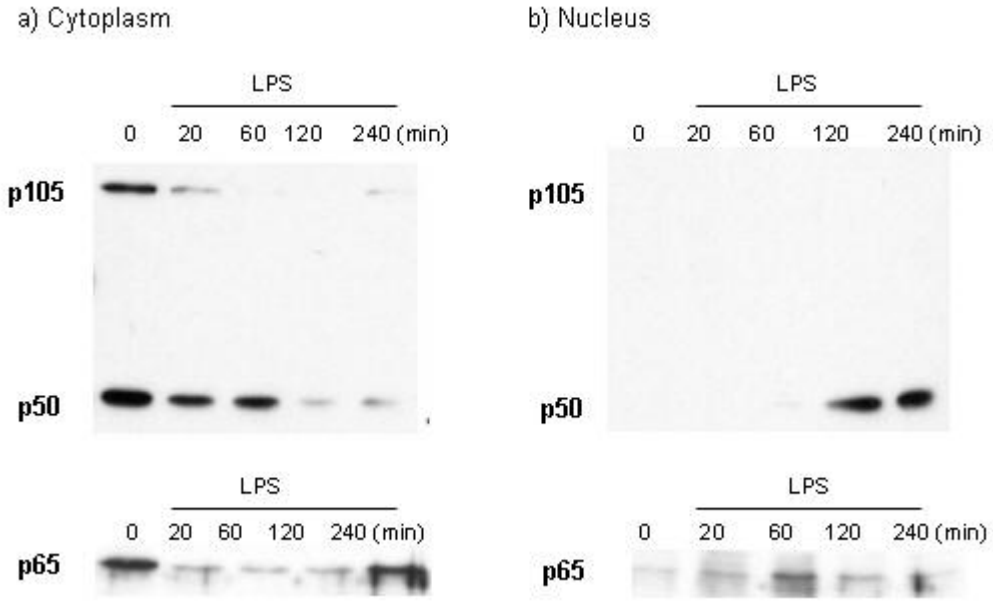


Figure 5. Translocation of both p50 and p65 NF-κB subunits occurs with sustained LPS stimulation in HUVECs.
Primary cultured HUVECs were stimulated with LPS (100 ng/μl) for different time courses and p50, p105, and p65 protein levels were measured in both cytoplasmic (a) and nuclear extracts (b). Time expressed in minutes. These figures demonstrat that NF-κB p50 and p65 subunits located in the cytoplasm was gradually translocated into the nucleus with LPS stimulation. p105 protein existed in resting cytoplasm but gradually disappeared in the early stages of LPS stimulation.

The I-type NFKB1 promoter has higher DNA-protein binding activity

To determine whether the NFKB1 I/D promoter polymorphism affected DNA-protein binding activity between the polymorphic promoter region and nuclear proteins extracted from HUVECs, we conducted the electrophoretic mobility shift assay.

As mentioned earlier, the NFKB1 I/D polymorphic site is located 94-bp upstream of the transcription initiation site of the gene and has either an ATTGATTG (wild, Insertion allele (I)) or ATTG (variant, Deletion allele (D)). Therefore, we synthesized six oligonucleotide probes having different sequences: (1) insertion allele (I), (2) deletion allele (D), (3) size-matched deletion allele which includes a 4-bases of genomic sequence (gcgt) at the 5' end (D1), (4) substitution of the first ATTG with arbitrary sequences (M1), (5) substitution of the second ATTG with arbitrary sequences (M2), and (6) a single nucleotide substitution of the first thymidine of the second repeat to adenine (ATTG → AaTG, M3). The sequences for the oligonucleotide probes are as below:

Insertion (I)	:	TCCCCGACCATTGATTGGGCCCGGC
Deletion (D)	:	TCCCCGACCATTGGGCCCGGC
Deletion variant (D1)	:	*gcgt*TCCCCGACCATTGGGCCCGGC
M1	:	TCCCCGACC*cagt*ATTGGGCCCGGC
M2	:	TCCCCGACCATTG*cagt*GGCCCGGC
M3	:	TCCCCGACCATTGA*a*TGGGCCCGGC

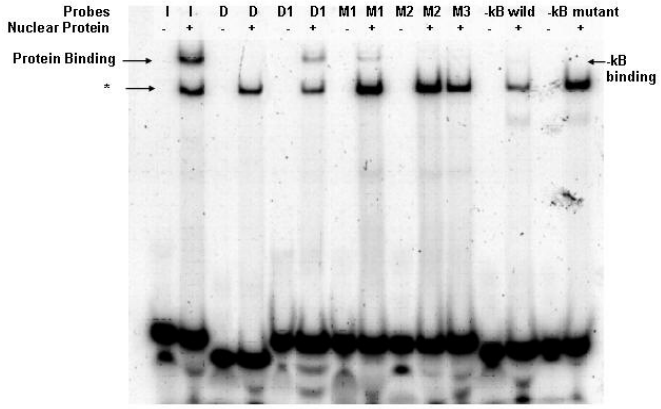
Using the wild (I), variant (D or D1) or mutant (M1, M2, or M3) 25-base pair oligonucleotides representing the polymorphic regions, we found two complexes forming between nuclear extracts from unstimulated HUVEC and the probes. A faster mobility

complex (marked as *) was deemed non-specific as it was formed with all probes, including a κB mutant probe. A second slower mobility complex was also formed. This complex was specific as it formed between the I probe but not the κB or mutant κB probes. Also, the I probe had higher binding activity compared to the D or the size-matched D1 probes, and the mutant M2 and M3 probes did not show binding activity. <Figure 6a>

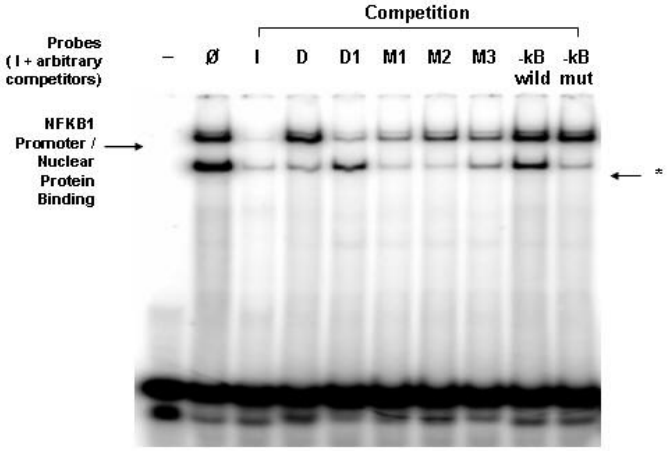
Competition assays, using excess nonradioactive oligonucleotide probes (50 times higher concentration), confirmed that the I allele forms the specific complex with higher affinity than the D allele (compared I to D and D1 competition). Oligonucleotides with the 5' ATTG (M1) abolished and a single point mutation in the 3' ATTG competed for binding, while an oligonucleotide with the 3'ATTG abolished did not compete. Overall, these results showed that a protein(s) present in HUVEC binds to the I/D region of the NFKB1 promoter, and preferentially binds to the I allele sequence. <Figure 6b>

We investigated the uncharacterized transcription factor that binds to the sequence using the Transcription Element Search Software on the World Wide Web (TESS software, University of Pennsylvania, PA). Promoter sequence analysis revealed that the TGATTGG sequence in the polymorphic region of the I allele is in complete homology with the AP-1 binding site[122]. <Figure 6c>

a)



b)



c)

Possible AP-1 binding site

I -type sequence: tccccgaccat*TGATTGG*gcccggc
D-type sequence: tccccgacc----attgGgcccggc

Consensus AP-1 binding sequence: TGA(C/G)TCA
AP-1 binding site variants : TGATTGG, TGACTCA, TGAATCA

Figure 6. I-type NFKB1 gene promoter has higher protein binding activity.
Nuclear protein extract was prepared from basal HUVECs. Olgonucleotide probes included insertion and deletion alleles and various mutations of the NFKB1 promoter polymorphic regions (I:TCCCCGACCATTGATTGGGCCCGGC, D: TCCCCGACCATTGGGCCCGGC, D1:gcgtTCCCCGACCATTGGGCCCGGC, M1: TCCC CGACCcagtATTGGGCCCGGC, M2: TCCCCGACCATTGcagtGGCCCGGC, M3:T CCCCGACCATTGAaTGGGCCCGG C) . a) Protein binding activities of the six NFKB1 promoter mimic oligonucleaotide probes. b) Protein binding activity of the I-type NFKB1 gene promoter with competitors using excess nonradioactive I-type, D-type, D1, M1, M2, M3 probes (50 times higher concentration). A wild consensus or mutant –κB binding sequence was used for a positive control. 3) the I and D allele sequence is similar to the AP-1binding element sequence. −: free nuclear protein +: nuclear protein added *: non-specific bands may result from probe-dimers or self-complementarity due to the GC rich regions at the 3' franking end of the probes

p50/p105 NFKB1 I/D promoter polymorphism regulates NFKB1 gene expression

To determine whether the NFKB1 I/D polymorphism regulates transcriptional activity of the NFKB1 promoter, we conducted reporter gene assays under lipopolysaccharide (LPS), oscillatory shear stress (OSS), and unidirectional LSS stimulations in HUVECs. In these experiments, the HUVECs were transiently transfected with either chimeric NFKB1 I- or D-type promoter-LUC reporter gene plasmid constructs 18-24 hour before the stimulation. In addition, basal cytoplasmic p50/p105 protein levels were measured in II and DD genotyped HUVECs to investigate whether the polymorphism affected protein expression levels.

Based on a previous study[91] that demonstrated different promoter activities between NFKB1 I and D type promoters in LPS-stimulated HeLa and HT-29 cell lines, the transiently transfected HUVECs were subjected to 6 or 24 hours of the LPS (500 ng/μl) treatment. Both promoters were activated with the LPS treatment by 25 to 33 fold vs. control. The I-type promoter showed significantly higher activity than D-type promoter after LPS treatment. <Figure 7a>

In the shear stress experiments, the results depended on the type of shear stress applied. The I- and D-type promoters showed similar basal activities and no significant difference under OSS (P=0.239). However, unidirectional LSS induced the D-type promoter (1.4-fold, p=0.024) and downregulated the I-type promoter below the level observed under static conditions (0.78±0.14 fold vs. I-static). <Figure 7b> In addition, the magnitude of the induction was relatively small (range 0.49 – 1.83 folds) compared to the LPS stimulation experiment (range 15 to 35 fold in 6 and 24 hour stimulations, respectively).

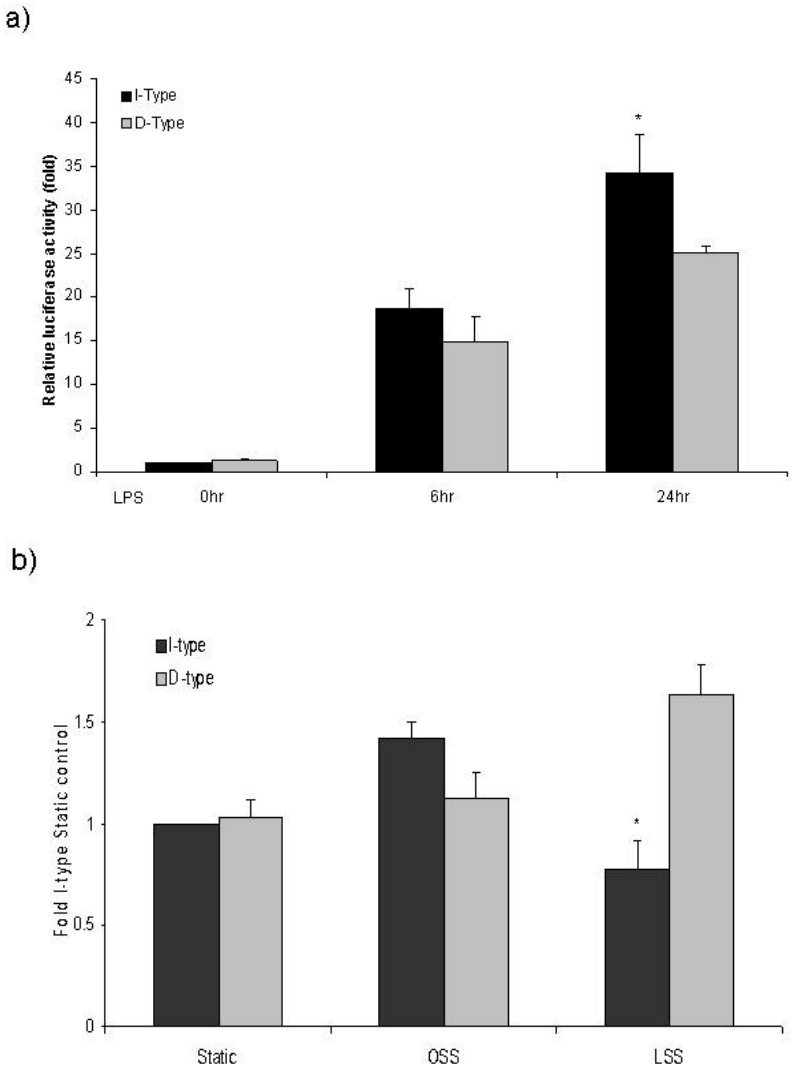


Figure 7. I-type NFKB1 promoter has significantly higher activity than the D-type promoter after LPS treatment.

NFKB1 I- or D-type promoter with luciferase reporter gene constructs were transiently transfected into HUVECs and the transactivities were investigated under the LPS and three different fluid mechanical conditions. a) Data represent the results of statistical analysis of fold increase in adjusted luciferase activity over the control (0 hr) observed at the 6 or 24 hour LPS stimulations. b) Data represent the fold increases in adjusted luciferase activity over the control (static) observed in the 24 hour OSS or LSS stimulations. Results from three independent experiments were used for each analysis. Data are presented as means with error bars representing the SEM. hr, hour; I, insertion; D, deletion; LPS, lipopolysaccharide; Static, static fluid mechanical condition; OSS, oscillatory Shear Stress; and LSS, Laminar Shear Stress *P <.05 vs. D-type

Since the above results suggested that the NFKB1 I/D promoter polymorphism affects the NFKB1 promoter activity, we investigated whether the NFKB1 polymorphism also affected protein expression levels in HUVECs. To test this, we obtained 17 primary-cultured HUVECs from different donors and identified II and DD homozygote cells. We tested the level of p50-p105 proteins in the different passage of these cells (passages 4th to 7th). Higher p50-p105 protein levels were observed in the II homozygote cells compared to the DD homozygote cells. <Figure 8> These results showed that HUVECs with the I-type promoter had higher protein expression levels of NFKB1 gene products, and ultimately, a greater number of copies of the NF-κB complex in the cytoplasm, through which signaling transduction for downstream gene expression is likely to be facilitated in these cells.

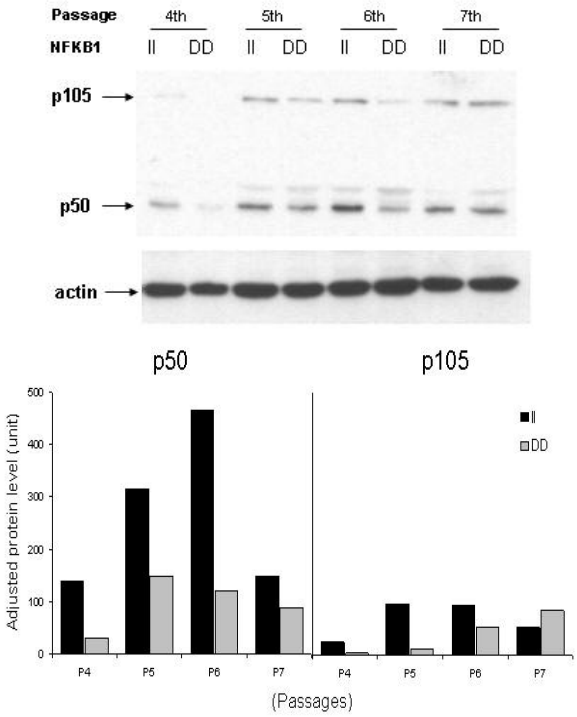


Figure 8. II homozygote cells have higher basal p50/p105 protein levels compared to the DD homozygote cells.
Cytoplasmic levels of p105/p50 subunit in HUVECs having either II or DD genotype under basal conditions. Actin used as an internal control for equal sample loadings. II: insertion/ insertion vs. DD: deletion/deletion.

The II homozygote HUVECs are more sensitive to unidirectional LSS with respect to eNOS protein expression

Previous studies have demonstrated that the NF-κB signaling pathway, generally a proinflammatory transcription factor, is responsible for upregulation of the eNOS in response to LSS.[33, 60] Furthermore, we found higher levels of NFKB1 gene products in NFKB1 I/I cells than D/D cells. Therefore, we tested whether the NFKB1 promoter polymorphism affected the level of eNOS gene expression in HUVECs in response to unidirectional LSS.

The II or DD homozygote HUVECs were subjected to 24 hours of unidirectional LSS or static conditions and total cell extracts were prepared. We excluded eNOS -786 CC homozygote cells for this experiment according to a previous report showing a lack of eNOS gene expression in response to LSS in these cells.[17] There was no difference in basal eNOS level between II or DD homozygote cells. However, there was a greater eNOS protein levels in II homozygote cells compared to the DD homozygote cells (P=0.058) <Figure 9b> These results suggest that NFKB1 II homozygote cells are more responsive to unidirectional LSS in terms of the induction of the eNOS gene expression, likely due to the increased amounts of p50 and p105 in the cells.

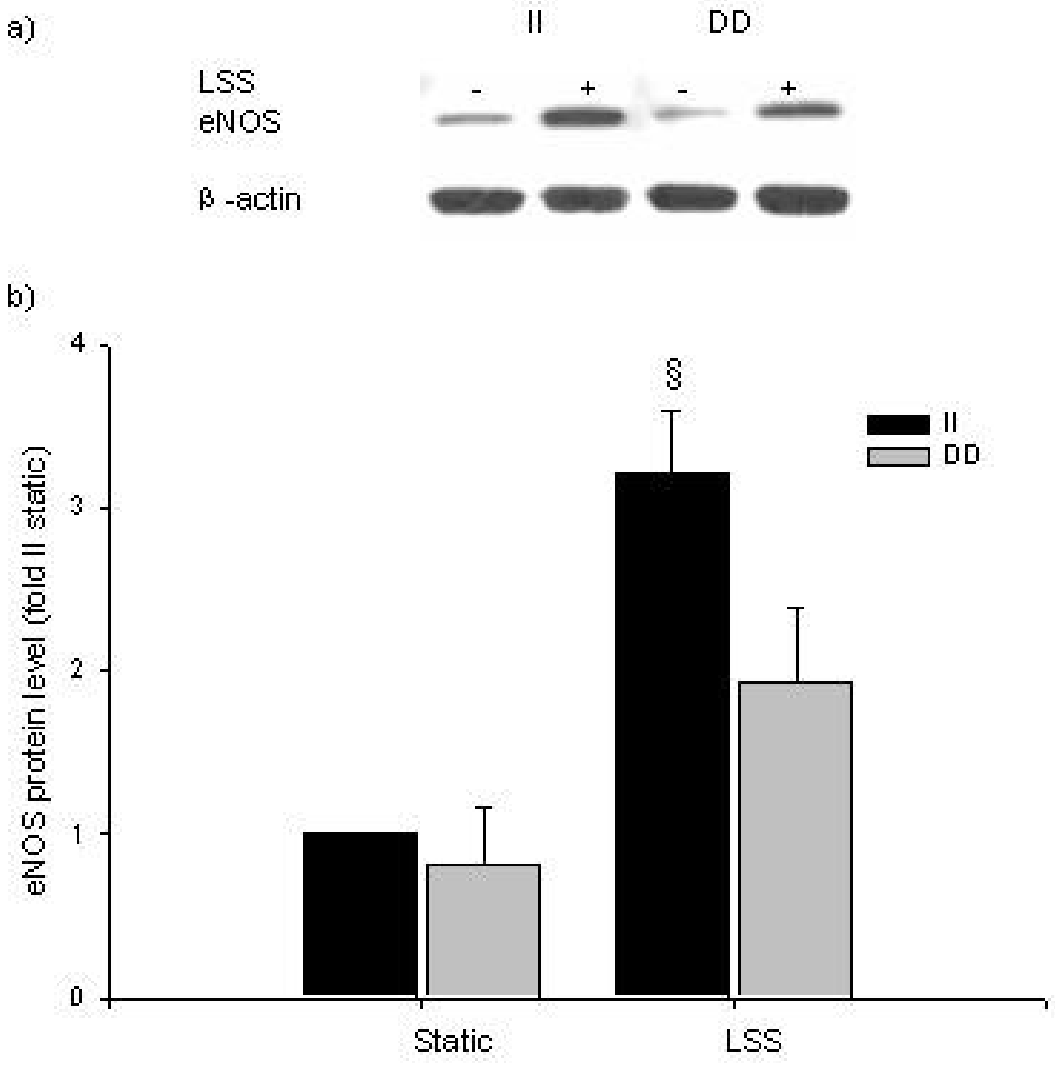


Figure 9. II homozygote cells are more responsive to unidirectional LSS in terms of the induction of eNOS gene expression.

Primary cultured HUVECs were genotyped for the NFKB1 I/D polymorphism. II and DD homozygous were used for the present experiment. The HUVECs were subjected to the unidirectinal LSS (15 dyne/cm^2) for 24 hours using a cone-plate apparatus; and eNOS protein levels were measured in total extracts. a) An example of immunoblot assay performed for eNOS protein levels in the static or 24 hour LSS stimulated II or DD genotyped HUVECs. b) Summary of statistical anaylsis (n=6) for eNOS protein levels expressed in fold increase over II homozygote cells under static conditions. All eNOS protein levels were adjusted to the actin protein levels. LSS, laminar shear stress § P=0.058 vs. DD-LSS (Mean±SEM)

The I-allele is associated with higher reactive hyperemic forearm blood flow before exercise training

To determine the association of the NFKB1 I/D polymorphism with the adaptability of endothelial function in response to exercise training, genotyped Pre- and Stage I-hypertensive subjects underwent 6 months of standardized aerobic exercise training, and reactive hyperemic FBF measurements were performed before and after the exercise training using the venous occlusion plethysmography method. Mean resting FBF was calculated from three separate measures. After 5 minutes of upper arm arterial occlusion, reactive hyperemic FBF was recorded for 7 cardiac cycles every 15 seconds during the 3 minute reactive hyperemic period. All data related to the reactive hyperemic FBF were adjusted for demographic (age, gender and ethnicity) and biologic (body weight, mean BP and BMI) confounding factors.

Subjects' general characteristics are shown in Table 1. In our study population, genotype frequencies for the NFKB1 I/D promoter polymorphism were 21.4 %, 55.4% and 23.2% for II, ID and DD, respectively. These genotype frequencies were similar to those observations of previous studies[13, 91]. The 36 samples used for the final (after exercise training) FBF analysis included the 27 subjects who completed baseline (before exercise training) FBF testing plus 9 subjects who only completed the final FBF testing.

Table 1. Characteristics of the study subjects in the human exercise training intervention study

	before (n=47)	*after (n=36)*	*Cohort (n=27)*
Gender (f/m)	24 / 23	16 / 20	12 / 15
Ethnicity (w/nw)	24 / 23	18 / 18	13 / 14
Age (yrs)	58.5 ± 0.8	58.7 ± 0.7	58.6 ± 1.3
Height (cm)	171.0 ± 1.4	170.5 ± 1.3	171.5 ± 2.0
Weight (kg)	84.4 ± 2.1	84.1 ± 1.9	84.0 ± 2.3
SBP (mmHg)	132.8 ± 1.6	132.6 ± 1.9	132.7 ± 2.4
DBP (mmHg)	87.9 ± 0.9	87.1 ± 1.0	87.3 ± 1.3
MBP (mmHg)	102.7 ± 1.1	102.1 ± 1.2	102.3 ± 1.5
BMI (kg/m^2)	29.0 ± 0.6	28.6 ± 0.7	28.7 ± 0.8
Vo_{2max} (ml/kg/min)	26.5 ± 0.6	29.8 ± 0.9	30.2 ± 1.1
Baseline FBF (ml/min/100ml)	2.1± 0.1	2.3± 0.2	2.2± 0.2
Maximum FBF (ml/min/100ml)	9.7 ± 0.6	9.9 ± 0.6	10.4 ± 0.5
Minimum FVR ((mmHg·ml/min/100ml)	11.8 ± 0.7	10.8 ± 0.7	11.4 ± 0.7
Total AUC (ml/100ml)	10.2 ± 0.5	10.9 ± 0.8	10.8 ± 0.7
NFKB1 I/D (n, %)	II: 12 (26%)	II: 8 (22%)	II: 8 (30%)
	ID: 26 (55%)	ID: 23 (64%)	ID: 18 (67%)
	DD: 9 (19%)	DD: 5 (14%)	DD: 1 (3%)

FBF data are adjusted LS mean ± SEM. All the other data are unadjusted mean ± SEM except for frequency data. before, before exercise training; after, after exercise training; cohort, subgroup used for change values; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; BMI, body mass index; Vo2max, maximal oxygen consumption; FBF, forearm blood flow; FVR, forearm vascular resistance; AUC, area under curve; f, female; m, male; w, white; nw, non-white.

Table 2 shows the adjusted LS mean ± SEM values for the resting and reactive hyperemia parameters in the NFKB1 I/D genotype groups. Before exercise training, resting FBF ranged from 0.84 to 5.61 $ml \cdot min^{-1} \cdot 100ml^{-1}$ (mean 2.06 $ml \cdot min^{-1} \cdot 100ml^{-1}$) in all subjects. After cuff deflation, FBF was dramatically increased by 443.1 % in the total population ($P<0.001$). Accordingly, the FVR was significantly decreased by 76.8 % during the time when peak reactive hyperemic FBF occurred ($P<0.001$). Similar response patterns were also observed after exercise training.

There were no age and gender main effects observed in reactive hyperemic FBF response before or after exercise training. Before exercise training, there was a tendency for a greater reactive hyperemic FBF response in II and ID genotype groups compared to the DD genotype group (genotype main effect, $P=0.122$). <Figure 10a> When the II and ID genotype groups were combined into an I-allele carrier group and compared to the non-carrier group, a significant genotype main effect was observed ($P=0.039$), and likewise, subsequent post-hoc tests revealed that the total FBF during the first minute of reactive hyperemia was significantly higher in the I-allele carrier group ($P=0.05$). <Figure 11a> After exercise training, no significant genotype main effect was observed among the three genotype groups ($P=0.275$). <Figure 10b and 11b>

In our *in vitro* experiments, the I allele of the NFKB1 I/D promoter polymorphism was associated with higher gene expression level as well as higher eNOS expression under unidirectional LSS in HUVECs. Therefore, it was hypothesized that the I allele would have higher adaptability in endothelial function in response to aerobic exercise training.

Table 2. Summary of reactive hyperemic forearm blood flow before and after exercise training

		NFKB1 -94 ins/del Genotype Group		
		II	**ID**	**DD**
Resting				
FBF ($ml \cdot min^{-1} \cdot 100ml^{-1}$)				
	before	1.85±0.24	2.20±0.16	1.91±0.28
	after	1.84±0.36	2.32±0.20	2.74±0.43
FVR ($mmHg \cdot ml \cdot min^{-1} \cdot 100ml^{-1}$)				
	before	61.2±7.40	52.6±4.82	61.4±10.89
	after	64.3±9.10	49.3±5.04	37.0±10.74
Reactive Hyperemia				
Peak FBF ($ml \cdot min^{-1} \cdot 100ml^{-1}$)				
	before	10.3±1.03**	10.2±0.67**	8.5±1.17**
	after	10.1±1.16**	10.7±0.64**	8.9±1.37**
% change in FBF				
	before	524.3±75.8	412.9±49.8	392.2±86.8
	after	569.9±107.8	413.7±59.7	250.9±127.3
Minimum FVR ($mmHg \cdot ml \cdot min^{-1} \cdot 100ml^{-1}$)				
	before	10.7±1.23**	11.5±0.80**	13.2±1.80*
	after	10.4±1.23**	10.2±0.69**	11.7±1.46**
% change in FVR				
	before	-80.4±4.63	-74.2±3.01	-75.7±6.81
	after	-79.8±4.13	-77.0±2.29	-65.2±4.87
Total AUC ($ml \cdot 100ml^{-1}$)				
	before	11.0±0.98	11.0±0.64	8.7±1.12
	after	8.9±1.45	11.8±0.80	12.1±1.71
Flow debt ($ml \cdot 100ml^{-1}$)				
	before	9.3±1.20	11.0±0.81	9.6±1.42
	after	9.2±1.80	11.6±1.00	13.7±2.13
Excess flow ($ml \cdot 100ml^{-1}$)				
	before	5.89±0.75	5.28±0.49	3.34±0.85
	after	3.87±1.00	5.35±0.55	4.55±1.18
Repayment (%)				
	before	70.6±9.54	54.4±6.39	40.5±10.9
	after	58.4±13.35	51.1±7.33	36.6±15.5
Duration (sec)				
	before	161.7±10.6	147.5±6.9	138.5±12.1
	after	131.7±12.6	163.9±7.0	139.2±14.9

Data are adjusted LS mean ± SEM. Samples sizes for the genotype groups before and after exercise training were II (n=12), ID (n=26), DD (n=9) and II (n=8), ID (n=23), DD (n=5), respectively. % change, percent change from resting. FBF, forearm blood flow; FVR, forearm vascular resistance; AUC, area under curve. *P ≤ 0.05 **P ≤ 0.01 vs. corresponding values at resting

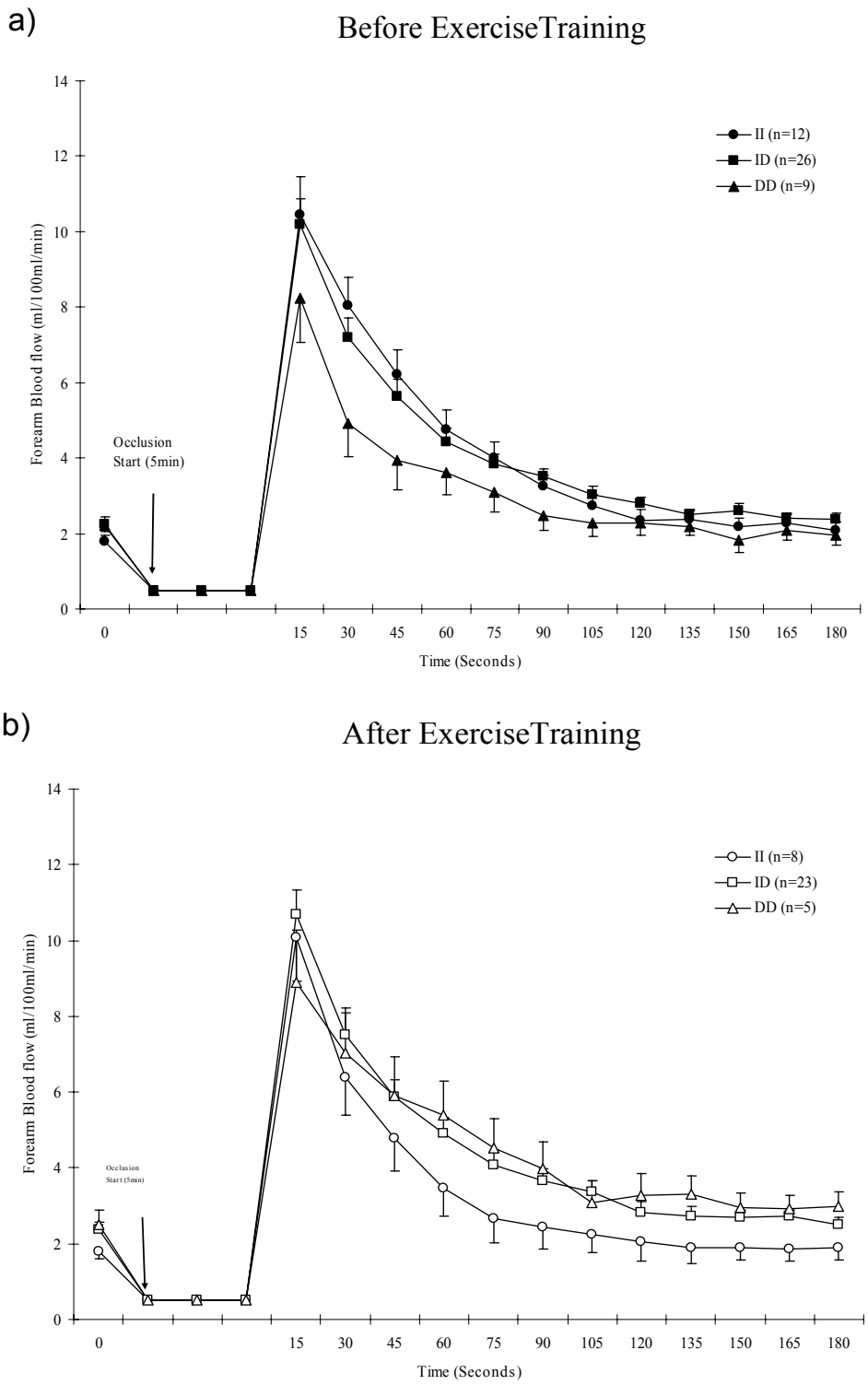


Figure 10. Difference in reactive hyperemic FBF response before and after exercise training among II, ID and DD genotype groups.
Data are adjusted LS means with error bars representing SEM. Age, gender, ethnicity, weight, mean blood pressure, body mass index were used as covariates. I: insertion, D: deletion.

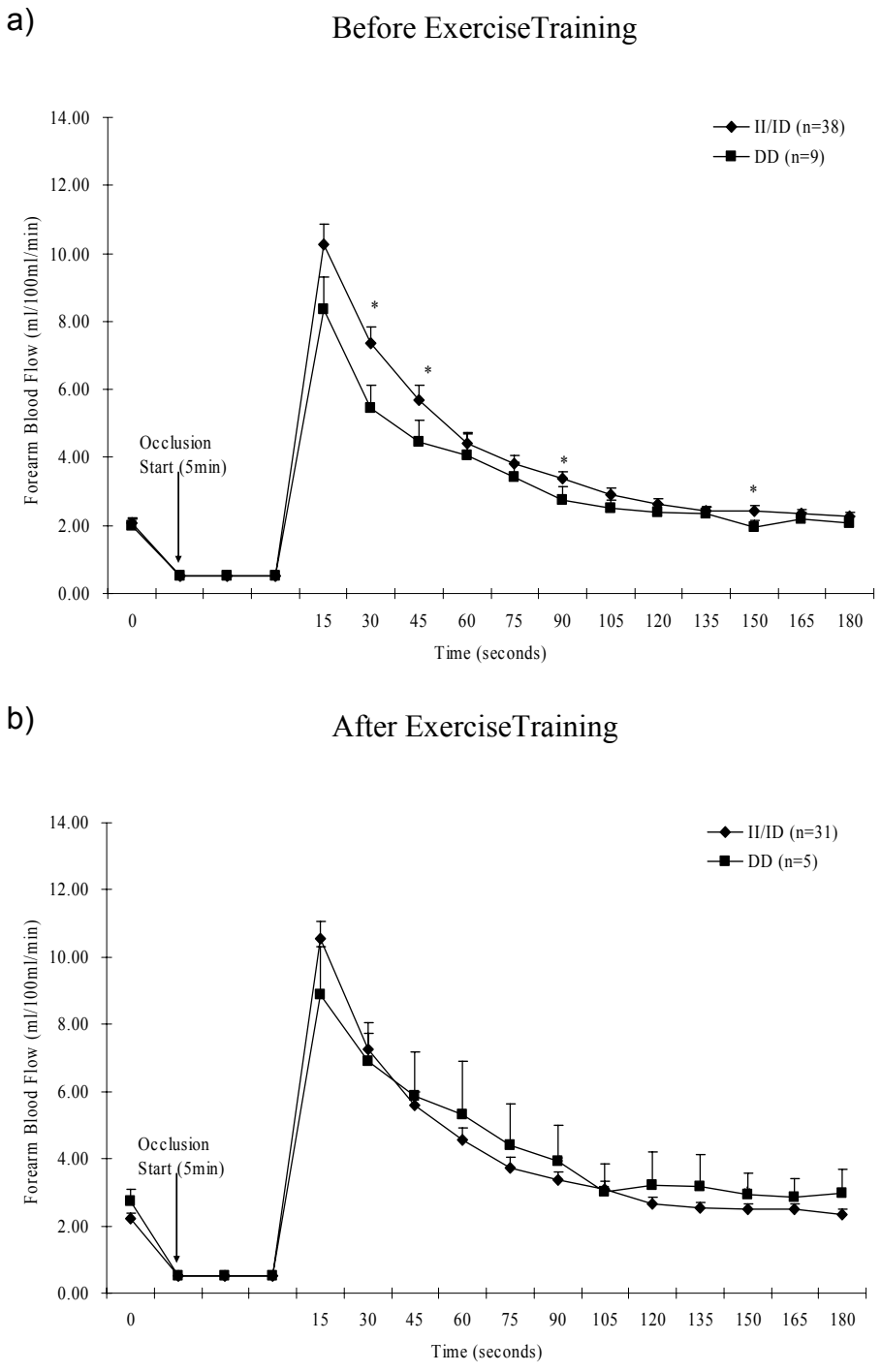


Figure 11. I-allele carrier group has higher reactive hyperemic FBF response than DD homozygote group before and after exercise training.
Data are adjusted LS means with error bars representing SEM. Age, gender, ethnicity, weight, mean blood pressure, body mass index were used as covariates. I: insertion, D: deletion.

In this analysis, subjects (n=26, 58.7 ± 1.3 yr) who completed both baseline and final FBF testing were used, and only II and ID genotype groups were compared due to the small sample size in the DD homozygote group (n=1). Change values were calculated for peak FBF, minimum FVR, and total AUC. All data related to the change value were adjusted for age, gender, ethnicity, and baseline values (peak FBF, minimum FVR, and total AUC values before exercise training). None of the initial subject characteristic values in the cohort were significantly different between the genotype groups; and VO_{2max} was significantly increased after exercise training in both genotype groups (both $P<0.001$). In the population, peak FBF, minimum FVR, and total AUC were not significantly changed after exercise training. No significant differences in the change values were observed between the genotype groups. <Table 3> In all subjects, inter-individual variation in these variables was large such that percent changes in peak FBF, minimum FVR, and total AUC ranged from -40.1 to 94.1 %, from -50.7 to 51.74 %, and from -55.9 to 150.8 %, respectively.

Table 3. Percent changes in reactive hyperemic forearm blood flow reponses after exercise training

	II	ID
%Δ FBFpeak	7.45 ± 10.05	9.89 ± 6.43
%Δ FVRmin	-2.01 ± 7.88	-6.11 ± 5.07
%Δ total AUC	-5.12 ± 15.05	12.09 ± 9.41

Data are adjusted LS mean ± SEM. For this analysis, II and ID genotype groups were compared due to lack of DD homozygote subjects (n=1). Samples sizes for the genotype groups were II (n=8) and ID (n=18), respectively. %Δ, percent change from before exercise training; FBFpeak, peak forearm blood flow; FVRmin, minimum forearm vascular resistance; AUC, area under curve.

DISCUSSION

The endothelial isoform of nitric oxide synthase (eNOS) is a vascular enzyme that plays a substantial role in maintaining endothelial function since nitric oxide (NO) plays a crucial role in the regulation of vascular tone and the function and structural integrity of the vessel wall. Nuclear factor kappa B (NF- κB) is an essential transcription factor in eNOS gene expression in the endothelial cell response to laminar shear stress (LSS). Recently, a functional promoter polymorphism has been reported in the NFKB1 gene which encodes for the p50 and p105 NF- κB isoforms[91]. However, the functional polymorphism has not been tested with respect to eNOS gene expression in endothelial cells. Therefore, in this study we investigated the functional significance of the -94 ATTG insertion (I) / deletion (D) promoter polymorphism in human vascular endothelial cells.

The major findings of the present study were that 1) a protein present in human umbilical vein endothelial cells (HUVEC) preferentially binds to the I allele sequence compared to the D allele; 2) the I allele had significantly higher promoter activity than the D allele; 3) the II homozygote cells had higher p50/p105 NFKB1 protein levels than the DD homozygote cells; 4) the II homozygote cells showed a significantly greater increase in eNOS protein levels than the DD homozygote cells under unidirectional LSS; and 5) the I-allele carrier group had a higher reactive hyperemic FBF response before exercise training. However, the NFKB1 I/D polymorphism was not significantly associated with differential changes in endothelial function following exercise training.

p50/p65 NF-κB complex activation under unidirectional laminar shear stress

Previous studies showed that shear stress increases nuclear translocation of the NF-κB, and elevates the transcriptional activity of promoters containing the -κB binding element in bovine aortic endothelial cells[92, 97, 168]. In the present study, we confirmed that in HUVECs, the p50/p65 NF-κB complex translocated into the nucleus under a physiological level of unidirectional LSS (15 dyne/cm^2) generated by a cone-and-plate apparatus. This result agrees with previous findings reported by Hay et al. [67] that p50/p65 NF-κB complex translocation occurred in response to 15 dyne/cm^2 of LSS generated by a parallel plate flow chamber system incorporated into a closed loop perfusion device.

Shear-induced NF-κB activation and its role in downstream eNOS gene expression is an extensively studied area, and some upstream signaling mechanisms have been proposed. Rapid (~5minutes) and transient activation of the inhibitor kappa B kinase alpha subunit (IKKα) has been observed in endothelial cells with a similar degree of LSS stimulation (12 dyne/cm^2) [10] to what we used (15 dyne/cm^2) in the present study, and the induction of the IKKα was prevented by treatment of c-Src and MEK 1/2 inhibitors suggesting that these signaling molecules accounted for upstream IKKα activation[33]. In addition, degradation of the inhibitor kappa B alpha (IkBα) subunit was observed at 30 minutes of unidirectional LSS. Together, the activation of the NF-κB complex depends on the upstream IKKα activation, and consequent dissociation of the IkB complex from the NF-κB complex.

Cytoplasmic p50/p105 protein and eNOS gene expression

In the present study, the most interesting findings were that the II homozygote cells

had higher cytoplasmic p50/p105 protein level than DD homozygote cells, and accordingly, that the II homozygote cells demonstrated a greater increase in eNOS protein level than DD homozygote cells after 24 hour of unidirectional LSS.

Several lines of evidence suggest that NF-κB, commonly known as a proinflammatory transcription factor, is predominantly responsible for increasing transcription of the eNOS gene in response to LSS. In endothelial cells, the activation of NF-κB plays a critical role in the intracellular signal transduction induced by flow shear stress. Resnick et al.[153] identified a *cis*-acting shear stress responsive element (SSRE, 5'-GAGACC-3') in promoter regions of shear responsive genes. Supershift assays using p50 and 65 antibodies demonstrated that the translocated NF-κB under flow shear stress binds to the SSRE in the promoter of the target genes.[67] Davis et al.[33] identified the SSRE (5'-GAGACC-3') at position -990 to -984 base pairs upstream of the transcription start site of the eNOS gene and demonstrated that the shear activated p50/p65 NF-κB bound to the identified binding sequence. Moreover, Grumbach et al.[60] showed that a NF-κB inhibitor, Panepoxydone, completely prevented the increase in eNOS mRNA level in endothelial cells exposed to the shear stress.

The present study could not provide direct evidence as to whether or not the higher eNOS gene expression in the II homozygote cells was due to the higher basal cytoplasmic p50 level. Future studies should determine whether the time course pattern of the nuclear translocation of the p50/p65 proteins levels is different between the II and the DD cells under LSS. Also, a dose-response study on the effect of the I allele on the p50 protein level should be conducted by including the ID heterozygote cells to confirm the current finding.

We also found that p105 NFKB1 protein level was also affected by the NFKB1 promoter polymorphism. Whether the upregulation of the p105 protein affected the NF-κB signaling and the downstream eNOS gene expression is not known. The p105 NF-κB protein is considered a member of IκB family because of the presence of seven ankyrin repeats in its C-terminus region[154]; on the other hand, the p105 protein is considered as a precursor protein for p50 protein. It has been demonstrated that the p105 protein is constitutively processed via proteasomes, as opposed to degradation, through a cotranslational mechanism[104]. However, the proteolysis of the precursor protein that generates p50 is limited by the presence of a glycine-rich region (GRR) between amino acids 376 and 404 that serves as a stop signal for proteolysis[105, 137]. Moreover, multiple reports have demonstrated IKKβ-dependent, and IKKα-independent, phosphorylation of p105 C-terminal serines including Ser 923 and Ser 927, followed by inducible degradation[98, 157]. However, there is no definitive evidence that this degradation of p105 has a functional role. Rather, it has been suggested that phosphorylation of the p105 results in polyubiquitination and degradation of the protein through $SCF\beta^{-TrCP}$ binding[26].

With lipopolysaccharide (LPS), a potent activator of NF-κB, we observed that the cytosolic p105 protein level was reduced early (~30 minutes) and completely disappeared with 60 minutes of treatment. Although it remains controversial whether p105 can also undergo inducible processing, the present data supports the notion that external stress (i.e. LPS) may trigger post-translational modification and inducible degradation of the p105 protein.

Protein binding activity and Promoter activity

Previous studies demonstrated that the I allele of the NFKB1 promoter showed higher protein binding activity and promoter activity than the D allele in immortalized cell lines (HT-29: colonic epithelial cells; and HeLa: cervical cancer cells)[91], and a significant association has been observed between the D allele and inflammatory bowel disease, specifically ulcerative colitis[13, 91].

In the present study, we confirmed the functional properties in primary cultured HUVECs. We observed that NFKB1 promoter-reporter gene constructs containing the ATTG insertion (I) allele showed significantly increased promoter activity in the primary cultured HUVECs following 24 hours of treatment with LPS. We further found that nuclear protein extracted from HUVECs preferentially and specifically bound to the ATTG (I) containing oligonucleotides. Conversely, the ATTG deletion (D or D1) showed weak or non-binding activity to the nuclear proteins. These results suggested that the NFKB1 I/D promoter polymorphism may affect promoter activity of the NFKB1 gene; and the specific polymorphic region may contain a core binding sequence of an unidentified nuclear protein. Future studies should address whether the up-regulation of NFKB1 promoter activity observed in I the allele was due to the nuclear protein binding to the I-type promoter and weakly to the D-type promoter.

There was no significant difference in basal promoter activities between I- and D-type promoters. This result do not agree with our finding that the II homozygote cells had higher basal p50/p105 cytoplasmic protein levels compared to the DD homozygote cells. A definitive explanation of the discrepancy could not be made by this study since it is beyond of its scope. One possible explanation is that the gene expression level was regulated by an interaction between a protein binding to the polymorphic site and other

transcription factors which bind to a cis-element which is distant from the transcription initiation site so that it was not included in the reporter vectors we used for the promoter assays.

Promoter sequence analysis revealed that the TGATTGG sequence in the polymorphic region of the I allele is in complete homology with the AP-1 binding site previously found in the polyoma virus enhancer[122]. The AP-1 binding sequence variant has yet to be reported in humans. In contrast, the AP-1 binding site was abolished in the D allele (ccATTGG). This supported the current findings of a lack of binding activity in the D allele sequence. However, this assumption could not explain the presence of binding activity in D1 and M1, which showed relatively weak binding. We speculated that the binding activity is size dependent (no protein binding occurs when probes were 21 base pairs or less); or the alteration of the sequence created novel binding sites for other nuclear proteins. Additional sequence analysis suggested that D1, 5'-gcgtTCCCCGACCATTGGGCCCGGC-3', contains potential binding sites for CCAAT-binding Protein 1 (CP1); in M1, substitution of 5' ATTG to cagt created the Sp1 binding site (TCCCCGACCc).

Endothelial function and exercise training

A healthy endothelium serves to (1) inhibit monocyte and platelet adhesion; (2) maintain a profibrinolytic and antithrombotic activity; (3) prevent vascular smooth muscle cell proliferation; and (4) modulate the vasodilatory response. All these protective functions of the endothelium are achieved by the activation of eNOS and ultimately, the release of NO. The result of the present study demonstrated that I allele was significantly

associated with a higher baseline endothelial function measured by the reactive hyperemic FBF response in the Pre or Stage I hypertensive population.

Because NFKB1 is also a strong candidate gene for numerous inflammatory diseases and the risk for immune-mediated conditions, the NFKB1 -94 ATTG I/D polymorphism has been studied in several immune diseases such as arthritis[14, 115], cancer[106], and ulcerative colitis[13, 91, 136]; and an association has been observed in some, but not all, of these studies[13, 91, 106, 115]. However, there is a paucity of association studies on the relationship between other vascular disease conditions or risk factors and shear stress responsive genes that are regulated by the NF-κB signaling pathways. Therefore, the NFKB1 I/D polymorphism should be tested in these cardiovascular pathophysiologic conditions, particularly since we have provided evidence that this polymorphism modulates the protein expression level of its gene product and the downstream eNOS gene expression levels in response to LSS, and appears to be an important risk factor for endothelial dysfunction.

After exercise training, the association between the NFKB1 polymorphism and endothelial function was no longer evident. Similar results have been observed in other studies[135], and this may be due to the downregulation of physiological phenotypes that result from the deleterious alleles and due to the other salutary effects of exercise training. This notion is conveyed by Dr. Frank Booth who states that "…disruptions in cellular homeostasis are diminished in magnitude in physically active individuals compared with sedentary individuals due to the natural selection of gene expression that supports the physically active lifestyle displayed by our ancestors."[12].

Reactive hyperemia refers to the phenomenon of increased blood flow that follows relief of ischemia and is a result of extended dilation of conduit arteries and resistance arterioles. Several factors have been implicated in the genesis of reactive hyperemia, including mechanical[15] and neurogenic[99] mechanisms, endothelium-derived nitric oxide[29, 40, 121], adenosine[15], and membrane-bound ion channels[30, 132]. Therefore, the measurement of reactive hyperemia is not solely endothelium-dependent. However, several infusion studies using an eNOS inhibitor (i.e. N-monomethyl-L-arginin, L-NMMA) or a pharmacological agonist for eNOS activator (i.e. acetylcholine, Ach) have revealed that 1) the endothelium-derived NO accounts predominantly for the reactive hyperemic vasodilatory response[29, 121]; and 2) there is a strong correlation observed between the peak reactive hyperemic FBF and the acetylcholine induced peak vasodilation ($r=0.89$, $P<0.001$)[73].

Given the rationale that; 1) flow shear stress in the endothelial cell is increased during aerobic exercise; 2) NF-κB plays a crucial role in intracellular signaling transduction for eNOS gene expression in response to flow shear stress; and 3) the NFKB1 I/D polymorphism transcriptionally regulates p50 NFKB1 gene expression, we hypothesized that the polymorphism may affect the endothelial function response to aerobic exercise training. However, we found that the NFKB1 I/D polymorphism did not associate with change values for peak FBF, minimum FVR and total AUC in response to aerobic exercise training. The change values after the standardized exercise training were highly variable among the individuals, which probably resulted from many other genetic and environmental factors. Perhaps this result might also be attributed to the small sample size and/or a weaker genetic effect.

The ability to detect interactions between genes and environmental factors is determined by the precision with which the environmental exposure and phenotype variables are measured as well as the magnitude of effect and sample size[188]. In our study, rigorously screened older subjects underwent a dietary stabilization period and a highly standardized exercise training program for six months. In addition, the interpretation of the FBF measures were performed by two technicians and cross checked while blinded to the subjects' profiles.

The primary criticism of gene association studies is a lack of reproducibility, especially when there is a lack of supporting functional data at the cellular or molecular level. It might be suggested that human blood vessels should be used. However, it is an inherent limiting factor in human research that resistance blood vessel biopsies are difficult to obtain. Moreover, even if they are obtained in studies, the vessels are obtained from subjects with significant CV disease where the samples are typically obtained during surgical treatment (i.e. angioplasty or coronary artery bypass graft).

The present approach was considered to be the best way to provide functional data for a gene polymorphism related to exercise training-mediated vascular adaptations. Thus, the current complementary study design may provide a major breakthrough approach to study exercise science and vascular research. Furthermore, this study is an example of a translational physiology study in vascular biology and exercise physiology research.

Summary

The present study identified a significant association between NFKB1 -94 ATTG deletion (D) allele and decreased reactive hyperemic FBF response before exercise

training, which was consistent with the effect of NFKB1 on downstream eNOS gene expression found in the HUVECs. These results have potential clinical implications for endothelial dysfunction that are related to the development and progression of atherosclerosis and cardiovascular diseases. Since more than 20% of the population is homozygous for the D allele, genotyping of this polymorphism may provide an option for more effective health management by increasing the diagnostic capacity and improving the conventional risk factor profile (i.e. endothelial function) in affected individuals. Additionally, our findings provide insight into the molecular mechanisms involved in the intracellular signaling transduction process of eNOS gene expression and function of the NFKB1 gene promoter region.

CONCLUSIONS

Hypothesis #1: The NFKB1 I/D promoter polymorphism has functional genetic properties that respond to *in vitro* unidirectional LSS in HUVECs.

Nuclear translocation of p50/p65 NF-κB complex was observed in response to the unidirectional LSS. The NFKB1 I/D promoter polymorphism showed functional genetic properties that responded to unidirectional LSS such that: 1) NFKB1 promoter-reporter gene constructs that contained the ATTG insertion (I) allele showed significantly increased promoter activity in the primary cultured HUVECs; 2) accordingly, the nuclear protein extracted from HUVECs bound preferentially and specifically to the ATTG (I) containing oligonucleotides; 3) the II homozygote cells had higher p50/p105 NFKB1 protein levels than the DD homozygote cells; and 4) the II homozygote cells showed a significantly greater increase in eNOS protein levels than the DD homozygote cells under the unidirectional LSS.

Hypothesis #2: The NFKB1 I/D polymorphism is associated with differential changes in endothelial function following exercise training.

Although the I-allele carrier group had higher reactive hyperemic FBF response before exercise training, which was consistent with the molecular assay data, the NFKB1 I/D polymorphism was not significantly associated with the differential changes in endothelial function following exercise training.

Endothelium-Dependent Vasodilation, Nitric Oxide, and Hypertension

Endothelial dysfunction

Over the last two decades, vascular endothelial function has been emerged as a strong marker for monitoring cardiovascular (CV) health on the basis that impairment of the endothelial function is the earliest event in the process of developing atherosclerosis. Endothelial dysfunction has been frequently associated with increased levels of all common CV risk factors predisposing to atherosclerosis, such as age[19, 37, 174, 190], obesity[170], post-menopause[173], hypertension[108, 143-145, 176], hypercholesterolemia[16, 28, 69], diabetes[119, 120, 185], smoking[18, 69], and physical inactivity[48]. Moreover, prognostic implication of the endothelial dysfunction was also reported in various diseases states such that impaired endothelial function predicts future cardiovascular events (i.e. cardiac death, unstable angina, myocardial infarction, coronary bypass grafting, ischemic stroke, or peripheral artery revascularization) in the population experiencing coronary artery disease[62, 68, 161, 171], chest pain[129], hypertension[148], and noncardiac vascular complications[55]. Besides to be considered as a useful biological marker, studies have demonstrated that the impaired endothelial cell function contributes to the manifestation of atherogenic lesions, vasoconstriction, vasospasm[116], plaque rupture, intimal growth, and, in turn, coronary and cerebrovascular events.

In the presence of the endothelial dysfunction, improvement in the endothelial function was observed with various pharmacological treatments, which are generally related to lower the CV risk factors, such as lipid-lowering drugs (i.e. statins[86]), weight-

control pills (i.e. Orlistat[9]), anti-hypertensives (i.e. angiotensin converting enzyme inhibitor[77] or angiotensin II receptor blocker), and hormone replacement therapy[75, 158], and also was evident with nutritional and behavioral modifications such as weight loss[43, 159], low-fat diet, supplementary[41] or natural antioxidant intake, smoking cessation, and physical activity[48, 56, 102, 172].

Independent of reducing CV risk factors, the underlying mechanisms, by which those pharmacological and nonparmacological treatments enhanced protective function of the endothelium, also include enhancing anti-atherogenic gene expressions and vascular redox state, and reducing systemic and local oxidative stress, and, the most importantly, increase nitric oxide (NO), a key endothelium-derived relaxing factor.

Endothelial vasodilatory function

Endothelial vasodilatory dysfunction presents early in the pathogenesis of vascular disease, and contributes to the manifestation of atherogenic lesions, vasospasm, plaque rupture, intimal growth, and, in turn, coronary and cerebrovascular events. Moreover, forearm endothelial vasodilator dysfunction has been shown to be a marker of future cardiovascular events. Thus, a better understanding of the mechanisms responsible for the loss in endothelial vasodilator function associated with insulin resistance may lead to new targets for therapeutic intervention.

Reactive hyperemia refers to the phenomenon of increased blood flow that follows relief of ischemia and is a result of extended dilation of conduit arteries and resistance arterioles. Several factors have been implicated in the genesis of reactive hyperemia,

including mechanical[15] and neurogenic[99] mechanisms, endothelium-derived nitric oxide[29, 40, 121], adenosine[15], and membrane-bound ion channels[30, 132].

Muscle contraction during physical exercise increases local muscle blood flow by metabolic vasodilation of feeding and resistance arteries. To determine mechanism underlying the immediately increased blood flow, studies used venous occlusion plethysmography technique before, immediately after and recovery of a hand grip exercise session. Nitric oxide contributes to the resting blood flow (vascular tone)[39, 167, 187] as well as blood flow during recovery period[167]; however, nitric oxide may not play a significant role in the increased exercising muscle blood flow[39, 167, 187].

Peripheral Resistance and Blood Pressure regulation

Pressure required to deliver blood through the circulatory bed is provided by the pumping action of the heart and the tone of arteries. This oversimplified mechanism can be illustrated by a basic equation: Blood Pressure (BP) = Cardiac Output (CO) X Peripheral Resistance (PR). Each of these primary determinants is, in turn, determined by exceedingly complex series of factors. Hypertension has been ascribed to abnormality of virtually all of the factors which induces increased CO and/or PR. (Kaplan, 2002: *'Clinical Hypertension'* textbook)

The increased CO could logically arise in two ways: either from an increase in fluid volume or from an increase in contractility from an overactivity of sympathetic nerve system. However, increased CO has been shown only in young patients with secondary and does not persist in established hypertension in middle aged to older hypertensives.

The typical hemodynamic finding in the established hypertension is an elevated PR and normal CO.[27]

According to Poiseuille's law, vascular resistance is positively related to both the viscosity of blood and the length of blood vessel, and negatively related to the third power of the lumen radius. Because neither viscosity nor length is much, if at all, altered, and because small changes in the luminal radius can have a major effect, it is apparent that the increase vascular resistance seen in established hypertension reflects changes in the caliber of the small resistance arteries and arterioles and ultimately increase PR.[46] Maintenance of primary hypertension is mainly due to decreased lumen size or radius. The persistently elevated PR, a hemodynamic hallmark of primary hypertension, may be reached through a number of different paths. These may converge into either structural hypertrophy of the vessel wall and functional vasoconstriction.

Nitric Oxide

Nitric Oxide (NO) is the endothelium-derived relaxation factor. In 1980, Furchgott and Zawadzki[50] showed that the normal relaxing response to acetylcholine in vessels constricted by norepinephrine was abolished if the endothelial lining was rubbed off, depriving the cells of an endothelial-derived relaxing factor. Seven years later, Palmer et al.[142] identified the endothelium-derived relaxing factor as NO, now known to be the primary endogenous vasodilator. Nitric oxide is produced in the process convert L-arginine to L-citruline, which catalyzed by the dioxygenase, NO synthase.[140] The synthesis of NO is controlled by endothelial NO synthase availability and is induced by calcium-mobilizing agents and fluid shear stress.[58] The intravascular half-life of NO is

approximately 2 msec, but its extravascular half-life is up to 2 sec, depending on tissue oxygen concentration.[179] Basal NO release is stimulated by several mediators, such as acetylcholine, bradykinin, and substance P, and by mechanical stimuli, such as shear stress[50, 84]. NO also has preferred characteristics as a signal messenger: fast, because it is not stored in vesicles; short lived, easily permeable through and between cells; and economically produced from an abundant and recyclable substrate.[111] Basal generation of NO keeps the arterial circulation in an actively dilated state.[182] When the inhibitor of NO synthesis, N-monomethyl-L-arginine, is infused, BP rises.[152] Under basal conditions, whole-body NO production is diminished in patients with essential hypertension, and impaired NO release may play a role in hypertension and atherosclerosis.[47]

NO bioactivity and Oxidative Stress

The term oxidative stress implies a state in which cells are exposure to high concentrations of oxygen molecule or chemical derivatives of oxygen, called reactive oxygen species. In the normal metabolic process, oxygen undergoes a series of univalent reduction leading to producing superoxide anion, hydrogen peroxide and water.

Reactive Oxygen Species (ROS) plays several important role in cellular level including mediating signaling transduction, regulating gene expression, inducing lipid peroxidation, and modulating extracellular matrix remodeling.[44] The most particular importance to hypertension and vascular biology is the interaction between superoxide and NO. Because superoxide and NO are radicals that contain unpaired electrons in their outer orbits, they can undergo extremely rapid, diffusion-limited radical-radical reactions with rates similar to those of superoxide and superoxide dismutases (SODs). These

nonenzymatic reactions can be 10,000 times faster than reactions between superoxide and common "scavenger" antioxidants such as vitamins A, E, and C.

A major product of reaction between superoxide and NO is peroxynitrite anion (OONO-). Peroxynitrite is a weaker vasodilator compared to NO, and thus this reaction markedly impairs the vasodilator capacity of NO. Many of the beneficial effect of NO (inhibition of platelet aggregation, vascular smooth muscle cell growth, monocyte adhesion, etc.) are lost when OONO- is present. The rapidity of the reactions between superoxide and NO, and superoxide and SODs suggests that their interactions could alter the amounts or activities of available superoxide, SOD, or NO. Indeed this seems to be the case. In the normal vessel, the balance between NO and superoxide favors the net production of NO, permits a state of basal vasodilatation, and the maintenance of normal BP.[66] The critical balance of superoxide and NO is altered in the setting of numerous common disease states. These include atherosclerosis, hypertension, diabetes, cigarette smoking, and aging. Treatment of vessels or animals with ecSOD markedly improves endothelium dependent vascular relaxation.[1] Likewise, infusion of antioxidant vitamins transiently improves endothelium dependent vasodilatation as has been shown in forearm vessels in human subjects with diabetes.[180]

The nitric oxide synthases can produce large amount of superoxide when deprived of their critical cofactor (tetrahydrobiopterin) or their substrate (L-arginine). In this state, referred to as NOS uncoupling, electron flow through the enzyme results in reduction of molecular oxygen to form superoxide rather than formation of NO. In aortas of mice with DOCA-salt hypertension, superoxide production from NO synthase is markedly increased and tetrahydrobiopterin oxidation is evident. In this model, initial production of ROS

from the NADPH oxidase leads to oxidation of tetrahydrobiopterin, uncoupling of eNOS, decreased NO production and increased superoxide production from endothelial NO synthase. Treatment of mice with oral tetrahydrobiopterin reduces vascular superoxide production, increases NO production, and blunts the increase in BP.

Endothelial Dysfunction and Hypertension

Due to its critical role in vasodilation of NO, testing of vasodilatory capacity using various methods including plethysmography, brachial artery ultrasound scanning, and quantitative coronary angiography combined with infusion of combinations of NO stimulant (Acetylcholine) and other dilator without stimulating NO synthesis (Nitroprusside) or NO blocker (L-NMMA) is often used to investigate endothelium-(in)dependent vasodilation.[183] Whereas increased pressure responsiveness was examined frequently in the past, most recent work has measured the impairment of vasodilation in response to reactive hyperemia or NO-mediated forearm vasodilation.[107]

Hypertensives have been shown to have an impaired vasodilatory capacity in brachial arteries,[145] coronary arteries,[38] renal circulation,[74] and small arteries and arterioles.[103] For example, the impaired vasodilation was seen in the responses (usually measured as forearm blood flow) to NO stimulants (usually acetylcholine) but not to endothelium-independent vasodilators such as nitroprusside. The impairment also demonstrated by ultrasonography in response to reactive hyperemia.[85] The impairment may reflect more than just reduced synthesis of NO but is not related to decreased availability of particular substrate, L-arginine.[145] This evidence for a role of defective NO-mediated vasodilation in the pathogenesis of hypertension has been further

strengthened by its recognition in the still-normotensive children of hypertensive parents.[117, 175] Oxidative stress and subsequent breakdown of NO may be responsible for much of the endothelial dysfunction of hypertension.[65]

Endothelial Vasodilatory Function and Exercise Training

There are several studies showing effects of exercise training on vascular NO function in healthy individuals. Studies have typically examined basal release of NO inferred from the effects of NOS inhibition and/or NO released in response to agonist such as bradykinin, acetylcholine or methacholine. Green et al.[59] demonstrated that four weeks of handgrip exercise training increased peak vasodilatory capacity after ischemic stimulus but vasodilatory responses to methacholine and sodium nitroprusside (endothelium-independent vasodilator) was not altered. In contrast, improved NO productions, measured by L-NMMA infusion[94] and plasma NOx level,[112] with 4-8 weeks of aerobic exercise training have been shown in young healthy subjects and this effect persists during few weeks of detraining. Goto et al.[57] tested effects of different intensity of exercise training (12 weeks at mild (25%)-, moderate (60%)-, and high (75%)-intensity) on FBF and showed that acetylcholine induced FBF was significantly increased in the moderate intensity group but not in the mild- or high- intensity groups. Miyachi et al.[125] reported that blood flow in the ascending aorta during exercise after 8 weeks of aerobic training was significantly larger than that found before training.

The effects of exercise training on endothelial-dependent vasodilation can be characterized by that it is a systemic response by increased vascular shear stress as a result of elevation in blood flow, heart rate, pulse pressure, and blood viscosity, and that

endothelium-dependent dilation may be altered persistently in the period between exercise bout and account, at least in part, for the reduced total peripheral resistance and BP.[93] Furthermore, long-term exercise training enhances the endothelium-dependent vasodilation reserve. For example, in athletes,[94] the basal NO production was not significantly different compared with sedentary individuals, where forearm vascular resistance decreased greater after acetylcholine administration.

Exercise Training has been also shown augment endothelial vasodilatory capacity in hypertensive patients. Higashi et al.[78] tested endothelial-dependent (reactive hyperemia) and endothelial-independent vasodilation (sublingual nitroglycerine) before and after 12wks of exercise training in patients with mild- to moderate- hypertension. After 12 wks of exercise training, maximal FBF response during reactive hyperemia response to ischemic stimulus increased where changes in FBF after sublingual nitroglycerin administration were similar before and after 12 wks of exercise training. By the same research group,[76] it has been proven that the effect of exercise training on augmented vasodilatory capacity in hypertensive patients was attributed to endothelial-dependent vasodilatory mechanism by acetylcholine and L-NMMA infusion study.

Physical activity reduces morbidity and mortality from cardiovascular diseases[114, 139] although the precise mechanisms responsible for this salutary effect have not been fully uncovered. Studies showed that aerobic exercise improves endothelial function at all ages, at all stage of cardiovascular disease. Regular physical training improves peripheral endothelium-dependent vasodilation in patients with chronic heart failure[82] and coronary artery disease[64].

Nuclear Factor kappa B and Signal Transduction

Nuclear factor kappa B (NF-κB) is a ubiquitous dimeric transcription factor which involves in various expressions of genes that related to inflammation, cell adhesion, cell division, apoptosis, and stress response. Acute exercise induces NF-κB activation through the mitogen-activated protein kinase (MAPK)-mediated signaling pathway including p38 and extracellular signal-regulated kinase (ERK).[33] In endothelial cells, NF-κB pathway is involved in transducing an extracellular signal, which associated with mechanical force mediated by flow LSS, into nucleus for eNOS gene expression.

NF-κB Transcription Factor Family and Its Activation

NF-κB involves in intracellular signaling cascade of various gene expressions. NF-κB family includes p50/NFKB1, p52/NFKB2, p65/RelA, RelB, and c-Rel. In biological systems, these family members presents and acts as homo- or hetero-dimers. NF-κB is frequently observed as a form of a p50/p65 heterodimer, encoded by the genes NFKB1 and NFKB2, respectively.[20]

In a quiescent cell, NF-κB complex is retained in the cytoplasm by binding to a family of protein inhibitor, IκBs.[7] The formation of the NF-κB/IκB complex masks a critical nuclear localization domain on a conserved N-terminus region of NF-κB proteins, called the Rel homology domain, and this prevents translocation of NF-κB dimers into the nucleus. The IκB family includes IκBα, IκBβ, IκBε, and Bcl 3. Each subunit contains multiple copies of an amino acid sequence known as the ankyrin repeat.

Once the cell is stimulated by appropriate stimuli, such as cytokines, immunoglobulins, UV light, lipopolysaccharide, oxidative stress and other stresses, IκB

kinase (IKK) phosphorylates IκB complex at Ser32 and Ser36 on IκBα subunit followed by ubiquitination and proteosome-dependent degradation of the IκB complex.[2, 6] Dissociation of IκB from NF-κB results in exposing a nuclear localizing domain of NF-κB complex so that the NF-κB complex is translocated into nucleus and bind to κB-binding site of a target gene to initiate gene transcription. Activated NF-κB complex translocated into the nucleus and binds DNA at κB-binding motifs such as 5'-GGGRNNYYCC-3' or 5'-HGGARNYYCC-3' (where H is A,C, or T; R is an A or G purine; and Y is a C or T pyrimidine).[52] Then, the activated NF--κB is down-regulated through multiple mechanisms including the well-characterized feedback pathway whereby newly synthesized IκB α protein binds to nuclear NF--κB and exports it out to the cytosol.

IκB Kinase: Upstream Signaling Molecule for NF-κB Activation

In the process of activation of NF-κB complex, IKK plays a critical role so that the upstream signaling transduction pathway has been given an increasing attention. IKK comprises three subunits: IKKα, IKKβ, and IKKγ. IKKα/IKK1 and IKKβ/IKK2 are catalytic, while the third, called IKKγ or NF-κB essential modulator (NEMO) is constitutive. IKK1 and IKK2 are substrates for members of the MAP kinase kinase kinase family. Both NF-κB inducing kinase (NIK) and MAPK/extracellular signal-regulated kinase kinase 1 (MEKK1) can activate IKK1 and IKK2 by phosphorylating specific serine residues.[52, 138] NIK binds to the adaptor protein TNF-receptor-associated factor 2 (TRAF2) and preferentially phosphorylates IKK1, while MEKK1 activated

IKK2.[128] IKKs may be also downstream targets for TAK1, PKB, PKC (ζ and θ), and Akt.[36, 138]

Ozes et al.[138] showed that AKT1 is involved in the activation of NF-κB by TNF-alpha, following the activation of phosphatidylinositol 3-kinase (PIK3). The Akt1-induced NFKB1 activation is mediated by phosphorylation of IκBα at threonine 23, which can be blocked by dominant-negative NIK. Conversely, NIK-induced activation of NFKB1 is mediated by phosphorylation of IKK1 at serine 176, which can be blocked by an AKT1 mutant lacking kinase activity. Considering these results, it is indicated that both AKT1 and NIK are necessary for the activation of NFKB1 by TNF-alpha through phosphorylation of IKK1. Platelet-derived growth factor promotes cellular proliferation and inhibits apoptosis. Romashkova[156] showed that PDGF activates the RAS/PIK3/AKT1/IKK/NFKB1 pathway. In this pathway, NFKB1 induces putative antiapoptotic genes. Zhong et al. [192] demonstrated that transcriptionally inactive nuclear NFKB in resting cells consists of homodimers of either p65 and p50 complexes with the histone deacetylase HDAC1. Only the p50-HDAC1 complexes bound to DNA and suppresses NFKB-dependent gene expression in unstimulated cells.

NF-κB, Mechanotransduction and eNOS Gene Expression

In endothelial cells, activation of NF-κB plays a critical role in the intracellular signaling transduction induced by flow shear stress. The term for this process is mechanotransduction. LSS stimulates mechanoreceptors on vascular endothelial cell membrane and alters the expression levels of several genes related to endothelial function. Rensnick et al.[153] identified a *cis*-acting shear stress responsive element (SSRE)

in promoter regions of the flow-sensitive PDGF-β chain gene and it was later shown to act as a non-consensus binding site for NF-κB [92].

Hay et al. [67] found that shear stress induces gene transactivation by NF-κB p50/p65 heterodimer through proteosome-dependent degradation of $I\kappa B_\alpha$ and $I\kappa B\beta_1$ in endothelial cells. Some components of the signaling pathway involved in the activation of NF-κB by flow have been identified in bovine aortic endothelial cells. Bhullar et al. [10] showed that degradation of IκBs by activated IKKs are critical steps in the process. The study also demonstrated that induction of IKK 1 / 2 activity by shear stress is inhibited by pretreating cells with a inhibitor of the $\alpha_v\beta_3$ integrin, pointing to a role for this integrin in the mechanotransduction process.

Davis et al.[33] indicated that LSS increases eNOS gene expression by NF-κB p50/p65 heterodimer binding to the GAGACC sequence present in the human eNOS promoter. This study also showed that exposure of endothelial cells to LSS resulted in IKK phosphorylation via MEK1/2 and tyrosine kinase c-Src signaling molecules which are upstream of NF-κB activation.[32]

Therefore, while NF-κB activation is generally viewed as a pro-inflammatory stimulus, these observations indicate that its transient activation by vascular shear stress may increase the expression of eNOS, which will increase the bioactivity of NO and could convey vasodilatory characteristics. Taken together, NF-κB activation in endothelial cells may be an important intracellular step in improving vasodilatory capacity with exercise training, by inducing gene expression of eNOS.

NF-κB and Acute Exercise

There are several papers providing evidence for an involvement of NF-κB activation in exercise stimulated intracellular signaling transduction. Ji et al.[89] reported that one hour of treadmill running increase NF-κB binding activity and p50 protein content in rat muscle nuclear extracts. This study also measured IκB pathway from cytosolic extracts and found that $I\kappa B_\alpha$ and IKK contents were decreased, whereas phospho- $I\kappa B_\alpha$ and phospho-IKK contents were increased in exercise group compared to control.

Ho et al.[80] reported that IKK 1 / 2 phosphorylation and NF-κB activity increased in muscle tissue throughout the time course (5-60 min) and 1-3 hours following the exercise. With the contraction of isolated muscles, additional kinases were activated, which include p38 and extracellular-signal regulated protein kinase (ERK). Using inhibitor assays, this study also confirmed that p38 and ERK are involved in the upstream signaling pathway to activate the NF-κB in muscle after exercise. Although it has been clearly demonstrated that fluid shear stress induces NF-κB activation[67], none of studies are yet to report whether NF-κB is also activated and participated in the signaling cascade triggered by exercise in vasculature.

NFKB1 Gene Polymorphisms

The human NFKB1 gene, localized to 4q23-q24, encodes two proteins, a 105 kilodalton (kDa) non DNA-binding cytoplasmic molecule (p105) and a 50 kDa DNA-binding protein (p50) that corresponds to the N terminus of p105 [72]. Recently, a functional promoter polymorphism in the NFKB1 gene that affects NFKB1 transcription has been described[91]. The NFKB1 -94 ATTG insertion (I)/deletion (D) (NFKB1 I/D) polymorphism is a common functional promoter polymorphism. The genotype

frequencies of the NFKB1 I and the NFKB1 D are 61% and 39%, respectively, in the normal population. The polymorphism is located between its transcription factors binding sites, AP-1 and κB, in the promoter region. In immune cells, a luciferase reporter gene analysis showed that the NFKB1 I allele had a two-fold higher NFKB1 gene promoter activity than the NFKB1 D allele[91]. However, it has not been tested whether the NFKB1 I/D polymorphism is a functional in endothelial cells.

Laminar Shear Stress and Endothelial Function

At the cellular level, exercise acts as a physiological stressor that disturbs resting homeostasis. Endurance aerobic exercise can be translated into several distinct, but integrated stimuli which include oxidative stress, hypoxic stress, and mechanical stress through muscle contraction and increased vascular wall shear force. Among them, in the vasculature, the exercise stimulus can be defined as endothelial wall shear stress.

Vascular Endothelial Cell

Most of the various humoral and mechanical factors that control vascular function and structure work through the endothelium, the source of a host of mediators that maintain a balance of opposing physiologic and pathologic effects. The endothelium is the guardian of the vessel wall, providing an antithrombotic non-sticky surface for blood flow, synthesizing molecules that regulate vessel tone and diameter, and allowing nutrients to pass from the blood to the underlying smooth muscle.

Vascular endothelial cell, located at the interface between the circulating blood and the blood vessel, are exposed to shear stress in any moment resulting form the tangential

force exerted by the flowing fluid on the vessel wall. The magnitude and pattern of the shear stress acting on endothelial cells depend on blood flow, blood viscosity, and the vascular geometry, which varies with the location in the vascular tree.

Within the vascular tree, there are gradients of shear stress that typically occur near vessel bifurcations or branches, in the regions of arterial narrowing or in the areas of extreme curvature. These anatomical regions are traditionally known as proatherogenic.[113] Direct models of the low or oscillatory shear stress regions have revealed shear values on the order of +/- 5 dyne/cm^2 (1 dyne = 10 μN/cm^2) compared with greater than 10 dyne/cm^2 in the atheroprotective or LSS regions.[90]

Vascular Shear Stress: Different Patterns

The magnitude of the shear stress can be estimated by Poiseuille's law, which states that shear force is proportional to blood flow and viscosity, and inversely proportional to the third power of the internal radius.

Endothelial surface of blood vessel is constantly exposed to hemodynamic shear stress. Measurements using different methods show that the shear stress ranges from 1 to 6 dyne/cm2 in the venous system and between 10 and 70 dyne/cm2 in the arterial vascular network.[113] Within the vascular tree, there are gradients of shear stress that typically occur near vessel bifurcations or branches, in the regions of arterial narrowing or in the areas of extreme curvature such as carotid bulb. The complex flow pattern is associated with low/oscillatory shear stress (+/- 5 dyne/cm^2) that exhibit in predilection for atherosclerosis lesions. In contrast, the straight parts of the arterial tree, which are generally spared from atherosclerosis, blood flow is more laminar (>15 dyne/cm^2).[90]

Based on *in vitro* analysis, under high fluid shear stresses (>15 dyne/cm2) vascular endothelial cells enter a quiescent, anti-proliferative, antioxidant, and anti-thrombotic state which is reflected by the downregulation of a number of atherogenic factors.[113] For example, high fluid shear stress results in the downregulation of vascular cell adhesion molecule 1 (VCAM-1) upregulation of antioxidant genes (SOD1, SOD2, and GPX-1), downregulation of vasoconstrictive factors (ET-1, ACE) and upregulation of vasodilatory factors (eNOS and COX-2). In contrast, endothelial cells exposed to low or oscillatory shear stress are thought to enter a procoagulant and prothrombotic state. Specifically, such condition have been shown to cause the upregulation of ET-1, endothelin converting enzyme (ECE), angiotensin converting enzyme (ACE), and PDGF α and β.[118]

Laminar Shear Stress during Exercise

A bout of endurance exercise increases heart rate, pulse pressure, and blood flow in circulatory system. These changes in systemic hemodynamic components can be translated into elevated the pulsatile stretch and shear stress on endothelium. Shear stress is a tangential force exerted at a vector that is parallel to the long axis of the vessel (i.e. 15 dyne/cm^2), where pulsatile stretch is fluctuation of arterial pressure acting on perpendicular to the longitudinal axis of vessel (i.e. 15±10 dyne/cm^2).[70] The magnitude of shear stress can be modified by typical physiological conditions that affect increase in blood flow or flow pulsatility such as reactive hyperemia and exercise. For example, during ischemic stimulus-induced reactive hyperemia, peak wall shear stress was up to 40 dyne/cm^2 in brachial arteries.[54] During exercise, magnitude of shear stress in large conductance arteries are 12 dyne/cm^2 (7 dyne/cm^2 during rest) and 16 dyne/cm^2 (12

dyne/cm^2 during rest) at the supraceliac level and the infrarenal level, respectively.[22] It is not surprise result of which the increment is not quiet large in aortas because its naturally bigger radius. We can carefully scrutinize that the increased shear stress during a bout of exercise in small and resistance arteries may be bigger scale than those numbers even taking in count of the capacity of vasodilatory response.

The effect of exercise in the properties of shear stress can be found not only in the magnitude but also in the pattern of flow. Cheng et al.[23, 24] showed that, using magnetic resonance imaging, that flow and shear oscillations present at rest were eliminated during exercise. These result agreed with previous observations of which great reduction in flow reversal and retrograde during exercise.[127, 147] In addition, Taylor et al.[178] measured hemodynamic conditions in the abdominal aorta using a three dimensional computer model of a healthy human aorta, and concluded that moderate levels of lower limb exercise can eliminate the flow reversal and regions of low shear stress that exist under resting conditions.

Laminar Shear Stress and Mechanotransduction

The ways by which shear stress is translated into various endothelial responses involved multiple mediators. The shear stress has short- and long- term effects on vascular tone through various pathways including ion-channel and receptor mediated signal transduction. The mechanosensors on the endothelium that sense changes in shear stress are poorly defined at the molecular level, but at the cellular level a timescale of cell-signaling pathways has been carefully described. The endothelium response to shear stress probably achieved by calcium influx. These changes in intracellular calcium

concentration drive changes in potassium channel activation, the generation of inositol triphosphate and changes in G-protein activation to inform the cellsignaling cascade within endothelial cell. These signaling cascades within the endothelial cell are activated within a period of several minutes up to one hour and include activation of the mitogen-activated protein kinase (MAP kinase) signaling cascade and the translocation of the nuclear factor – kappa B (NF-κB) from the cytosol in to the nucleus. These signal transduction cascade induce downstream gene expression.[87]

Some of these gene have a particular consensus of nucleotides in the 5' region of the gene, which is known as the shear stress responsive element (SSRE). Mutation of this limited cassette of bases can result in the loss of sensitivity of gene expression in response to shear stress. The mechanism by which flow augments the expression of eNOS involves SSRE within the promoter region of the gene. The consensus sequence for the SSRE in the eNOS promoter is GAGACC, a putative transcription factor binding site that is common to the promoter regions of many endothelial genes that are responsive to shear stress including tissue factor, intercellular adhesion molecule (ICAM), Transforming growth factor - beta1(TGF-β), platelet derived growth factor – beta(PDGF-β), and endothelin-1(ET-1).[153]

eNOS gene expression and Exercise Training

A number of studies support the hypothesis that increases in blood flow associated with each bout of exercise generate a shear stress signal for vascular adaptation. Long-term changes in flow (like an exercise) exert their effects on endothelium-dependent vasodilation by modulating the expression of eNOS. The expression of mRNA for eNOS

synthase is unregulated in cultured endothelial cells exposed to LSS.[34, 189] Similarly, the magnitude and nature of shear stress have a major impact on endothelial cell NOS gene expression. Noris et al.[133], in cultured HUVECs, demonstrated that LSS dose dependently up-regulate eNOS gene expression, showing that turbulent shear stress has no effect on the NOS expression.

Delp et al.[34] demonstrated that increases in blood flow caused by exercise increased eNOS protein contents and endothelium-dependent vasodilation.

Laughlin et al.[101] showed that exercise training induce increase eNOS protein level in non-uniform manner throughout the coronary artery in pig. The increase was observed in small artery (300-1000 μm), in resistance artery (150-300 μm), and all sizes of arterioles (large: 100-150 μm; intermediate: 50-100 μm; and small: >50 μm) but not in conduit artery (2-3 mm). This result supports their previous findings which exercise training improved endothelium dependent vasodilatory capacity in small artery but not in conduit artery.

Genetics in Endothelial Vasodilatory Function

Heritability in Endothelium Dependent Vasodilation

Benjamin et al.[8] estimate heritability of flow mediated dilation from the Framingham Offspring Study. In the study, endothelial vasodilatory function was measured by the flow mediated vasodilation during reactive hyperemia using a ultrasound in 2883 Framingham Heart Study participants. Arterial blood flow was interrupted for 5 minutes by cuff placed on the proximal forearm by inflating up to 200 mm Hg or 50 mm Hg above individual systolic BP. Percent and actual flow mediated dilation (%FMD and

FMD) were assessed between 55 and 65 seconds after cuff deflation. The study showed that the heritability of FMD was 12% to 14% suggesting the modest contribution of genetic factors to the variability in endothelial vasodilatory function.

Even though only one study is available to date for the estimated heritability of endothelial vasodilatory function, genetic contribution to endothelial vasodilatory function has been supported by earlier studies demonstrating that young individuals with a family history of CVD have diminished endothelial function.

Clarkson et al.[25] studied endothelial vasodilatory function in 50 offspring of patients with premature coronary artery disease (CAD). FMD was measured by brachial artery diameter using ultrasound in response to reactive hyperemia, and to sublingual nitroglycerine. In the family history group, FMD during reactive hyperemia was impaired whereas FMD after nitroglycerine administration was comparable with control groups. These result suggested that reduced FMD was due to endothelial dysfunction.

Schachinger et al.[160] tested coronary blood flow response to acetylcholine (endothelial- dependent) and to papaverine or adenosine (endothelial-independent) in 150 patients having chest pain or PTCA of single vessel disease. The study demonstrated that coronary blood flow responses to acethylcholine were reduced in a dose-dependent manner in patients with a positive family history. Also, it is evident by multivariate analysis that the positive family history was a predictor of coronary blood flow increase to acetylcholine along with hypercholesterolemia and age.

A study from Gaeta et al.,[51] investigated whether changes in brachial-artery reactivity measured by FMD during reactive hyperemia are present in adolescents and young adults with a parental history of premature myocardial infarction. In this study, 80

healthy young offspring of parents with(n=40) and without(n=40) a history of premature myocardial infarction underwent the measurement of the brachial-artery vasodilatory response after 4.5 minutes of arterial occlusion. The group of offspring of parents with premature myocardial infarction had lower FMD than the control groups.

Gene Association Studies and Endothelial Vasodilatory Function

According to the Pub-Med search results, 17 articles, to date, have been published, which investigating associations of specific gene polymorphism with endothelial vasodilatory function. The studies included 12 polymorphisms from 10 candidate genes. The genes tested for the association were endothelial nitric oxide synthase(eNOS)[95, 181], angiotensin converting enzyme(ACE),[4, 146] angiotensinogen(AGT)[96], angiotensin II receptor type 1(AT1R)[96], aldosterone synthase (Cyp11B2), G-protein β3(GNB3)[124], neuropeptide Y(NPY)[88], β2-adrenergic receptor (β2AR)[81], p22phox and paraoxonase-1 (PON1)[177] genes. Among the 12 polymorphisms, 7 polymorphisms have been reported to be associated with a measurement of endothelial vasodilatory function. The associated polymorphisms include eNOS 4a/b, ACE I/D, AGT T174M, AT1R A1166C, NPY Leu7Pro, β2AR Gly16Arg and PON1 Q192R. In contrast, 3 polymorphisms in each of eNOS, AGT, and CYP11B2 genes have been shown no associations with the endothelial function, whereas 2 polymorphisms, GNB3 C825T and p22phox C242T, have been demonstrated inconsistent results.

In the studies, endothelial vasodilatory functions were measured mostly in the brachial arteries. However, some of the studies measured the vasodilatation in hand veins, lower limb, or coronary arteries. Lack of a uniformed standard method to measure

the endothelial vasodilatory function can be a problem to integrate the results from different studies. To measure endothelium-dependent vasodilation, flow mediated dilation during reactive hyperemia and intravenous infusion of acetylcholine are most frequently used methods, where, in some other studies, bradykinin, metacholine, calcium ionophore, paraverine, and adenosine were used. For endothelium-independent vasodilation, nitroglycerine (NTG) or sodium nitroprusside (SNP), and glyceryl trinitrate (GTN) were used.

APPENDIX A –Limitations of the Study

Delimitations

1. Subjects were participants in a large exercise intervention study, the Gene Exercise Research Study, which investigate the genetic influence on changes in BP with endurance exercise training.
2. Subjects were sedentary (aerobic exercise <2 times/wk, <20 min/session, sedentary job); 50 - 75 yrs of age; nonsmoker, nondiabetic; not on lipid-lowering medications; and no evidence of chronic obstructive pulmonary disease.
3. Subjects had Pre or Stage I Hypertension (systolic BP: 120-159; diastolic BP: 80-99 without medication)
4. Subjects were recruited from the Washington D.C. metropolitan area surrounding the University of Maryland, College Park, MD through advertisements and public service announcements.
5. Endothelial function was measured by venous occlusion plethysmography method during reactive hyperemia.
6. All women were postmenopausal for more than past 2 years. Those who were using or not using hormone replacement therapy (HRT status) were instructed to continue their HRT status throughout the study period.
7. Human Umbilical Vein Endothelial Cells were obtained from the Emory Skin Diseases Research Center, Department of Dermatology, Emory University School of Medicine

8. Unidirectional shear stress experiment was conducted at the Vascular Mechanics Laboratory, Department of Cardiology, Emory University School of Medicine using custom designed and hydrodynamically evaluated cone-and-plate apparatus.

Limitations

1. The concept that one gene will contribute substantially to changes in vasodilatory capacity response to exercise training was an oversimplification. However, the rationale for selecting the candidate gene was (1) both exercise and LSS activate NF-κB, (2) both exercise training and LSS induce eNOS gene expression, (3) NF-κB pathway is important to induce the eNOS gene expression inresponse to LSS, and (4) there is strong evidence that the NFKB1 I/D promoter polymorphism transcriptionally regulates the NF-κB level.
2. Exercise training produced other stimuli on endothelial cells and improves endothelial function through other mechanisms. This study focused on LSS as an important stimulus on endothelial cells during exercise.
3. Specific aim 1.4. (Determine whether the NFKB1 I/D promoter polymorphism affects intracellular levels of eNOS protein under unidirectional LSS in HUVECs) was addressed by using HUVECs with different genotypes (II and DD). 17 HUVEC cell lines from different people were genotyped to collect for HUVECs with the specific genotypes. It assumed that the collected HUVEC cells were not identical related to their other genetic factors. However, the HUVECs were also genotyped for a recently reported functional eNOS polymorphism (eNOS T-786C) to prevent possible confounding effects of the polymorphism on current findings. Although genetically identical human endothelial cell line are commercially available, using primary cultured HUVECs met the aim of the current study design of comparing HUVECs with different genotypes.

4. The present study did not include a control group. For subjects who fall into the Stage 1 Hypertension categories, lifestyle modifications, including increasing physical activity, were recommended (JNC VII). The recommendation for physical activity was at least 30 min per day, most days of the week. Subjects in the present study underwent a 6 mo exercise training intervention. Since there was no possible alternative treatment as part of this study, then to include a hypertensive group and not treat them would have been unethical.
5. Mixed ethnic population was used to improve a statistical power in this study.

APPENDIX B – Definition of terms

Endurance aerobic exercise training: A systemic physical conditioning designed to enhance circulatory and respiratory efficiency that involves vigorous sustained exercise.

Signal transduction: The cascade of processes by which an extracellular signal interacts with a receptor at the cell surface, causing a change in the level of a second messenger and ultimately effects a change in the cell's functioning (for example, inducing gene expression).

Mechanotransduction: The process by which cells convert mechanical stimuli into a chemical response. It can occur in both cells specialized for sensing mechanical cues such as mechanoreceptors, and in parenchymal cells whose primary function is not mechanosensory.

Genetic polymorphism: The occurrence in a population (or among populations) of several phenotypic forms associated with alleles of one gene or homologs of one chromosome.

Genome: The entire genetic content contained in a haploid set of chromosomes in eukaryotes

Gene polymorphism: A DNA sequence variation, occurring when a single or a set of nucleotides in the genome is altered. A variation must occur in at least 1% of the population to be considered a common gene polymorphism.

The insertion/deletion polymorphism: A gene polymorphism that arises by insertion or deletion of one or more nucleotides into a DNA sequence.

Endothelium: The endothelium is the layer of thin, flat cells, that lines the interior surface of blood vessels, forming an interface between circulating blood in the lumen and the rest of the vessel wall. Endothelial cells, a type of epithelial cells, line the entire circulatory system, from the heart to the smallest capillary.

Shear stress: The components of stress at a point that act parallel to the plane in which they lie. In the vascular system, shear stress refers to a tangential force exerted at a vector that is parallel to the long axis of the vessel.

Laminar shear stress: The shear stress induced by laminar flow which is nonturbulent flow of a viscous fluid in layers near a boundary.

Flow shear stress: The blood flow-mediated friction force acting on the interface between the circulating blood and the endothelium of the vessel wall. In the vessel tree, flow shear stress exists in exceedingly complex manner with combination of laminar and oscillatory shear stress which are mediated by laminar and turbulent flow, respectively.

Unidirectional laminar shear stress: The laminar shear stress continuously exerted one direction only. This type of shear stress can be simulated in an *in vitro* system using cone-and-plate apparatus.

Dyne: The unit to express a level of shear stress. A centimeter-gram-second unit of force, equal to the force required to impart an acceleration of one centimeter per second per second to a mass of one gram. (1 dyne = 10 $\mu N/cm^2$)

Endothelial function: Broadly, the function of endothelium in vascular tone regulation, immune cell filtration, angiogenesis and preventing atherosclerosis. Endothelial dysfunction, or the loss of proper endothelial function, is a hallmark for vascular

diseases, and often leads to atherosclerosis. In vascular biology, endothelial function often refers to the endothelium-dependent vasodilatory function which is mediated by nitric oxide production and its bioactivity. Forearm blood flow and flow-mediated dilation measured by plethysmography and ultrasound during reactive hyperemia are widely used to measure the endothelial function.

Reactive hyperemia: Hyperemia after a transient ischemic period.

Signal profiling: Monitoring the binding of transcription factors to enhance elements and screen for the induction of key signaling pathways.

Protein expression: The process by which a gene's information is converted into the protein structures.

Promoter: A DNA molecule to which RNA polymerase and other transcription factors bind, initiating a gene to be transcribed

Transient transfection: Temporary introducing recombinant DNA into eukaryotic cells, such as endothelial cells.

APPENDIX C – Institutional Review Board Approvals for Human Subjects & Recombinant DNA Experiments

UNIVERSITY OF MARYLAND
INSTITUTIONAL REVIEW BOARD

2100 Lee Building
College Park, Maryland 20742-5121
301.405.4212 TEL 301.314.1475 FAX

Reference: IRB HSR Identification Number 04-0319

June 21, 2004

MEMORANDUM

Notice of Results of Final Review by IRB on HSR Application

TO: Dr. Michael Brown
Joon Y. Park
Department of Kinesiology

FROM: Dr. Phylis Moser-Veillon, Co-Chairperson
Dr. Marc Rogers, Co-Chairperson
Institutional Review Board

PROJECT ENTITLED:

"Functional Analysis of NFKB1 Gene Variation in Hypertensives: Exercise Training Intervention and Human Endothelial Cell Shear Studies"

The Institutional Review Board (IRB) concurs with the departmental Human Subjects Review Committee's (HSRC's) preliminary review of the application concerning the above referenced project. The IRB has approved the application and the research involving human subjects described therein. We ask that any future communications with our office regarding this research reference the IRB HSR identification number indicated above.

We also ask that you not make any changes to the approved protocol without first notifying and obtaining the approval of the IRB. Also, please report any deviations from the approved protocol to the Chairperson of your departmental HSRC. If you have any questions or concerns, please do not hesitate to contact us at irb@deans.umd.edu. Thank you.

ADDITIONAL INFORMATION REGARDING IRB/HSRC APPROVALS

EXPIRATION OF IRB APPROVAL—Approval of non-exempt projects expires one year after the official date of IRB approval; approval of exempt projects expires three years after that date. If you expect to be collecting or analyzing data after the expiration of IRB approval, please contact the HSRC Chairperson in your department about submitting a renewal application. **(PLEASE NOTE: If you are not collecting data from human subjects and any on-going data analysis does not increase the risk to subjects, a renewal application would not be necessary.)**

STUDENT RESEARCHERS — Un less otherwise requested, the IRB will send copies of approval paperwork to the supervising faculty researcher (or advisor) of a project. We ask that such persons pass on that paperwork or a copy to any student researchers working on that project. That paperwork may be needed by students in order to apply for graduation. **PLEASE BE ADVISED THAT THE IRB MAY NOT BE ABLE TO PROVIDE COPIES OF THAT PAPERWORK, particularly if several years have passed since the date of the original approval.**

Enclosures (where appropriate), will include stamped copy of informed consent forms included in application and any copies of the application not needed by the IRB; copies of this memorandum and any consent forms to be sent to the Chairperson of the Human Subjects Review Committee

IND.

IRB-1 – Page 1 of 4

MAP.

UNIVERSITY OF MARYLAND, COLLEGE PARK
INSTITUTIONAL REVIEW BOARD
APPLICATION FOR INITIAL REVIEW OF RESEARCH USING HUMAN SUBJECTS

Name of Principal Investigator or Faculty Advisor: Michael Brown — Tel. No. 301-405-2483
(NOT a student or fellow; must be UMD employee)

Name of Co-Investigator: _____ — Tel. No. _____
(NOT a student or fellow)

E-Mail Address of P.I. mdbrown@umd.edu — E-Mail Address of Co-P.I. _____

Where should IRB send approval letter? Joon Y. Park, Dept. of Kinesiology, HHP Bldg, Rm. 2126

Student Investigator Name & E-mail Address: Joon Y. Park, joonpark@umd.edu
(Student, Fellow, Post-Doctoral Fellow)

Student Identification No. 212 - 41 - 1937 Check here if student dissertation research project: ☒

Department: Kinesiology — Project Duration (mm/yyyy – mm/yyyy): 07/01/04 - 06/30/06

Project Title: Functional Analysis of NFKB1 Gene Variation in Hypertensives: Exercise Training Intervention and Human Endothelial Cell Shear Studies

Funding Agency: American Heart Association — UM Proposal # (s): _____

CONFLICT OF INTEREST: Investigators ☐ do ☒ do not have a real or potential conflict of interest. See question #7, Page 2.
MEMBERS OF HEALTH CENTER: Investigators ☐ are ☒ are not members of Health Center. See question #8, Page 2.

Please attach a copy of your responses to items 1 – 8 of the instructions (on page 2 of this document), including all related documents, such as questionnaires, interview questions, surveys, etc.

OPTIONAL: Please indicate whether this research should be exempt or non-exempt from further human subjects review and indicate which of the six exemption reasons (described on page 3 of this document) justifies an exemption status:

☒ Exempt (list all possible categories) #4 ☐ Non-Exempt

If exempt, please briefly describe the reason (s) for exemption. Your notation is simply a suggestion to the IRB.

6-18-04 — Michael D. Brown
Date — Principal Investigator/Faculty Advisor's signature

_____ — _____
Date — Co-Investigator's signature

6/19/04 — Joon Y. Park
Date — Student Investigator's signature

6-19-04 — Marc A. Rogers
Date

REQUIRED SIGNATURE: Must be signed on line above by either Unit Head (Chairperson or Director) or Designee or Departmental Human Subjects Review Committee Chair.
Please print name and title on line below.

PLEASE ATTACH THIS COVER PAGE TO EACH SET OF COPIES.
SEND THREE (3) COPIES WITH ONE CONTAINING ORIGINAL SIGNATURES
To inquire about the status of applications, post e-mails to irb@deans.umd.edu

IRB-1 11/2003

UNIVERSITY OF MARYLAND
DEPARTMENT OF ENVIRONMENTAL SAFETY

3115 Chesapeake Building 338
College Park, Maryland 20742-3133
301.405.3960 TEL 301.314.9294 FAX

12 April 2006

MEMORANDUM

TO: Joon Young Park
Department of Kinesiology

FROM: Janet Peterson
Biological Safety Officer

SUBJECT: *NFKB1 Gene Promoter polymorphism and Unidirectional Laminar Shear Stress: Implications for NF-κB activation, eNOS Protein Expression and Endothelial Function*

The experiments in your dissertation involving recombinant DNA (cloning the promoter-exon 1 region of the human NFKB1 gene into the pGL3-Basic firefly luciferase expression vector propagated in *E. coli* K12) are exempt from the requirements of the *NIH Guidelines for Research Involving Recombinant DNA Molecules* according to section III-F-6. Therefore they do not require registration and review by the University of Maryland Institutional Biosafety Committee.

Please don't hesitate to contact me at peterson@umd.edu if you have further questions.

CONSENT TO ACT AS A SUBJECT IN AN EXPERIMENTAL STUDY

Project Title: **ACE genotype, blood pressure, and exercise training in hypertensives**

I state that I am over 18 years of age and wish to participate in a program of research being conducted by Dr. James Hagberg in the Department of Kinesiology, University of Maryland.

The purpose of this study is to determine the role that genetics may play in determining how my blood pressure changes with exercise training. This research project will require visits to University of Maryland College Park and the Baltimore VA Medical Center. The specific tests, their requirements, and time commitments are described below.

I already completed a telephone interview that determined that I am not physically active, 50 - 75 years of age, not a diabetic, have no evidence of lung disease, have an appropriate body weight for my height, and have no other medical problems that would keep me from exercising vigorously. It is also probable that I have a blood pressure that is in the Prehypertensive or Stage 1 hypertension range (Systolic blood pressure: 120 – 159; Diastolic blood pressure: 80 – 99). Furthermore, if I am a woman, I must be postmenopausal, defined as no menstrual cycles for at least the last 2 years. I understand that if I am a woman and change my hormone replacement therapy regimen during the study, my participation in the study will be terminated.

The Flow Chart on the next page indicates the different testing sessions and time required by this study. I understand that if I qualify and complete this study my total involvement will last approximately 9 months. Two of the testing visits are performed at the Baltimore VA Medical Center. I understand that I will also be asked to sign a University of Maryland Baltimore consent form for the tests conducted in Baltimore.

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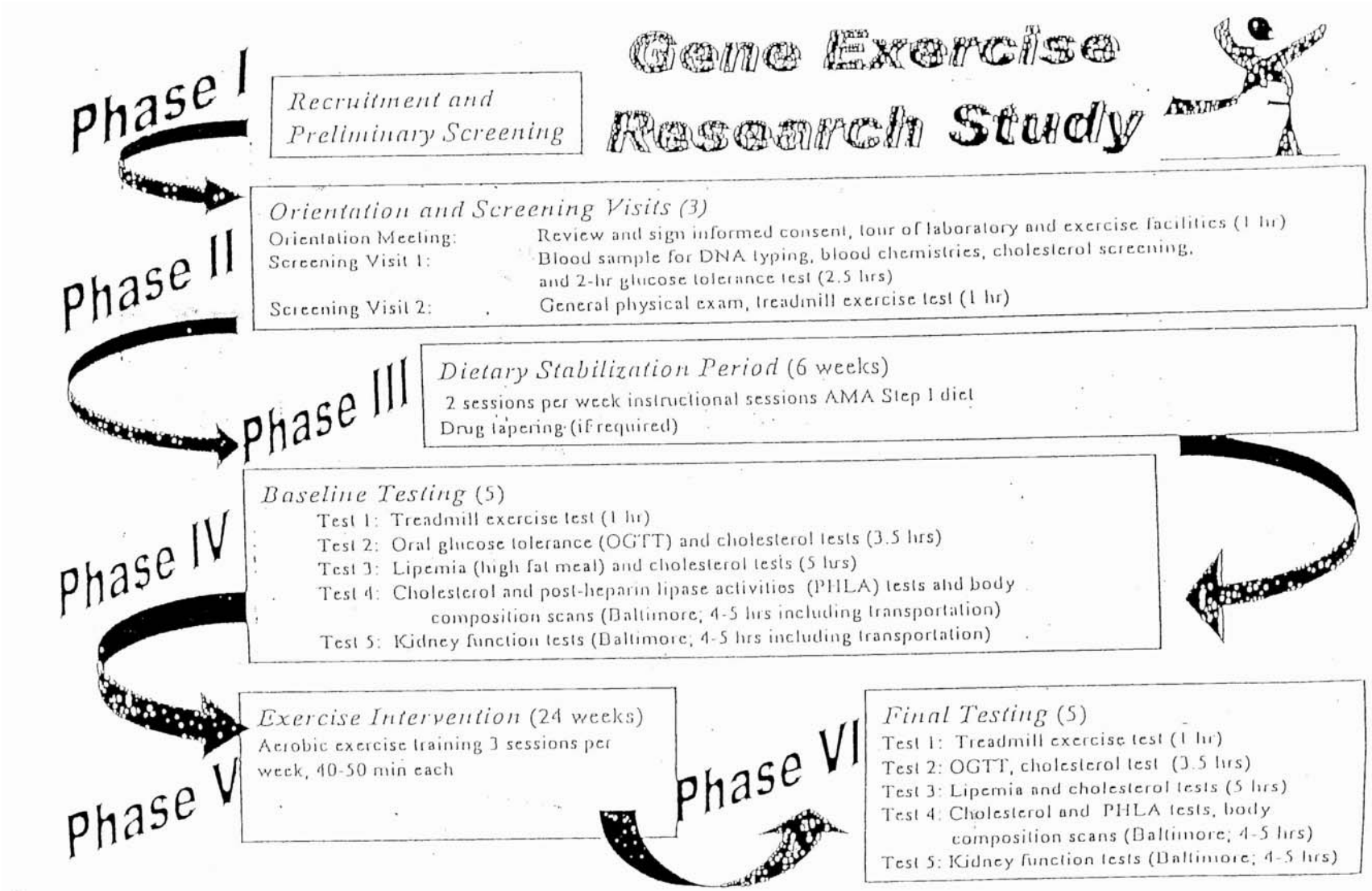
Gene Exercise Research Study
Phase I
Recruitment and Preliminary Screening
Phase II
Orientation and Screening Visits (3)
Orientation Meeting: Review and sign informed consent, tour of laboratory and exercise facilities (1 hr)
Screening Visit 1: Blood sample for DNA typing, blood chemistries, cholesterol screening, and 2-hr glucose tolerance test (2.5 hrs)
Screening Visit 2: General physical exam, treadmill exercise test (1 hr)
Phase III
Dietary Stabilization Period (6 weeks)
2 sessions per week instructional sessions AMA Step 1 diet
Drug tapering (if required)
Phase IV
Baseline Testing (5)
Test 1: Treadmill exercise test (1 hr)
Test 2: Oral glucose tolerance (OGTT) and cholesterol tests (3.5 hrs)
Test 3: Lipemia (high fat meal) and cholesterol tests (5 hrs)
Test 4: Cholesterol and post-heparin lipase activities (PHLA) tests and body composition scans (Baltimore; 4-5 hrs including transportation)
Test 5: Kidney function tests (Baltimore; 4-5 hrs including transportation)
Phase V
Exercise Intervention (24 weeks)
Aerobic exercise training 3 sessions per week, 40-50 min each
Phase VI
Final Testing (5)
Test 1: Treadmill exercise test (1 hr)
Test 2: OGTT, cholesterol test (3.5 hrs)
Test 3: Lipemia and cholesterol tests (5 hrs)
Test 4: Cholesterol and PHLA tests, body composition scans (Baltimore; 4-5 hrs)
Test 5: Kidney function tests (Baltimore; 4-5 hrs)

I understand that I will complete 1 Orientation and 2 initial screening visits. For my first visit, the study will be explained to me, my medical history will be reviewed, and I will provide my written informed consent. This visit will last about 60 minutes. On my first screening visit, I will report to the laboratory in the morning after an overnight fast and a blood sample will be drawn for blood chemistries and for isolation of my DNA. I will have my height, weight, and blood pressure measured. I understand that I may be excluded from the study if this initial blood sample shows elevated levels of glucose or creatinine in my blood. A blood sample will also be drawn 2 hours after I drink a sugar solution. This visit will last about 2 ½ hours. I understand that a total of 3 tablespoons of blood will be drawn during this visit. I understand that I will be excluded from the study at this point if I have a low red blood cell count, evidence of kidney or liver disease, evidence of diabetes, or if my blood pressure is too high or too low.

I understand that if I remain qualified to this point, I will undergo a treadmill exercise test to determine if I have heart disease. A physical examination will precede the exercise test. I will have my resting blood pressure measured. I will then complete a test on an exercise treadmill where the treadmill speed and grade will increase every 3 minutes until I cannot continue or symptoms of heart disease develop. Blood pressure, heart rate, and electrocardiogram (electrical activity of my heart) will be recorded before, during, and after the test. During this test I will have a noseclip on my nose and I will breathe through a mouthpiece so that the air that I breathe out can be analyzed. I understand that this visit will last about 1 hour and that I will be excluded from the study at this point if I have evidence of heart disease.

I understand that if I meet these requirements to enter the study and if I am taking medications to control my blood pressure, I give my permission for my private physician to be contacted to obtain their approval for me to stop taking these medications for the remainder of this study. I understand that I will be slowly withdrawn from these medications according to the plan my physician provides and that my blood pressure will be measured weekly for the remainder of the study. I also understand that if my blood pressure is too high (Systolic blood pressure: >159; Diastolic blood pressure: >99) for three consecutive weeks at any time during the study, I will be excluded from further participation in the study and referred back to my physician. If I am in the exercise training portion of the study, I understand that if this happens I will complete all Final Testing (see below) and then be referred back to my physician. I also understand that a physician from the University of Maryland School of Medicine is directly involved in this study and that he can be contacted for any medical questions, but only as they concern my involvement in this study.

I understand that if I meet these requirements to enter the study, I will undergo 6 weeks of instruction in the principles of an American Heart Association low-fat and low salt diet and must follow this diet for the remainder of this study. This program consists of two 40 minute classes each week for the 6 week duration of the program. During the final 3 weeks of this dietary program, I understand that I will have my blood pressure measured weekly for 3 weeks. I understand that my blood pressure must average in the range of 120 – 159 for systolic or 80 – 99 for diastolic blood pressure for me to continue in the study.

Initials _____

After this I will undergo Baseline Testing that includes the following tests that will be completed in 7 testing sessions (5 at the University of Maryland, College Park and 2 at the Baltimore VA Medical Center). I will have blood drawn on 2 occasions from a vein in my arm in the morning after an overnight fast to measure my cholesterol levels; these visits will each last about 20 minutes. I understand that a maximum of 2 tablespoons of blood will be drawn during these visits. I understand that I will also undergo a second exercise test on a treadmill to measure my cardiovascular fitness. This test will start at 70% of the highest heart rate achieved on my first exercise test and the treadmill grade will increase by 2% every 2 minutes. Blood pressure, heart rate, and electrocardiogram will be monitored before, during, and after the test. The test will be stopped when I can no longer continue. During this test I will have a noseclip on my nose and I will breathe through a mouthpiece so that the air that I breathe out can be analyzed. I understand that this visit will last about 1 hour. I also understand that my dietary habits will be measured by having me record for 7 days all of the food items that I eat. I understand that I will collect my urine for 24 hours in a container that must be refrigerated so that the amount of salt I eat in my diet can be measured; I also understand that my blood pressure will be monitored throughout this 24 hour period with a cuff around my upper arm and a "Walkman-size" controller worn at my waist. I also understand that I will undergo a 3 hour glucose tolerance test where I will come to the laboratory in the morning after an overnight fast, have a small catheter inserted in an arm vein for blood sampling, and have blood samples drawn before and for every 30 minutes after I drink a glucose solution. Additional samples will be drawn before this test to measure hormone levels in my blood that affect my blood pressure, immunological (disease-fighting), and blood clotting systems. I understand that a maximum of 7 tablespoons of blood will be drawn for this portion of the study. I understand that on another occasion after an overnight fast, I will have blood samples drawn before and every 30 minutes for 4 hours after drinking 1- 2 cups of a high-fat liquid meal. These blood samples also will be drawn through a small catheter inserted into my arm vein. The high-fat meal is made of heavy whipping cream with small amounts of chocolate, sugar, and powdered milk and tastes like a rich chocolate shake. I understand that 10 tablespoons of blood will be drawn during this test and will be used to measure how my body absorbs and uses fat from a meal and how my blood clotting, and substances that affect hunger are affected by a fat meal. Before and after I drink the high-fat meal, I understand that I will breath through a mouthpiece while my nose is closed-off with a nose clip and the air that I breath out will be collected and used to determine how much fat I use for energy while sitting at rest. I also understand that I will complete a test that takes about 1 hour to measure the blood flow in my arm at rest and immediately after stopping my arm blood flow for 5 minutes with the use of a blood pressure cuff. I understand that all of these tests listed above will be done at the University of Maryland College Park.

I understand that on a visit to Baltimore on a separate day, I will have my kidney function measured at the Clinical Research Unit, Division of Nephrology, University of Maryland at Baltimore after an overnight fast. Before the test, I will drink 17 ounces of water over a 30-minute period. A Registered Nurse will then insert a small needle into veins in both of my arms. One line will be used to give the study medications and the other will be used to draw blood samples. Before the study drugs are given, I will provide a urine sample and a 0.7 ounce blood sample. During the test I will remain in a seated position except for when I provide urine samples.

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Next, I will receive the study medications, para-aminohippurate and iothalamate, which are markers used for estimating kidney function. Para-aminohippurate (5mg/kg body weight) and iothalamate (434 mg) will be given over five minutes. Then I will receive an additional small dose of para-aminohippurate and iothalamate by a slow, continuous infusion so that I will have the necessary amounts in my blood. Four blood samples (~0.7 ounces) will be drawn over the next 2½ hours and I will be asked to collect my urine every ½ hour for the next 2½ hours. This test will take approximately 3 hours. A total of 1.4 ounces of blood will be drawn during this test. I understand that I will undergo this test twice, once before and once after 6 months of aerobic exercise training. I understand that the amount of fat I have around my waist will be measured with a CAT scan while I lie quietly on a table. Another study will be done to measure my total body fat mass and total body muscle mass while I lie quietly on a table. I also understand that if I have elevated blood cholesterol levels, I will have blood samples drawn before and after a substance that temporarily stops blood from clotting is injected into my arm vein. The blood samples will be used to measure chemicals that affect my blood cholesterol levels. A total of 4 tablespoons of blood will be drawn at this visit. I understand that I will remain in the VA Medical Center for 2 – 3 hours after this test to make sure that all bleeding is stopped. I also understand that these tests will be done at the VA Medical Center in Baltimore. I understand that each of these visits will require approximately 4 – 5 hours including travel time.

I understand that the maximum total amount of blood that will be drawn during this Screening and Baseline Testing is 28 tablespoons over 2 – 3 months. This is approximately 90% of the amount of blood that is typically drawn during a single blood donation.

I understand that after completing this testing, for 6 months I will complete 3 exercise sessions each week supervised by study personnel. I understand that I will be instructed on appropriate warmup and stretching exercises to perform prior to each exercise training session. I will be taught to measure my heart rate and to use heart rate monitors to control how hard I am exercising. The first training sessions will consist of 20 minutes of light exercise. The amount of exercise and how hard I exercise will increase gradually until I am completing 40 minutes of moderate intensity exercise every session. Exercise modes include walk/jogging, stairstepping, and cycle, cross-country ski, and rowing ergometry. I will be asked to add a 45-60 minute walk to my exercise program on weekends after the first 10 weeks of the exercise program. I understand that this is not designed as a weight loss program and that if I lose more weight than expected from the amount of exercise that I complete, I will be counseled by a dietitian against restricting how much food I eat. I will also be asked to complete food records during the exercise training program and if major dietary changes have occurred, I will also be counseled by a dietitian to resume my original dietary habits. I understand that I may also be asked to collect my urine for 24 hours during the exercise training portion of the study.

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I understand that after completing 6 months of exercise training, I will have everything reevaluated that was measured before I began the exercise program. I understand that during this 4 weeks of Final Testing a maximum of 28 tablespoons of blood will be drawn; this is approximately 90% of the amount of blood that is typically drawn during a single blood donation.

I understand that if I qualify for this study that my DNA will be isolated from my blood and analyzed at a number of sites for differences in DNA that may affect how my blood pressure changes with exercise training. I understand that some of my DNA will also be frozen for future studies. However, these studies can only analyze my DNA at sites that might affect how my blood pressure, cholesterol levels, glucose and insulin levels, bone density, body composition, immunology (disease-fighting), and cardiovascular and blood clotting systems change with exercise training.

All information collected in this study is confidential, and my name will not be identified at any time. I understand that my DNA (genetic material) will be sent to collaborating genetics laboratories that are part of this study and that a sample of my DNA will be kept in the University of Maryland Department of Kinesiology laboratories. I also understand that samples of my blood will be sent to other collaborating laboratories for other blood measurements. I understand, however, that my DNA and blood samples sent to these laboratories will be identified only by a numeric code and that only investigators at the University of Maryland College Park will know whose name is associated with each coded number. I further understand that the list of names and codes will be retained at the University of Maryland College Park for up to 25 years.

I understand the following risks are associated with my participation in this study. (1) The risk of maximal exercise testing is approximately 1 nonfatal event in 10,000 tests and 1 fatal cardiac event in 70,000 tests. Risks will be minimized by having the test administered by a physician and personnel trained in such tests and emergency procedures. I will be screened with a resting electrocardiogram and a physical examination prior to this test. An emergency cart with the necessary drugs and a cardiac defibrillator will be present at all testing sessions. (2) There is minimal risk of bruising and infection associated with blood drawing. These risks will be minimized by using sterile techniques and by having experienced personnel draw all blood samples. (3) The risk of the body composition testing is the exposure to X-rays. The amount of X-ray exposure for this test is the same as that occurring during 30 minutes of any activity outside in the sun. (4) There is some risk associated with the elevated blood pressure that I have and some risk associated with stopping the medications I take to control my blood pressure. However, a 12 month lifestyle change program including diet and exercise is part of the medical recommendations for blood pressure control for individuals with levels of blood pressure similar to mine. In addition, I understand that my blood pressure will be monitored weekly and that this exceeds the blood pressure follow-up guidelines recommended for physicians. I also understand that if my blood pressure is too high for three consecutive weeks anytime during the study, my participation in the study will be discontinued and I will be referred back to my private physician.

Initials _____

I also understand that a physician associated with this project is available to deal with concerns related to my participation in this study. (5) The risk associated with the CAT scan to measure abdominal fat is the exposure to X-rays. The X-ray exposure is less than the maximum radiation dose individuals are permitted to be exposed to each year in their occupation. (6) There are no risks associated with the 24 hour urine collection. (7) The only risks associated with the measurement of the hormones in my blood that affect my blood pressure are those associated with blood drawing. (8) The risks associated with the glucose tolerance test and high fat meal are those related again to blood sampling, the possibility that my blood sugar may go too low levels at the end of the test, and the possibility of an upset stomach, primarily a stomach ache, after drinking the glucose or high-fat meal. I understand that I will be given a juice drink and small snack to minimize the chances of my blood glucose levels decreasing too much. (9) There are no risks associated with the genetic testing because I will not be provided with these results. (10) The risk of exercise training is the possibility of a heart attack or other cardiovascular event. A large physical activity center reported that 1 nonfatal cardiovascular event occurred in 1.7 million walk/jogging miles. These risks will be minimized because I will undergo a cardiovascular evaluation before beginning exercise training. Exercise sessions will be supervised by experienced personnel trained in emergency procedures. An emergency cart with the necessary drugs and a cardiac defibrillator will be present at all supervised exercise training sessions. (11) If I have elevated blood cholesterol levels, I understand that the risk associated with the test requiring the injection into an arm vein of a substance that temporarily stops blood clotting is bleeding. This risk will be minimized by excluding people with bleeding disorders, peptic ulcers, or other blood disorders from the study. The risk is further minimized by placing a bandage on the intravenous access site after the blood sampling and observing the subject for 2 – 3 hours. (12) The risks associated with the tests to assess kidney function are low as these are routine clinical tests. The risks are the side effects of the compounds put into my blood to assess kidney function; side effects include nausea, vomiting, facial flush, a generalized feeling of warmth, and allergic reactions. The risks are also those associated with blood drawing. These risks are minimized by administering these tests in a hospital setting with nurses experienced with these methods administering the test, so that if I should experience these side effects, medical personnel and equipment are readily available to respond and treat these symptoms. (13) The risks associated with the 24 hour blood pressure recording are the possibility of sleep disturbances in about 2% of volunteers. (14) The risks associated with the measurement of the blood flow in my arm are the result of stopping blood flow to the arm for 5 minutes. This causes substantial discomfort that ceases shortly after the blood pressure cuff is removed. I understand that if I can not tolerate the discomfort, this test will be terminated immediately on my request.

I understand that this study is not designed to help me personally, but may help the investigators to determine whom exercise might benefit the most. I understand that I am free to ask questions or to withdraw from participation at any time without penalty. I understand that I will earn $50 at the completion of Baseline Testing after the dietary stabilization period. I also understand that I will earn another $50 after 3 months of exercise training if I complete at least

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90% of my exercise training sessions. I also understand that I will earn another $100 after completing 90% of my training sessions for 6 months and all final testing. I understand that that the total amount that I earn will be paid to me at the completion of my participation in the study. I understand that if my participation in the study has to be terminated because I change my hormone replacement therapy regimen, I will only be paid for the portion of the study that I have already completed, that is, which of the stages above that I have completed.

In the event of a physical injury resulting from participation in this study, I understand that immediate medical attention is available at the Washington Adventist Hospital or the Baltimore VA Medical Center. However, I understand that the University of Maryland does not provide any medical or hospitalization coverage for participants in this research study nor will the University of Maryland provide any compensation for any injury sustained as a result of participation in this research study except as required by law.

Principal Investigator: James Hagberg, PhD. Department of Kinesiology, HLHP Building, University of Maryland, College Park, MD 20742-2611, telephone 301-405-2487.

Subject's signature	Date
Witness	Date
Investigator	Date

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