UNIVERSITY OF CALIFORNIA, SAN DIEGO

Effects of Hypertension, Acute and Long-term Exercise on Pseudopod Formation on Naïve Neutrophils and Plasma Matrix Metalloproteinase Levels and Activity

A Thesis submitted in partial satisfaction of the
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in

Bioengineering

by

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The thesis of Bassem Merit Shoucri is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego
2010

Dedication

This thesis is dedicated to my grandfather, Attia Youssef Mansour, who always expected an “A plus plus” from me. I would also like to thank my grandmother, Jeanette Mansour, and my parents, Merit and Sylvia Shoucri, whose wisdom, guidance, and love have provided me with purpose throughout my life. I would finally like to thank my brothers, Amir and Sherif, who ensure that I never take myself too seriously.

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ABSTRACT OF THE THESIS

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Leukocyte activation and matrix metalloproteinases (MMPs) have been associated with experimental forms of hypertension as a signature of inflammation. It is the objective of my thesis to investigate leukocyte activation and MMP activity in a cohort of patients newly diagnosed with hypertension that are not on any medication.

My aims are to determine whether hypertensives exhibit signs of leukocyte activation on naïve neutrophils as determined by the formation of pseudopods and whether they exhibit elevated levels of MMPs in plasma.

Pseudopod formation has been identified as a cause of increased hemodynamic resistance and is associated with other forms of activation. Exercise interventions have successfully reduced blood pressure as well as plasma levels of known inflammatory markers in hypertension. Subjects with normal or elevated blood pressure participated;

hypertensives additionally partook in a 12-week exercise intervention. Naïve cells were stimulated by subject plasma, fixed, imaged, and analyzed. Significant correlations between pseudopod length and systolic ($R = 0.415$, $p = 0.018$), diastolic ($R = 0.402$, $p = 0.023$) blood pressure were identified. Pseudopod length post training was associated with a change in diastolic blood pressure.

Matrix metalloproteinases, a family of endopeptidases involved in vascular remodeling, are role players in a number of cardiovascular diseases known as risk factors in hypertension. Subjects performed acute exercise tasks before and after a lifestyle intervention. Acute exercise increased plasma levels of MMP-8 and -9 ($p < 0.001$). Training did not alter MMP-levels, or the response to acute exercise. Correlations between MMPs and markers of inflammation were found.

Chapter 1: Introduction

1.1 Hypertension

Hypertension, defined as systolic blood pressure (SBP) > 140 mmHg and/or diastolic blood pressure (DBP) > 90 mmHg, affects one in three adults in the United States, an estimated 74.5 million people over the age of 20, according to the National Health and Nutrition Examination Survey (NHANES) [1]. The worldwide prevalence of the disease is approximated at 1 billion people and it is responsible for an estimated 7.1 million deaths every year [2]. Hypertension is a leading cause of morbidity and mortality in the United States [3]. Between 1996 and 2006 both the death rate and the total number of deaths due to hypertension increased by 19.5% and 48.1%, respectively [1]. The estimated direct and indirect cost of hypertension in the United States for the year 2010 is $76.6 billion [1].

Epidemiological studies show that the risk of cardiovascular disease (CVD) increases linearly with blood pressure (BP) [2, 4]. For every 20 mmHg SBP or 10 mmHg DBP, mortality doubles for both ischemic heart disease and stroke [2]. More surprisingly, there is a dramatic increase in the incidence of cardiovascular events in patients with prehypertensive BP levels (120/80 – 140/90 mmHg) as compared to patients with optimal BP (< 120/80 mmHg) [2]. Data of this nature has established the importance of antihypertensive treatment in the fight against CVD, both on the clinical and research fronts.

1.2 Inflammation in Hypertension

Inflammation is a hallmark of hypertension, indicated by a number of elevated inflammatory markers [5-12]. Atherosclerosis, also classified as an inflammatory disease [10], is highly associated with hypertension, accounting for much of its associated morbidity and mortality. Hypertension exhibits multiple elevated levels of inflammatory processes connected to the pathogenesis of atherosclerosis, such as immune cell activation, elevated inflammatory cytokines and endothelial dysfunction [10].

Among the list of inflammatory markers elevated in hypertension are the pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) [5-8], as well as the acute-phase reactant C-reactive protein (CRP) [12], which is a sensitive indicator of systemic inflammation. It should be noted that while these proteins are used as markers for inflammation, their roles in the inflammatory response are quite varied and complex. For example, cytokines stimulate the production of acute-phase reactants in the liver, meaning that these markers are interdependent. Also crucial to the inflammatory process are selectins and integrins, cell adhesion molecules involved in leukocyte rolling and subsequent binding to the endothelium, prior to extravasation. Hypertensive patients have increased levels of soluble cell adhesion molecules, normally expressed on the endothelium, including inter-cellular adhesion molecule-1 (sICAM-1), vascular cellular adhesion molecule-1 (sVCAM-1), sP-selectin and sE-selectin [13-17], providing further evidence for the link between hypertension and inflammation.

1.3 Pharmacological Treatment of Hypertension

Few of the many pharmacological treatments that have been developed to reduce blood pressure (BP) have shown efficacy in diminishing the negative cardiovascular effects and mortality associated with hypertension [18, 19]. Studies have showed that angiotensin-converting enzyme (ACE) inhibitors fail to alter secondary stroke rates or the total number of vascular complications [19]. Diuretics have been found to reduce incidence of stroke and heart failure, but fail to alter rates of myocardial infarction and can predispose to diabetes [19, 20]. Indeed, a combination of diuretics and ACE inhibitors protects against vascular events and nonfatal stroke, but not fatal stroke or overall mortality [19]. No pharmacotherapy has reduced the negative cardiovascular effects or mortality associated with the progression of atherosclerosis in hypertension as compared to controls or placebo [19]. Still, BP reductions have been linked to positive outcomes such as a small decrease in mortality and reduced risk of stroke [21, 22]. However, the majority of treated hypertensive patients fail to successfully reduce their BP below hypertensive levels (140/90 mmHg) due to the inability of current pharmacological therapy to control BP levels, inflammation and due to non-adherence to pharmacotherapy [23-25].

1.4 Lifestyle Interventions in Hypertension

Lifestyle modification, such as diet and exercise programs, are established treatments for human hypertension and recommended for all hypertensive patients [2]. Similarly, a sedentary lifestyle remains a risk factor for cardiovascular disease [26, 27].

It follows that, given the inflammatory nature of cardiovascular disease, physical activity has been inversely correlated to markers of inflammation, including CRP, fibrinogen, and leukocyte count [28-30]. Physical fitness also inversely correlates with the cytokines IL-6 and TNF-α [31], as well as leukocyte adhesion molecule expression [32]. One study has shown that pharmacologically treated hypertensives also displayed an inverse relation between physical activity and CRP as well as soluble E-selectin (sE-selectin) in plasma, indicating the importance of exercise even in treated hypertension [33].

Exercise interventions can lead to significant BP reduction in hypertensives along with reduced levels of several inflammatory markers in the plasma [34]. BP reductions of 16/11mmHg, have been reported, depending on the frequency and intensity of the exercise [35-37]. Importantly, exercise interventions in hypertension show the same anti-inflammatory benefits indicated by cross-sectional physical activity reports, including decreased levels of CRP, IL-6, TNF-α, sICAM, and sVCAM [38-41]. The exact mechanisms through which exercise effectively treats hypertension are not fully understood, however they are likely multifaceted and interdependent.

1.5 Pseudopods

1.5.1 Hypertension and the Microcirculation

There are multiple research results suggesting that vasoconstriction along with restructuring of the arteries and arterioles are the main sources of BP elevation in arterial

hypertension [42-44]. In addition, there is evidence for the involvement of a capillary mechanism, by which leukocytes in the microcirculation have a profound effect on capillary resistance therefore on the blood pressure upstream in the arterioles and venules [45-52]. Organ perfusion experiments suggest that leukocytes provide roughly 22% of the whole blood resistance in spite of their accounting for less than 1% of total cell volume [45]. This effect requires an interaction between red cells and white cells in capillaries.

1.5.2 Leukocyte-Erythrocyte Interaction in the Microcirculation

Due to their greater size and rigidity, leukocytes travel through the capillaries slower than erythrocytes. This results in an accumulation ('traffic jam') of erythrocytes in the leukocyte's wake, a major disturbance of the position in the capillaries, which in turn causes a steep increase in the apparent viscosity of the erythrocytes leading to an elevation of microvascular resistance [46, 47]. This effect is dependent on the interaction between the two cell types and does not require leukocyte adhesion to the capillary endothelium [47].

1.5.3 Mechanical Consequences of Pseudopod Formation

This local increase in resistance is exacerbated if the leukocytes in the free circulation form pseudopods, an event rarely observed in healthy controls. Pseudopods are localized projections of the cytoplasm generated by actin polymerization and covered by the membrane. Mechanically, the effect of pseudopods in the microvasculature is drastic. The same aforementioned organ perfusion experiment shows that leukocytes

activated with formation of pseudopods account for 50-60% of the total resistance [45], nearly three times that of the unactivated leukocytes. Similarly, it has been shown that whole blood from spontaneously hypertensive rats (SHR), requires higher pressure than control animals to achieve the same flow rate. This finding is attributed to the elevated number of circulating leukocytes with pseudopods in the SHR [48, 53], given that this effect is diminished in the presence of a metabolite that induces pseudopod retraction [49].

1.5.4 Pseudopods and Inflammation

Pseudopods are involved in many of the inflammatory activities of leukocytes, including spreading on the endothelium, extravasation through the endothelium into the tissue and engulfing bacteria during phagocytosis. Pseudopod formation is also accompanied by the release of proteases and formation of oxygen free radicals [48, 54, 55]. Leukocytes that project pseudopods have enhanced expression of CD11a/CD11b integrin adhesion molecules and a heightened adherence to the endothelium, characteristics that enhance further the mechanical effects of leukocytes in the microvasculature [50, 51]. Studies in the SHR that show increased leukocyte count and increased percentage of leukocytes projecting pseudopods [48, 53] suggest an altered inflammatory process in hypertension, marked by increased cell activation, excessive adhesion to the endothelium, and a stronger inflammatory response [52, 54]. SHR exhibit increased endothelial ICAM-1 expression and increased adhesion resulting in vascular damage and subendothelial accumulation of monocytes/macrophages [56, 57]. This data is reflected in *in vitro* models of human hypertension, where peripheral blood

mononuclear cells (PBMCs) from hypertensive patients show increased endothelial adhesion [58, 59]. Still, there has been no study, *in vivo* or *in vitro*, of pseudopod formation as a mechanism for pressure elevation and a marker of inflammation in human hypertension.

1.6 Matrix metalloproteinases

1.6.1 Matrix Metalloproteinase Structure and Function

Matrix Metalloproteinases (MMPs), or matrixins, are a family of endopeptidases known to be involved in extracellular matrix (ECM) degradation. While the appropriate breakdown of the ECM is an essential biological function, especially in tissue repair and vascular remodeling, the inappropriate activity of MMPs have been associated with multiple pathologies including arthritis, cancer, fibrosis, and a number of cardiovascular disorders [69, 70].

There are 24 MMPs coded in the human genome that have been discovered; the entire family consists of 28 proteins. A typical MMP consists of, from N- to C-terminus, a propeptide, a catalytic domain, a linker peptide or 'hinge' region, and a hemopexin domain. Peptide lengths of the individual domains remain constant throughout the protein family with the exception of the variable hinge region. The propeptide (~80 amino acids) contains the "cysteine switch" that, along with the zinc binding motif of the catalytic domain, helps coordinate a catalytic zinc ion, rendering the proMMP inactive.

In order to activate the MMP, the propeptide must be cleaved. This cleavage happens at the "bait" region of the prodomain, a region particularly susceptible to protease activity. Once the propeptide is cleaved, the catalytic metalloproteinase domain (~170 amino acids) is exposed and active. The hemopexin domain (~200 amino acids) can serve as a binding-site for endogenous inhibitors, such as Tissue Inhibitors of Metalloproteinase (TIMPs) [69]. An MMP:TIMP ratio in favor of ECM degradation is characteristic of vascular remodeling and atherosclerosis [70].

1.6.2 MMP-8, Neutrophil Collagenase, Collagenase-2

Depending on the organization of the four domains and the preference of substrate MMPs are further classified as gelatinases, collagenases, stromelysins, matrilysins, membrane-type MMPs, or they remain uncategorized. MMP-8 (85 kDa) is a collagenase, which can cleave interstitial collagens I, II, and III. Typically, it is released by activated neutrophils during inflammation. MMP-8 knockout mice do not express abnormalities in development or adult life, though they are susceptible to skin tumors, implying that MMP-8 may protect against cancers [71]. MMP-8 has been found in macrophages as well as the smooth muscle cells and endothelium of atheromas, suggesting a role in inflammation [71].

1.6.3 MMP-2 and -9, Gelatinase A and B

MMP-2 (72 kDa) and -9 (92 kDa) are gelatinases, which digest gelatin, collagens IV, V, and XI, elastin and laminin. MMP-2, but not -9, can also cleave collagens I, II, and III, though at a lower activity than collagenases, as well as fibronectin, a number of

chemokines, and the prodomains of MMP-1 and -9. Structurally, MMP-2 and -9 differ from MMP-8 in their thrice-repeated fibronectin type II domain located in the catalytic region. MMP-2 has been linked to atherosclerosis, aneurysm, and myocardial infarction [70-72]. MMP-9 is also a suspected role player in atherosclerosis, specifically in the migration of smooth muscle cells [71].

1.6.4 MMPs in Hypertension

MMPs play a role in a number of cardiovascular pathologies also associated with hypertension [2, 4, 71]. Hence, there is ample research in search of the relationship between elevated BP and the MMP family. There have been mixed reports on MMP levels in human hypertensives. Hypertension has been associated with fibrosis of the arterial wall, a process implicating the involvement of MMPs [73, 74]. Several groups [69, 70] have shown increased MMP-2, -9, and TIMP-1 levels in addition to increased MMP-2 and -9 activity in hypertension. Contrary to this, other groups [76, 77] find MMP-2 and -9 depression, suggesting that decreased MMPs result in collagen build-up in the arterial wall. It is likely that conflicting reports stem from differences in experimental design, statistical power, and the presence or absence of co-morbidities and anti-hypertensive treatments within patient populations. Still, groups continue to show patterns of MMP over-expression in hypertensives. A recent study compared normotensive controls, uncomplicated hypertensives, and hypertensives with end-stage renal disease (ESRD) [78]. Hypertensives had an elevated MMP-9 concentration as compared to normotensives, while patients with ESRD had increased MMP-2 and -10 as compared to uncomplicated hypertensives. These results suggest that MMP-9 elevation

happens earlier in the progression of the disease while MMP-2 and -10 are elevated with organ injury.

1.6.5 MMPs and Receptor Cleavage

More recent work has focused on the possibility that MMPs in the plasma cleave substrates involved in the pathophysiology of hypertension and associated diseases. These substrates include a handful of vital membrane receptors including the insulin receptor, the β_2 adrenergic receptor (β_2AR), the vascular endothelial growth factor receptor-2 (VEGFR-2), the formyl peptide receptor (FPR), and the leukocyte integrin receptor CD18 [79-81, 93]. These studies have used the spontaneously hypertensive rat (SHR) as a model. As compared to their normotensive counterpart, the Wistar-Kyoto (WKY), SHR have elevated MMP-9 levels and activity [79]. When labeling for the extracellular domain of insulin receptor-α on fresh leukocytes, SHR have lower receptor density than WKY, though this density is significantly restored in the presence of a general protease inhibitor, doxycyclin [79]. The destruction of insulin receptors may explain insulin resistance in the SHR and imply links between human hypertension and the metabolic syndrome.

Similar results can be displayed for the β_2AR. Aorta and heart muscle of control Wistar rats was exposed to fresh plasma from Wistar, WKY, and SHR rats. Exposure to SHR plasma resulted in cleavage of the extracellular, but not intracellular, domains of the β_2AR as compared to controls [80]. This cleavage was prevented by the presence of MMP inhibitors (EDTA, doxycyclin). Since the β_2AR mediates vascular smooth muscle

cell relaxation, destruction of this receptor would lead to increased peripheral resistance and hypertension. The data is further supported by the fact that hypertensives show a reduced vasodilation in response to β_2AR agonist in both the SHR [82] and in human hypertension [83].

Vascular endothelial growth factor receptor-2 is involved in the proliferation and apoptosis of vascular endothelium. Depleted VEGFR-2 signaling is associated with apoptosis in the capillaries. SHR had lower expression of extracellular VEGFR-2 in cardiac arteries, arterioles, and capillaries as compared to WKY [81]. In addition, exposure of naïve cardiac tissue to SHR plasma resulted in lower VEGFR-2 density than tissue exposed to WKY plasma [81]. Again, as with the insulin receptor and β_2AR, density was restored in the presence of MMP inhibitor.

Finally, the formyl peptide receptor is a mechanosensor known to be involved in the mechanism by which fluid shear stress induces pseudopod retraction. Recent data [currently in review, 93] shows that SHR neutrophils express significantly less extracellular FPR and have an abnormal response to fluid shear stress. Treatment with MMP inhibitor (doxycycline) resulted in increased FPR density and improved shear response.

This data is in line with the Autodigestion Hypothesis [84], which states that several forms of proteases, including MMPs in plasma, may degrade the extracellular domains of a number of membrane receptors, resulting in loss of cell function. The

origin of elevated protease activity in the SHR is not known. Proteolytic activity may be over-expressed genetically or it may be derived from the digestive tract. Besides the SHR, ischemic shock in control animals has been used as a model for this theory, severe inflammation accompanied by a failure of the mucosal membrane of the small intestine [94]. It is yet to be studied how this hypothesis relates to hypertension and other related diseases in humans.

1.6.6 MMPs in exercise

Research on the role of exercise in MMP expression is limited. Multiple studies show an increase in MMP concentration following acute exercise. One such study showed increased plasma MMP-9 levels in both coronary artery disease patients and controls [85]. Another showed the same MMP-9 increase with serum concentrations, but only when the acute task was performed after an 8-week exercise intervention [86]. Roberts et al found that short-term diet and exercise interventions (2 and 3 weeks) in both overweight youth and adults with metabolic syndrome successfully reduced MMP-9 levels [87, 88]. A 16-week exercise intervention in a population of overweight patients with Type II diabetes mellitus resulted in decreased MMP-9 and increased TIMP-2 levels [89]. Since MMP-9 is involved in plaque formation, the reduction in its concentration has been associated with the beneficial effects diet and exercise have in reducing cardiovascular disease.

1.7 Specific Aims

The aims of the study are twofold.

(1) We examined the relationship between pseudopod formation on naïve neutrophils, plasma levels and activity of matrix metalloproteinases (MMPs) and BP in hypertensive and normotensive patients. Associations with levels of other inflammatory markers and a number of other physiological and demographic variables were investigated.

(2) We determined the effects of a lifestyle intervention aimed at lowering BP, VO_2 max, and BMI on pseudopod formation, MMP levels and activity.

1.8 Hypothesis

We hypothesize that there is a relationship between BP and pseudopod (PP) formation on naïve cells as well as plasma MMP concentrations and activity. PP formation, MMP levels and activity differ between the two BP groups and there is a continuous correlation between these markers and BP. We also hypothesize that a lifestyle intervention designed to reduce BP will reduce PP formation, MMP concentrations and activity. Finally, we hypothesize that MMP plasma concentrations increase with acute exercise, and that this increase will be attenuated following long-term lifestyle intervention.

Chapter 2: Pseudopods

2.1 Methods

2.1.1 Participants

Thirty-two healthy, sedentary volunteers (age 26-60 years, 19 female) were screened for heart, liver, or renal disease, diabetes, psychosis, severe asthma, pregnancy, and current use of prescription drugs. This study is under a parent study investigating the effects of acute stressors, psychological and exercise-induced, and long-term lifestyle intervention in non-medicated hypertensive patients. Subjects were instructed not to use any medication 1 week prior to testing, and to avoid vigorous exercise, caffeine, alcohol, and smoking 24 hours prior to testing.

Subjects were grouped according to screening BP; Normal BP (N=11, 6 female) < 120/80 mmHg (SBP = 119.9 ± 10.1 mmHg, DBP = 76.3 ± 7.8 mmHg); Elevated BP (N=21, 13 female) > 120/80 mmHg (SBP = 144.4 ± 9.4 mmHg, DBP = 86.9 ± 7.2 mmHg). Subjects in the elevated BP group, but not the normal BP group, participated in the 12-week lifestyle intervention.

2.1.2 Lifestyle Intervention

Hypertensive patients (N=21, 13 female) participated in a 12-week lifestyle intervention. Subjects were randomized into one of three groups: exercise, diet and exercise, or control intervention.

In both the exercise and the diet and exercise intervention arms, the goal of the exercise was to achieve 5 or more days per week of moderate-intensity cardiovascular physical activity for at least 30-60 min. Subjects met with a personal trainer twice a week at a local YMCA (Young Men's Christian Association, Mission Valley or La Jolla, San Diego, CA) and were encouraged to exercise an additional 3 times a week. Each supervised session included proper warm up, exercise, cool down, and stretching. Participants were asked to record all of their exercise activities including the type of exercise, the pace, the duration, their HR, their perceived exertion (1-10 scale), and whether or not they were with the trainer. Patients also filled out a strength-training log that details the part of the body they exercised, the duration or number of reps, as well as their perceived exertion. HR was monitored (Polar HR monitor FS1) throughout exercise tasks; subjects were advised to exercise within 60-75% of their previously measured maximum HR.

The diet and exercise intervention included all aspects of the exercise intervention with the addition of diet regimen provided by a registered dietician. The goal of the diet intervention was to promote a reduction in energy intake relative to expenditure of 500-1000 kcal/day. This was achieved through a lower energy density diet based on the

"Dietary Approaches to Stop Hypertension" (DASH) clinical study [60-62]. Dieticians (GCRC Nutrition Services Core, UCSD Medical Center, San Diego, CA) communicated with participants at least once a week via telephone or email to set goals and ensure successful implementation of the dietary plan. Adherence to the dietary intervention was monitored in several ways. First, participants were asked to complete a daily diary of the types of food they were eating, the method of cooking, and whether food was home-cooked or from a restaurant. Subjects were additionally required to complete three 24-hr dietary recalls at baseline, 6-weeks, and after the 12-week intervention using the Nutrition Data System (NDS University of Minnesota, Minneapolis, MN) and software for collection and analysis.

Subjects selected into the control group, spent 12-weeks without lifestyle intervention, after which they were randomly place into the exercise or diet and exercise group.

2.1.3 Neutrophil Isolation, Stimulation, and Imaging

Whole blood from a young and healthy donor was drawn into sodium heparin-coated tubes (BD vacutainers). Neutrophil isolation began with red blood cell sedimentation (6% Dextran, Sigma-Aldrich Corp., St. Louis, MO). The plasma layer was removed, layered onto 7.5 ml of Histopaque-1077 (Sigma), and then centrifuged (20 min, 600G, Sorvall Instruments RC5C, Allegra X-15R). The pellet was resuspended in 1X PBS, and carefully layered on top of a Percoll gradient (55% / 74%, Sigma). Following centrifugation (15 min, 600G), isolated neutrophils were extracted, washed with PBS,

spun down once more (10 min, 100G) and diluted in PBS to a concentration of 2.4 x 10^7 cells / ml.

Donor neutrophils (50 µl) in PBS were incubated with 50 µl of subject plasma at room temperature for 10 minutes. Negative (PBS) and positive (N-formyl-met-leu-phe (fMLP, Sigma)) controls were run with each batch of samples. The reaction was fixed with 50 µl of 0.5% glutaraldehyde (Sigma), and 150 µl of 1% Crystal Violet (Sigma) was added, bringing the final concentration of cells to 4 x 10^6 cells / ml.

Samples were loaded in duplicate onto a hemocytometer, and pictures of the first 100 cells were taken at 40X magnification (MiniVID USB Microscope Camera, ABBOTA Corp., Lake Hiawatha, NJ). Cells were analyzed using ImageJ (Version 1.42q, National Institute of Health, Bethesda, MD) for pseudopod formation and pseudopod length. Pseudopod formation was defined as the percentage of cells expressing pseudopods. Any cell with a pseudopod projection longer than 1 micron was considered activated (Figure 2-1). Pseudopod length was defined as the difference between the average maximum diameters of the first 5 unactivated cells and the first 40 activated cells (Figure 2-2).

2.1.4 Interleukin-6 Assay

A Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) was used to detect human plasma Interleukin-6 (IL-6) levels. 96-well polystyrene microplates coated with a mouse monoclonal antibody against IL-6 were used (R&D Systems, Inc., Minneapolis,

MN). 100 μL subject plasma (ethylenediaminetetraacetic acid (EDTA), BD vacutainers), standards (R&D), or controls (R&D) were added to 100 μL of assay diluent (R&D) and left to incubate for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 rpm (MTS 2/4 microtiter shaker, IKA Works, Inc., Wilmington, NC). The plate was washed with Wash Buffer (R&D) 6 times with the Multiwash III Microplate Washer (TriContinent Scientific, Inc., Grass Valley, CA) then excess fluid was removed by tapping plate on paper towel. 200 μL of polyclonal antibody against IL-6 conjugated to alkaline phosphatase was added to each well, followed by another 2 hours on the shaker. The wash cycle was repeated and 50 μL of substrate (Lyophilized NADPH, R&D) was added, followed by a 1 hour incubation on the benchtop. Then 50 μL of amplifier solution (R&D) was added, followed by 30 min incubation on the benchtop. 50 μL of stop solution (2N Sulfuric Acid, R&D) was then added and optical density was measured within 30 min on a spectrophotometer (Spectramax 340PC348, Molecular Devices, Sunnyvale, CA) at 490 nm with wavelength correction set to 650 nm. All samples, standards, and controls were run in duplicate.

2.1.5 C-reactive Protein Assay

An electrochemiluminescence detection method (MSD Vascular Injury Panel II Assay, Meso Scale Discovery, Gaithersburg, MD) similar to the sandwich ELISA was used to detect human plasma C-reactive Protein levels. 96-well MULTI-ARRAY plates coated with antibody against CRP were used (MSD). Subject plasma (EDTA, BD vacutainers) was diluted 200-fold in 1% Blocker A Solution (Bovine Serum Albumin, MSD). 200 μL of 5% Blocker A Solution was added to each well and allowed to

incubate at room temperature for 1 hour. Plates were washed with PBS with 0.05% Tween (PBST) 3 times (Multiwash III, TriContinent). 40 μL of Diluent V (MSD) was added followed by 10 μL diluted sample or calibrator, then incubated for 2 hours at room temperature on the shaker (IKA). The wash cycle was repeated and 25 μL of antibody against CRP conjugated to SULFO-TAG label (Ruthenium (II) tris-bypyridine-(4-methylsulfonate) NHS ester, Detection Antibody Reagent, MSD) was added followed by a 1 hour incubation at room temperature on the shaker. The wash cycle was repeated and 150 μL of 1X Read Buffer T (MSD) was added. Plates were immediately analyzed using the SECTOR Imager 2400 (MSD). All samples and calibrators were run in duplicate.

2.1.6 Statistical Analysis

Data were analyzed in SPSS (SPSS Inc., IBM, Armonk, NY). Differences between BP groups were analyzed by student t-test. Correlations to BP were analyzed using Pearson's Correlation. Predictors of individual BP responses to training were analyzed using a hierarchical linear regression. A value of $P<0.05$ was considered significant.

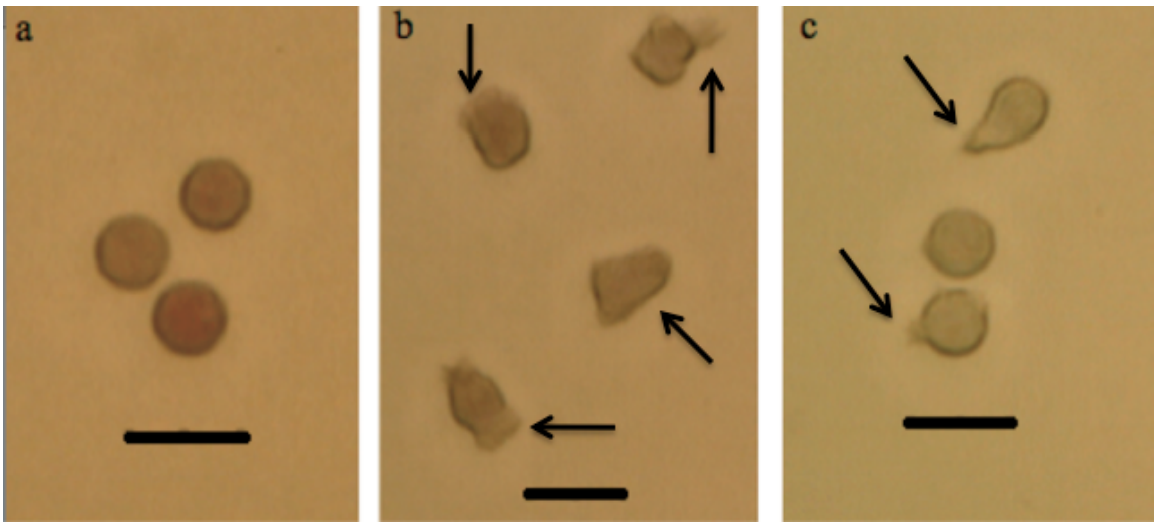

Figure 2-1. Naïve neutrophils projecting pseudopods. Images of naïve neutrophils stimulated by (a) negative control (1X PBS), (b) positive control (2 nM fMLP), and (c) subject plasma. Arrows point to pseudopods; black bar = 10 microns.

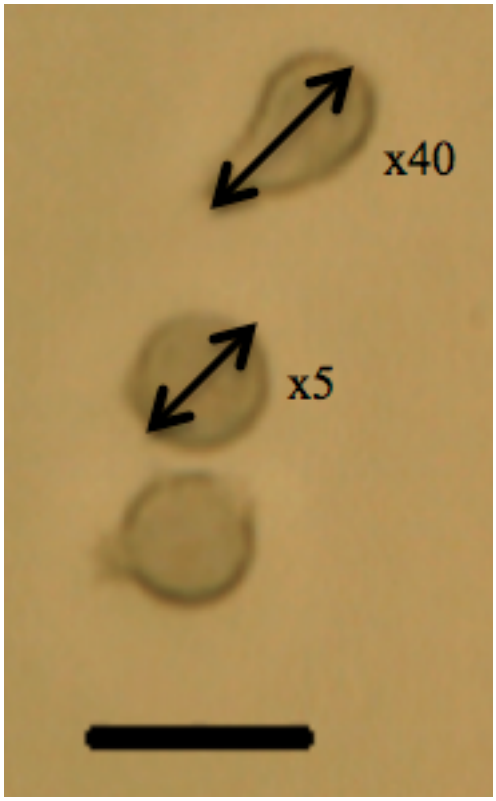

Figure 2-2. Calculation of pseudopod length. The first 40 maximum diameters of activated cells were averaged, the first 5 maximum diameters of unactivated cells were averaged. The difference between these two values was considered to be the average pseudopod length for a given sample. Approximate maximum diameters are shown by arrows; black bar = 10 microns.

2.2 Results

2.2.1 Demographics

Participants in the Elevated BP group were similar to those in the Normal BP group in regard to BMI, gender, and VO2peak, but were slightly older and as expected had higher resting SBP and DBP (Table 2-1).

2.2.2 Inflammatory Markers

Plasma IL-6 and CRP levels were assessed at baseline (Table 2-2). Neither CRP nor IL-6 showed significant difference among groups. The elevated BP group had significantly greater PP length than Normal BP group (2.48 ± 0.42 vs. 2.12 ± 0.54, $p = 0.05$).

2.2.3 Correlations with pseudopod length

Associations between PP length and physiological/demographic variables were determined using Pearson's Correlation (Table 2-3). The pseudopod length of naïve cells activated by the subject's plasma positively correlated with systolic ($R=0.415$, $p=0.018$) and diastolic ($R=0.402$, $p=0.023$) blood pressures (Figure 2-3, 2-4), but not with BMI, age, VO2peak, CRP or IL-6.

2.2.4 Adjusting for confounders

Age was significantly different between BP groups and showed a trend toward a significant correlation with PP length ($R = 0.326$, $p = .064$). Controlling for age as a

potential confounding variable, the correlation between PP length and BP was also not significant (SBP, R = 0.298, p = 0.103; DBP, R = 0.323, p = 0.076).

2.2.5 Effects of a lifestyle intervention on demographic, physiological variables

A summary of the effects of the exercise intervention on demographic and physiological variables is shown in Table 2-4. The intervention significantly decreased SBP and DBP, but no other variables.

2.2.6 Regression to assess individual response to intervention

While the intervention failed to reduce PP formation in naïve leukocytes by the plasma of the patient population as a whole, it was still postulated that individual response to the intervention could reflect changes in PP formation. A hierarchical linear regression was performed to predict training-induced change in BP with changes in PP length. Post-training PP length was predicted by change in diastolic (ß=-0.579, B=-0.140, R^2=0.227, p=0.018), but not systolic (ß=-0.024, B=-0.005, R^2=0.000, p=0.926), when using age and pre-training PP length as predictors in step one.

Table 2-1. Patient and Control Demographic Parameters

	Elevated BP (N=21)	**Normal BP (N=11)**	**t-test Sig. (p)**
Gender	8M, 13F	5M, 6F	--
BMI (kg/m^2)	29.0 ± 3.3	29.8 ± 3.6	0.552
Age (years)	49.3 ± 8.2	41.2 ± 8.7	0.014*
$VO_{2\text{-}Peak}$ (ml/kg/min)	28.5 ± 7.5	28.7 ± 8.9	0.965
SBP (mmHg)	144.4 ± 9.8	119.9 ± 10.1	0.000*
DBP (mmHg)	86.9 ± 7.2	76.3 ± 7.8	0.001*

Mean ± SD, *statistically significant in a comparison by student t-test.

Table 2-2. Markers of Inflammation in BP groups

	Elevated BP (N=21)	**Normal BP (N=11)**	**t-test Sig. (p)**
CRP (pg/ml)	5.35 ± 5.54	2.91 ± 3.01	0.205
IL-6 (pg/ml)	4.11 ± 2.99	2.55 ± 1.77	0.089
PP activation (%)	59.7 ± 18.3	63.4 ± 19.9	0.599
PP Length (μm)	2.48 ± 0.42	2.12 ± 0.54	0.051

Mean ± SD.

Table 2-3. Correlations between demographic, physiological variables and PP length

	Correlation to PP Length (R)	**Significance (p)**
BMI (kg/m^2)	-0.126	0.492
Age (years)	0.326	0.064
$VO_{2\text{-}Peak}$ (ml/kg/min)	-0.272	0.139
SBP (mmHg)	0.415	0.018*
DBP (mmHg)	0.402	0.023*
CRP (pg/ml)	0.101	0.589
IL-6 (pg/ml)	0.007	0.973

Mean ± SD, *statistically significant by Pearson's correlation.

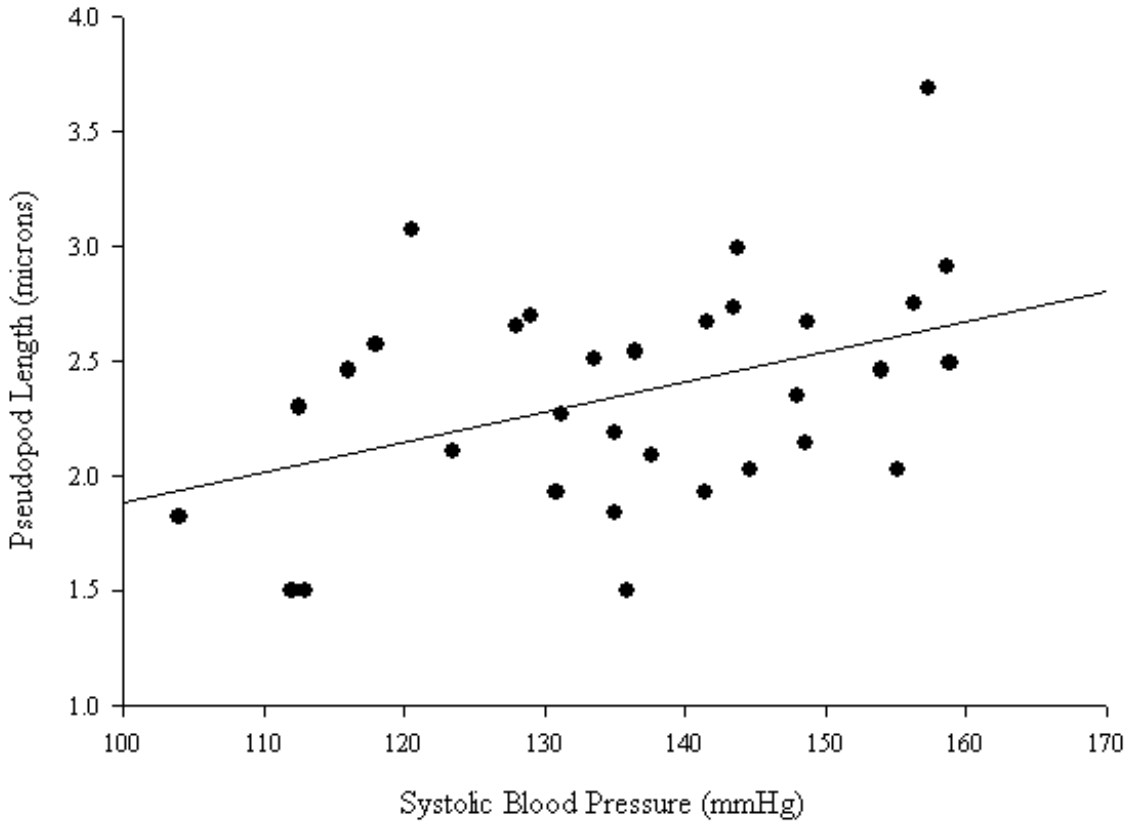

Figure 2-3. Pseudopod length vs. systolic blood pressure. Pearson's correlation R = 0.415, p = 0.018.

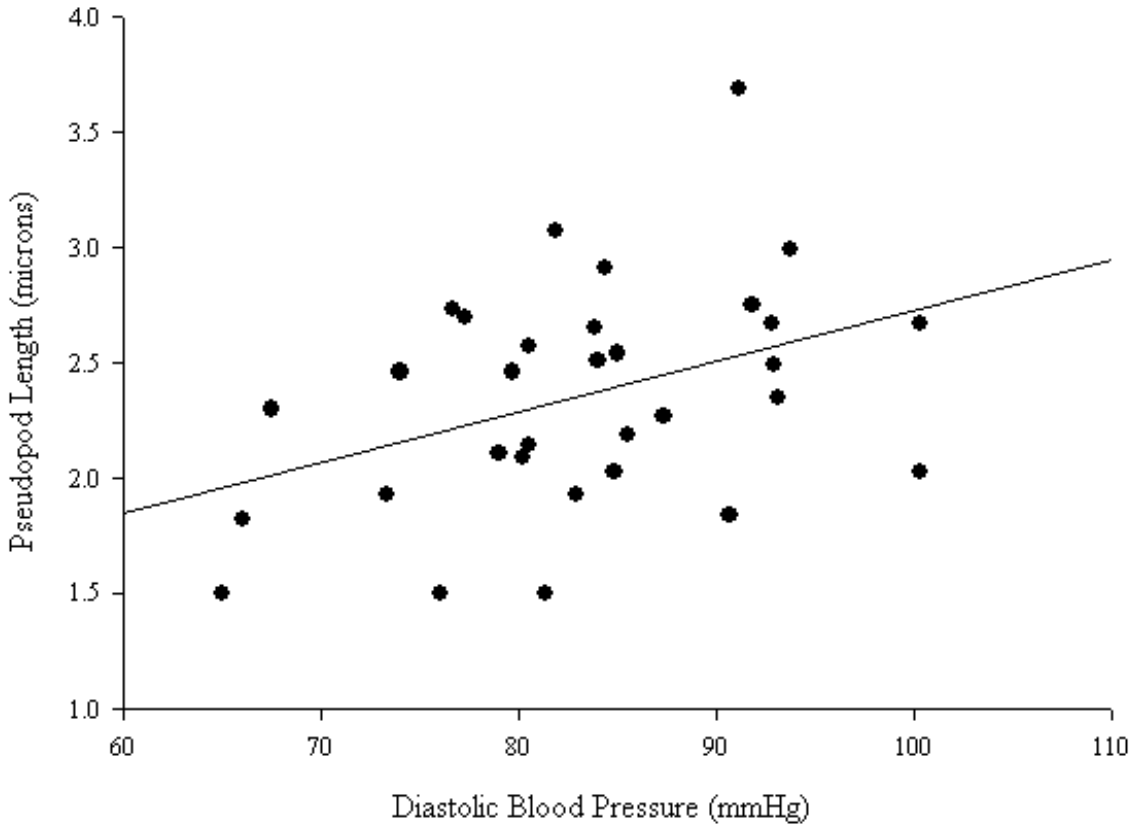

Figure 2-4. Pseudopod length vs. diastolic blood pressure. Pearson's correlation R = 0.402, p = 0.023.

Table 2-4. Clinical Indices and Pseudopod Length after 12-week Lifestyle Intervention

	Pre-Training	**Post-Training**	**Diff. (t)**	**Sig. (p)**
BMI (kg/m^2)	29.5 ± 3.0	29.3 ± 3.1	0.826	0.417
$VO_{2\text{-Peak}}$ (ml/kg/min)	26.8 ± 6.2	28.8 ± 6.9	-2.004	0.057
SBP (mmHg)	143.0 ± 10.8	135.4 ± 10.1	3.566	0.002*
DBP (mmHg)	86.8 ± 7.0	79.7 ± 8.7	4.372	0.000*
CRP (pg/ml)	4.99 ± 5.42	5.37 ± 5.50	-0.469	0.644
IL-6 (pg/ml)	3.59 ± 2.59	3.64 ± 4.48	-0.053	0.958
PP activation (%)	60.6 ± 18.4	67.6 ± 18.8	-1.904	0.071
PP Length (μm)	2.50 ± 0.43	2.47 ± 0.52	0.293	0.773

Mean ± SD, *statistically significant in a comparison by student t-test.

2.3 Discussion

2.3.1 Demographics

Enrolling normotensives in the same age group as hypertensives is a difficult task. As is evident in Table 2-1, in this study there is a significant difference in age between the two groups. When age was adjusted for the correlation between PP length and BP disappeared. However, since age did not correlate significantly with PP length (Table 2-3), we still regard the unadjusted correlation as relevant.

2.3.2 Inflammatory markers

Though differences were not significant ($p = 0.205$, $p = 0.089$), mean values for both CRP and IL-6 were higher in the hypertensive group. This reflects previous findings of increased inflammatory markers in hypertensives [8, 9, 12]. Increased PP length in the hypertensive group ($p = 0.05$) was also expected. As was hypothesized, increased PP length may account for a fraction of pressure elevation in the vasculature [45-52].

High levels of pseudopod formation (elevated BP: 59.7 ± 18.3%, normal BP: 63.4 ± 19.9%), high variance, and no significant difference between groups point to a uniform activation of all stimulated cells. It is important to note that no negative controls—cells stimulated with PBS—presented any significant activation (< 5%). This rules out a uniform activation in any step of the protocol prior to stimulation, since all cells were treated identically up to that point. In general, previous studies to carry out the same

protocol did not show activation levels as high as we have reported [63-66]. While pseudopod formation was compared to a large database of physiological data, data on some well-known agonists were not available, including IL-8 and endotoxin. Hence, the possibility of endotoxin contamination cannot be ruled out.

There are multiple ways to consider the origins of pseudopod formation. The presence of a pro-inflammatory mediator or the absence/inhibition of an anti-inflammatory mediator could result in pseudopod formation. Alternatively, proteolytic activity may result in pseudopods by way of cleaving receptors, degrading mediators, or releasing inflammatory epitopes from the ECM termed matricryptic peptides. In this latter mechanism, proteases may release cytokines, oxidative products, or other pro-inflammatory fragments. Data has shown that digestive enzymes in pancreatic homogenates are capable of releasing such inflammatory epitopes, resulting in pseudopod formation on naïve neutrophils [63]. These experiments were carried out in shock models, where a failure of the mucosal lining of the small intestine allowed digestive enzymes to escape into the plasma and cleave a host of proteins. The exact mechanism by which our sample plasma induced pseudopodia on naïve cells remains to be determined.

2.3.3 Correlation to pseudopod length

Due to the high levels of activation, the discrete data set (PP formation) was not sensitive enough for our experiment. Therefore, continuous data (PP length) was collected and analyzed. There are a known set of agonists (N-formyl-methionyl-leucyl-

phenylalanine (fMLP)), platelet activating factor (PAF), leukotriene B4 (LTB4), C5a anaphylotoxin (C5a), and interleukin-8 (IL-8) that induce pseudopod via G-protein coupled receptor (GPCR) activation (formyl peptide receptor, IL-8 Receptor, platelet-activating receptor) [67, 68]. In addition, it is known that the rate of pseudopod extension is proportional to the concentration of the stimulant [67]. Since all cells in our experiment were stimulated for the same amount of time (10 min), pseudopod length could indeed be used as a marker of inflammation.

There are certainly consequences for increased pseudopod length in the microcirculation, as can be seen in the work of Fukuda et al [49]. Increased maximum diameter of the leukocyte will result in increased resistance, raising the BP. It will also raise the probability of leukocyte trapping in the capillaries [68]. The linear correlation between PP length and BP is, therefore, in line with our hypothesis and measurements.

2.3.4 Effect of lifestyle intervention

Patients whose diastolic BP reacted positively to the lifestyle intervention (by decreasing) also tended to have a decrease in PP length. Again, if we take PP length as a marker of inflammation, this then reflects data already reporting a decrease in inflammation following exercise intervention [34]. Additionally, from our analysis of the mechanical effects of pseudopods in the microcirculation, a drop in blood pressure can be associated with a decrease in pseudopod size. While systolic BP is related to the contraction of the heart, diastolic BP is more related to the innate perfusion pressure of the vasculature, and possibly to the peripheral vascular resistance (once an independent

measurement of cardiac output is obtained). This may explain why SBP does not predict change in PP length, but DBP may do so.

2.4 Conclusion

We have shown that factors in human plasma causing naïve neutrophils to form PPs are present in plasma of normo- and hypertensives. However, neutrophils stimulated by hypertensive plasma are more likely to form larger PPs. Further investigation is required in order to identify what in the plasma is causing PP formation and why its presence (or absence) might produce larger or smaller pseudopods. Finally, training-induced change in diastolic BP predicted change in PP length after a 12-week exercise intervention. This may indicate that PP formation is a mediator in how chronic exercise lowers BP, or that exercise lowers BP and in that environment, diminishes PP growth.

Chapter 3: Matrix Metalloproteinases

3.1 Methods

3.1.1 Participants

Sixty-two healthy, sedentary volunteers (age 25-60 years, 30 female) were screened for heart, liver, or renal disease, diabetes, psychosis, severe asthma, pregnancy, and current use of prescription drugs. This study is under a parent study investigating the effects of acute stressors, psychological and exercise-induced, and long-term lifestyle intervention in non-medicated hypertensive patients. Subjects were instructed not to use any medication 1 week prior to testing, and to avoid vigorous exercise, caffeine, alcohol, and smoking 24 hours prior to testing.

Subjects were grouped according to screening BP; Normal BP (N=10, 4 female) < 120/80 mmHg (SBP = 120.8 ± 9.9 mmHg, DBP = 74.2 ± 5.4 mmHg); Elevated BP (N=52, 26 female) > 120/80 mmHg (SBP = 142.9 ± 10.3 mmHg, DBP = 86.1 ± 8.4 mmHg). Subjects in the elevated BP group, but not the normal BP group, participated in the 12-week lifestyle intervention.

3.1.2 Baseline acute exercise testing

All patients performed baseline exercise tasks supervised by a certified cardiopulmonary technician under the supervision of an investigator and a physician.

Subjects completed a VO_{2peak} test on a treadmill using the standard Bruce protocol where the speed and grade of the treadmill increased by 1.7 mph and 10% every 3 min. Expired gas was analyzed by a Sensormedics metabolic cart equipped with Vmax software, and ECG was recorded using Marquette Cardiosoft (GE medical systems, Milwaukee, WI). Oxyhemoglobin saturation was monitored using pulse oximetry (Ohmeda, Datex, Lousiville, CO) and perceived effort was recorder using Borg's 6-20 rating of perceived exertion (RPE) scale [90]. Approximate 1 week after the peak exercise test, subjects returned to the laboratory for a 20-min steady-state exercise task. A 21-gauge catheter was placed in a forearm vein for blood collection. Subjects rested quietly for 20-min; resting ECG, BP were recorded and blood samples were drawn (into tubes containing EDTA and sodium heparin, Becton, Dickinson, and Co. (BD) vacutainers, Franklin Lakes, NJ) and stored at -80°C. The exercise task consisted of a 2-min warm-up after which speed and incline were gradually increased until subjects reached 65-70% VO_{2peak} at which point the exercise continued for 20-minutes. Throughout the task ECG, HR, respiratory exchange ratio (R) and oxygen saturation levels were monitored; RPE and BP were recorded every 3-min. A post-exercise blood sample was collected during the last minute of exercise (BD vacutainers). After 20-min subjects continued to walk for a 2-min cool-down period, after which they were seated for the remaining 30-min recovery.

3.1.3 Lifestyle Intervention

Please see Section 2.1.2

3.1.4 MMP-2 Assay

An electrochemiluminescence detection method (MSD Vascular Injury Panel II Assay, Meso Scale Discovery, Gaithersburg, MD), similar to the sandwich ELISA method, was used to detect human plasma matrix metalloproteinase-2 (MMP-2) levels. 96-well MULTI-ARRAY plates coated with antibody against MMP-2 were used (MSD). Subject plasma (EDTA, BD vacutainers) was diluted 2-fold in Diluent 2 (MSD). 25 μL of Diluent 2 was added to each well and allowed to incubate at room temperature for 30 min on the shaker (IKA). After the addition of 25 μL sample or calibrator, plates were washed 3 times with PBS-T (Multiwash III, TriContinent). 25 μL of antibody conjugated to SULFO-TAG label (Ruthenium (II) tris-bypyridine-(4-methylsulfonate) NHS ester, Detection Antibody Solution, MSD) was added followed by a 2 hour incubation at room temperature on the shaker. The wash cycle was repeated and 150 μL of 2X Read Buffer T (MSD) was added. Plates were immediately analyzed using the SECTOR Imager 2400 (MSD). All samples and calibrators were run in duplicate.

3.1.5 MMP-8,9 Assay

A Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) was used to detect human plasma matrix metalloproteinase-8 and -9 (MMP-8,-9) levels. 96-well polystyrene microplates coated with a mouse monoclonal antibody against MMP-8 or -9 were used (R&D Systems, Inc., Minneapolis, MN). Subject plasma (sodium heparin, BD vacutainers) was diluted 20-fold (MMP-8) or 40-fold (MMP-9) in calibrator diluent (R&D). Samples, standards (R&D), or controls (R&D) were added to assay diluent (50 μL sample in 150 μL diluent for MMP-8, 100 μL in 100 μL for MMP-9, R&D) and left

to incubate for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 rpm (IKA). The plate was washed (Multiwash III, TriContinent) then excess fluid was removed by tapping plate on paper towel. 200 μL of polyclonal antibody against MMP-8 or -9 conjugated to horseradish peroxidase was added to each well, followed by another incubation on the shaker (2 hrs for MMP-8, 1 hr for MMP-9). The wash step was repeated and 200 μL of substrate solution (R&D) was added to each well, followed by 30 min incubation on the benchtop protected from light. 50 μL of stop solution (2N Sulfuric Acid, R&D) was then added and optical density was measured within 30 min on a spectrophotometer (Spectramax 340PC348, Molecular Devices) at 450 nm with wavelength correction set to 570 nm. All samples, standards, and controls were run in duplicate.

3.1.6 MMP Activity, Gelatin Zymography

Two 10% polyacrylamide gels with 0.8 mg/mL gelatin were prepared for each run-through. A subset of plasma samples (N=10, sodium heparin, BD vacutainers) were diluted 1:25 in 1X PBS. 7.5 μL diluted sample was added to 2.5 μL loading buffer (Comassie Blue, Sigma) before loading (Mini-PROTEAN Tetra Cell, BioRad, Hercules, CA), giving each well 0.3 μL of plasma sample. Protein marker (BenchMark, Invitrogen, Carlsbad, CA) was added in the first lane, MMP-2 standard (80 pg/mL, Calbiochem, EMD Group, Merck, Darmstadt, Germany) in the last, and samples in the middle. Gels were run in glycine buffer at a constant voltage (120 V, BioRad) for 1-1.5 hrs. The gels were then removed and agitated in renaturing buffer (2.5 % v/v Triton-X100, Fisher Scientific, Pittsburgh, PA) four times, adding fresh buffer every 15 min. Following

protein renaturing, gels were placed in developing buffer (0.6055% w/v Tris base, Sigma, 1.169% w/v NaCl, Fisher, 0.00007% w/v $ZnCl_2$, Sigma, 0.074% w/v $CaCl_2 \bullet 2H_2O$, Fisher, 0.02% w/v NaN_3, Sigma, pH 7.5) overnight at 37°C. Gels were stained (50% v/v methanol, Fisher, 20% v/v acetic acid, Fisher, 0.5% w/v Coomassie Blue) for at least 30 min, and then destained (50% v/v methanol, 10% v/v acetic acid) until clear bands were visible (Figure 3-1). Gels were scanned (CanoScan LiDE 200, Canon, Tokyo, Japan) and analyzed for integrative density in ImageJ (NIH).

3.1.7 IL-6, CRP Assays

Please see Sections 2.1.4, 2.1.5

3.1.8 Statistical Analysis

Results are presented as mean ± standard deviation. Data were analyzed in SPSS (SPSS Inc., IBM, Armonk, NY). Differences between BP groups were analyzed by student t-test. Correlations were analyzed using Pearson's Correlation. A repeated measures generalized linear model analyzed response to acute exercise and training. A value of $P<0.05$ was considered significant.

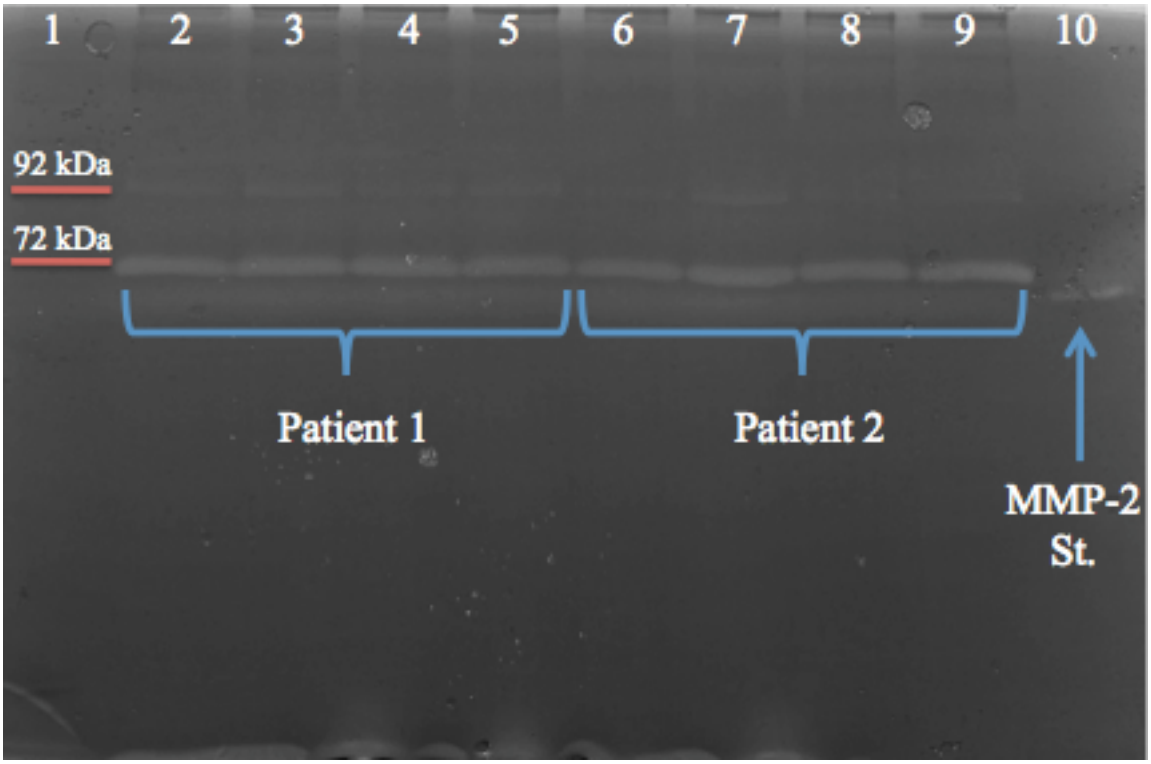

Figure 3-1. MMP activity bands. In the gelatin zymography protocol, active MMPs degrade gelatin leaving clear spots of no stain. The protein ladder is in well 1, plasma samples occupy wells 2-9, and the MMP-2 standard is in well 10. The brightest activity band is MMP-2 (72 kDa); the dimmer band above is MMP-9 (92 kDa).

3.2 Results

3.2.1 Demographics

Participants in the Elevated BP group were similar to those in the Normal BP group in regard to gender, BMI and VO2peak, but were slightly older and as expected had higher resting SBP and DBP (Table 3-1). All correlations were corrected for age.

3.2.2 Baseline Inflammatory Marker, Matrix Metalloproteinase Levels

Plasma IL-6, CRP, MMP-2, -8, and -9 levels were assessed at baseline (Table 3-2). Neither CRP nor IL-6 showed significant difference among groups. None of the baseline MMP levels were significantly different.

3.2.3 Correlations to MMP Levels

A partial correlation adjusting for age was used to search for associations between demographic, physiological variables and MMP plasma concentrations (Table 3-3). There was a negative correlation between MMP-2 and CRP that was significant for all points except baseline; after removing outliers the correlation still stands (Figure 3-2). MMP-9 and IL-6 levels did not associate with CRP before the intervention, but both significantly correlated after. Finally, MMP-2 and -9 positively correlated with MMP-8 at baseline ($p = 0.035$, $p = 0.017$) and MMP-8 and -9 correlate after acute exercise ($p = 0.002$).

3.2.4 MMP response to acute exercise

A repeated measures generalized linear model was used to assess the effect of acute exercise and training on MMP levels. Acute exercise resulted in significantly increased levels of MMP-8 and -9, but not -2 (MMP-8, $F(1,35)=34.587$, $p = 0.000$; MMP-9, $F(1,38)=29.653$, $p = 0.000$; MMP-2, $F(1,34)=3.232$, $p=0.081$; Figure 3-3, 3-4, 3-5).

3.2.5 MMP response to long-term intervention

There was no significant response in MMP concentration to long-term intervention, and no effect of the different intervention groups (MMP-2, $F(1,34)=0.083$, $p = 0.775$; MMP -8, $F(1,35)=0.267$, $p = 0.609$; MMP-9, $F(1,38)=0.656$, $p=0.423$). Additionally, there was no significant difference in MMP response to acute exercise before and after training.

3.2.6 MMP Activity

Activity was calculated as a fraction of the intensity of the baseline band (Table 3-5, Figure 3-6). There were no significant trends observed. When divided into exercise (N=6) and diet and exercise (N=4) groups, there will still no significant trends found (Figure 3-7).

Table 3-1. Patient and Control Demographic Parameters

	Elevated BP (N=52)	**Normal BP (N=10)**	**t-test Sig. (p)**
Gender	26M, 26F	6M, 4F	--
BMI (kg/m^2)	30.6 ± 3.7	34.3 ± 3.1	0.091
Age (years)	46.6 ± 9.0	38.6 ± 9.2	0.013*
$VO_{2\text{-}Peak}$ (ml/kg/min)	27.7 ± 7.3	32.2 ± 9.3	0.098
SBP (mmHg)	142.9 ± 10.3	120.8 ± 9.9	0.000*
DBP (mmHg)	86.1 ± 8.4	74.2 ± 5.4	0.000*

Mean ± SD, *statistically significant in a comparison by student t-test.

Table 3-2. Markers of Inflammation, MMP Concentrations in BP Groups

	Elevated BP (N=52)	**Normal BP (N=10)**	**t-test Sig. (p)**
CRP (pg/ml)	4.65 ± 5.08	3.09 ± 2.93	0.352
IL-6 (pg/ml)	4.67 ± 5.25	3.31 ± 2.33	0.426
MMP-2 (ng/ml)	140.7 ± 26.9	144.7 ± 19.4	0.697
MMP-8 (ng/ml)	1.74 ± 1.05	1.81 ± 0.79	0.855
MMP-9 (ng/ml)	27.8 ± 13.2	28.2 ± 12.7	0.941

Table 3-3. Demographic, Physiological Correlations to MMP Concentration

V1 Pre Ex	MMP-2	MMP-8	MMP-9	IL-6	CRP
MMP-2 (ng/ml)	1	0.320*	0.132	0.161	-0.200
MMP-8 (ng/ml)		1	0.358*	0.112	-0.007
MMP-9 (ng/ml)			1	0.133	-0.123
IL-6 (pg/ml)				1	0.012
CRP (pg/ml)					1
V1 Post Ex	MMP-2	MMP-8	MMP-9	IL-6	CRP
MMP-2 (ng/ml)	1	0.012	-0.103	0.477**	-0.307*
MMP-8 (ng/ml)		1	0.276	0.044	0.043
MMP-9 (ng/ml)			1	-0.035	0.033
IL-6 (pg/ml)				1	-0.052
CRP (pg/ml)					1
V2 Pre Ex	MMP-2	MMP-8	MMP-9	IL-6	CRP
MMP-2 (ng/ml)	1	-0.098	-0.065	-0.076	-0.543**
MMP-8 (ng/ml)		1	0.288	0.065	-0.027
MMP-9 (ng/ml)			1	0.119	0.400*
IL-6 (pg/ml)				1	0.499**
CRP (pg/ml)					1

R = Partial correlation coefficient.
*$p < 0.05$; **$p < 0.005$
Pre and post exercise (PreEx, PostEx) are before and after acute exercise tasks.
Visit 1 (V1) and Visit 2 (V2) are before and after lifestyle intervention.

Table 3-3 Continued

V2 Post Ex	MMP-2	MMP-8	MMP-9	IL-6	CRP
MMP-2 (ng/ml)	1	-0.079	0.063	-0.285	-0.445*
MMP-8 (ng/ml)		1	-0.130	0.196	0.012
MMP-9 (ng/ml)			1	0.142	0.434*
IL-6 (pg/ml)				1	0.492**
CRP (pg/ml)					1

R = Partial correlation coefficient.
*$p < 0.05$; **$p < 0.005$
Pre and post exercise (PreEx, PostEx) are before and after acute exercise tasks.
Visit 1 (V1) and Visit 2 (V2) are before and after lifestyle intervention.

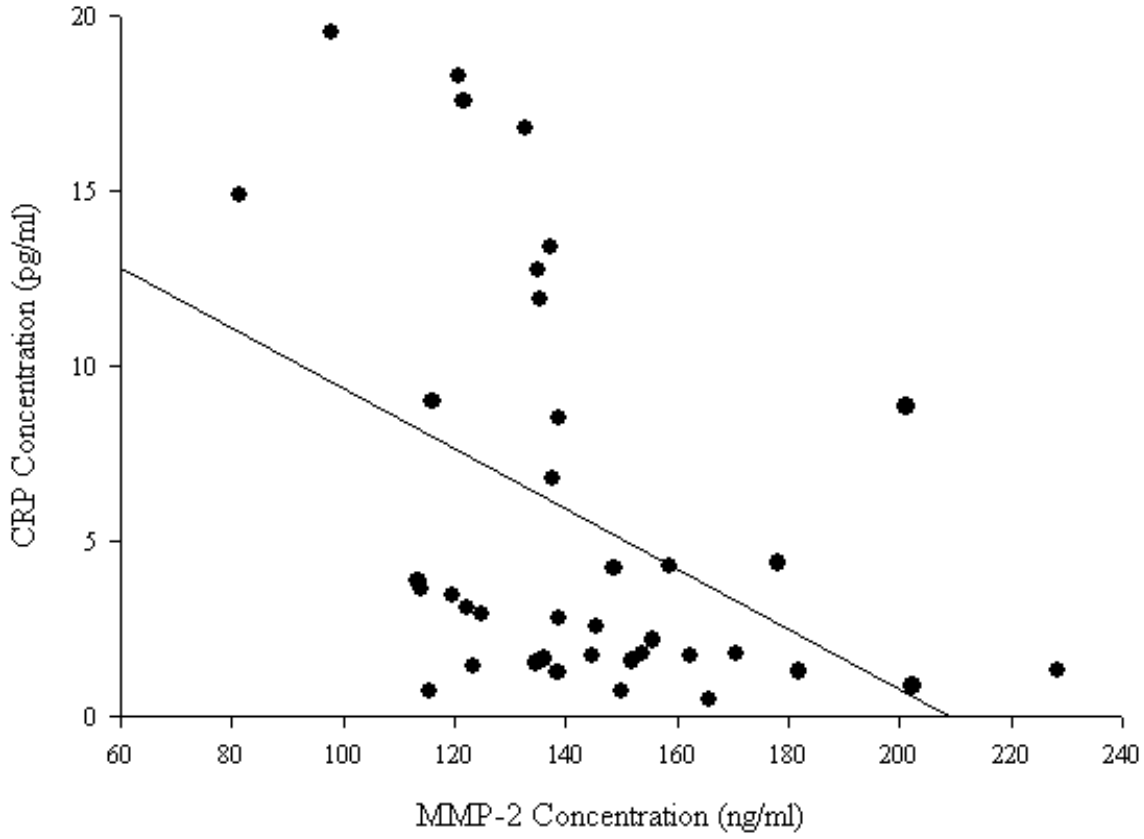

Figure 3-2. Baseline, post-intervention MMP-2 vs. CRP. There is a negative correlation between the gelatinase and CRP ($R = -0.543$, $p = 0.002$).

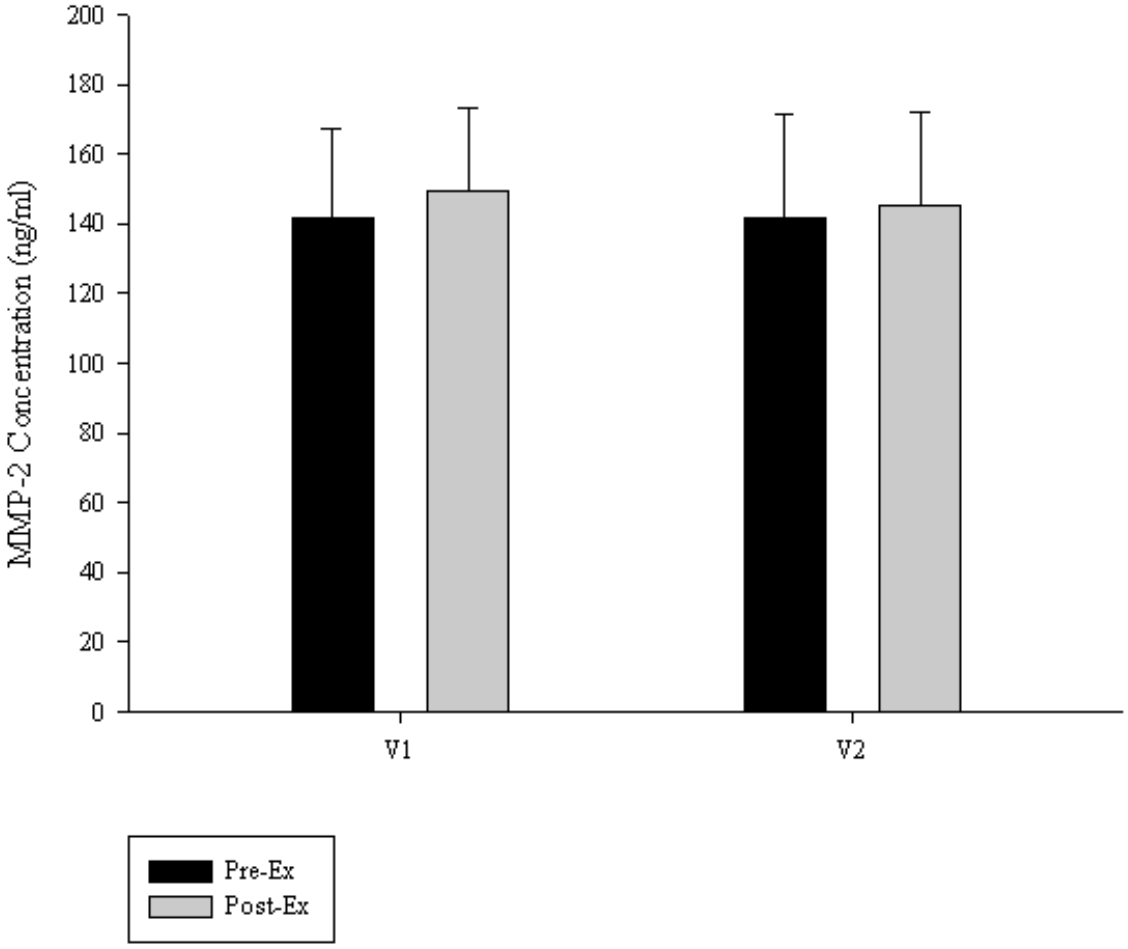

Figure 3-3. MMP-2 concentration. Concentration increased after acute exercise before (visit 1, V1) and after (visit 2, V2) training. Increase was not significant ($p = 0.081$). Mean ± SD.

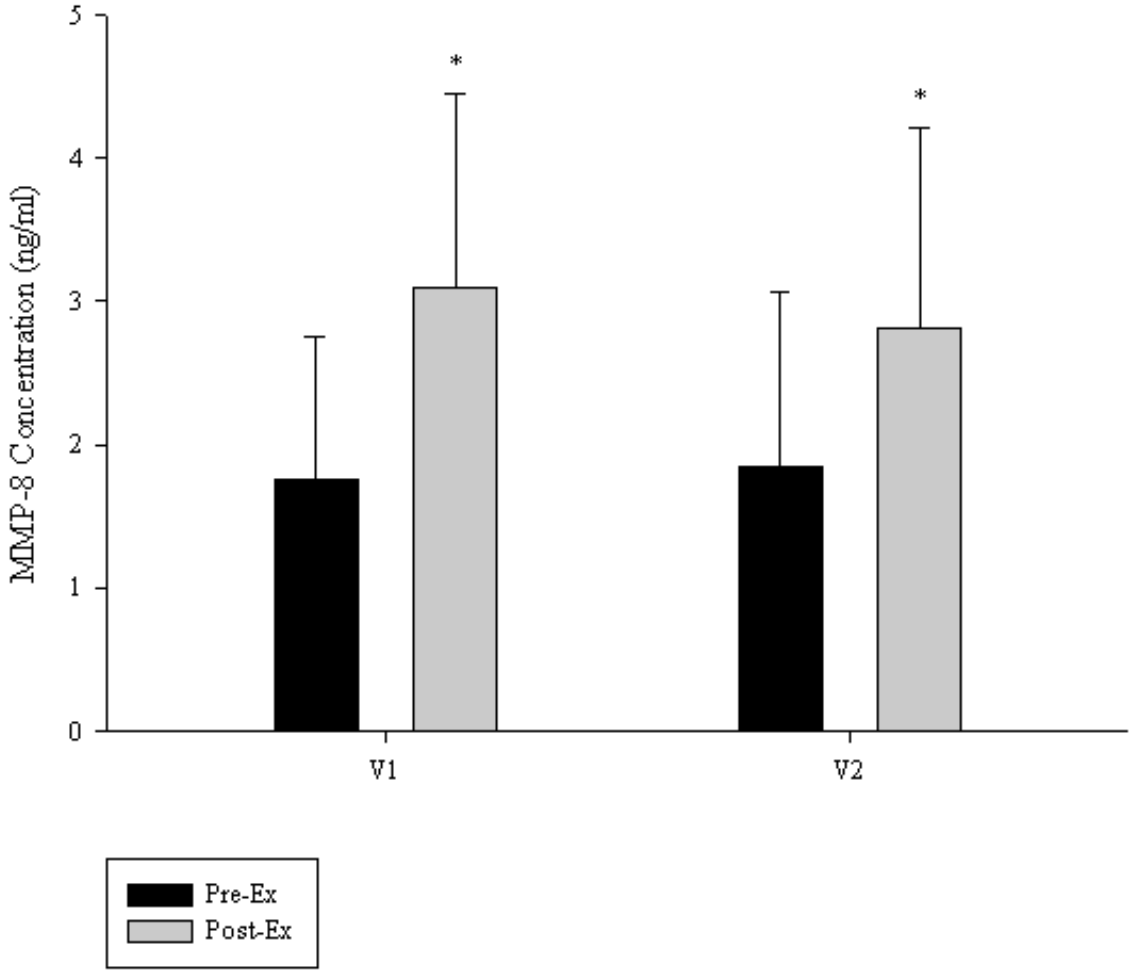

Figure 3-4. MMP-8 concentration. Concentrations increased after acute exercise, before and after training (V1, V2, *$p<0.001$). Mean ± SD.

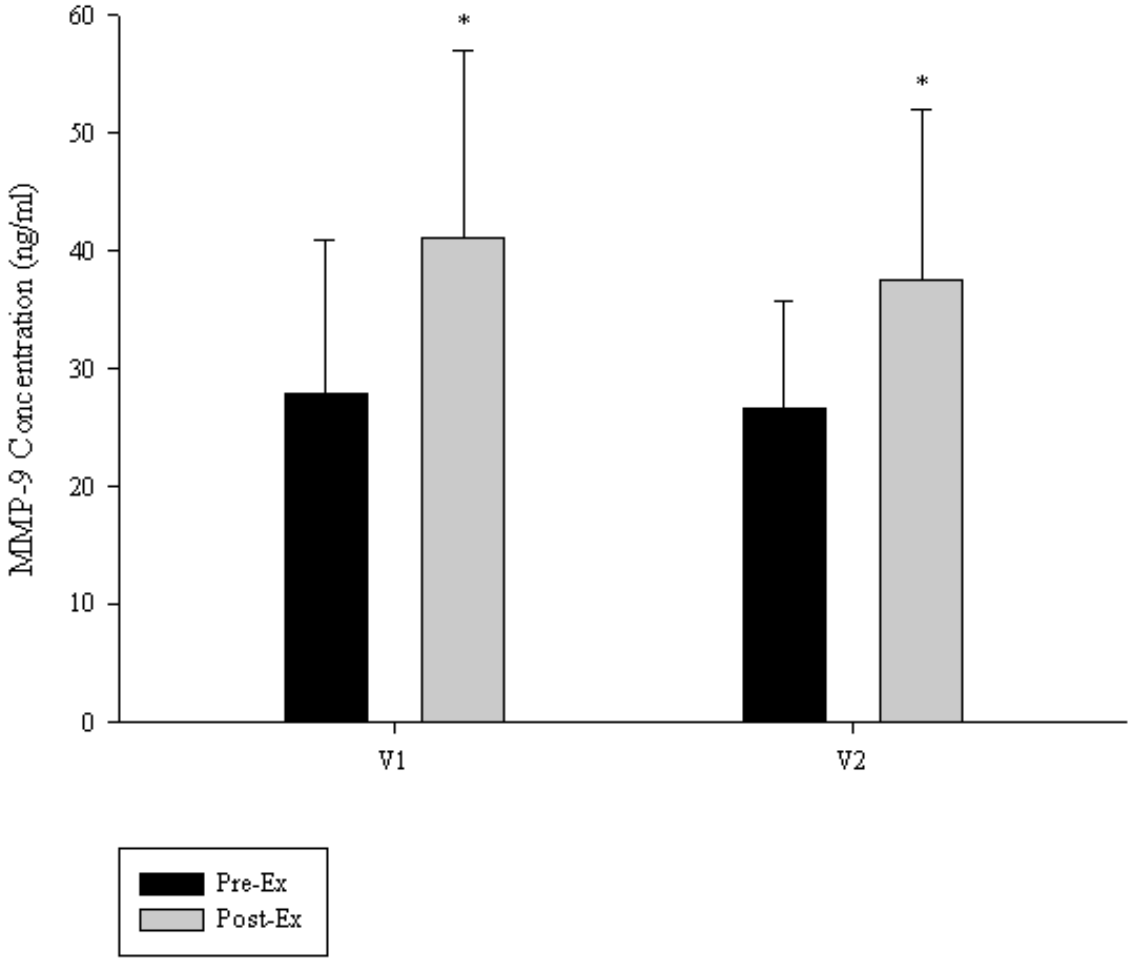

Figure 3-5. MMP-9 concentration. Concentrations increased after acute exercise, before and after training (V1, V2, *$p<0.001$). Mean ± SD.

Table 3-4. MMP Concentration Intra-assay, Inter-assay Coefficient of Variance (CVs)

	Intra-assay CV (%)						Inter-assay CV (%)
	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	
MMP-2	8.6 ± 8.5	4.9 ± 3.3	7.9 ± 7.5	3.2 ± 2.9	2.0 ± 2.3	---	5.3 ± 2.9
MMP-8	12.5 ± 9.5	8.4 ± 9.4	8.6 ± 9.7	5.7 ± 2.9	5.6 ± 4.5	14.9 ± 8.8	9.3 ± 3.7
MMP-9	12.3 ± 13.4	6.8 ± 6.4	4.2 ± 5.2	3.2 ± 2.9	7.7 ± 3.9	3.1 ± 3.3	6.2 ± 3.5

Mean ± SD.

Table 3-5. MMP activity before and after acute exercise (PreEx, PostEx) before and after a lifestyle intervention (V1, V2)

	V1 Pre-Ex	V1 Post-Ex	V2 Pre-Ex	V2 Post-Ex
V/(V1 PreEx)	1	0.992 ± 0.026	0.980 ± 0.044	0.985 ± 0.048

Mean ± SD.
Activity is expressed as a ratio of the time point relative to the baseline value.

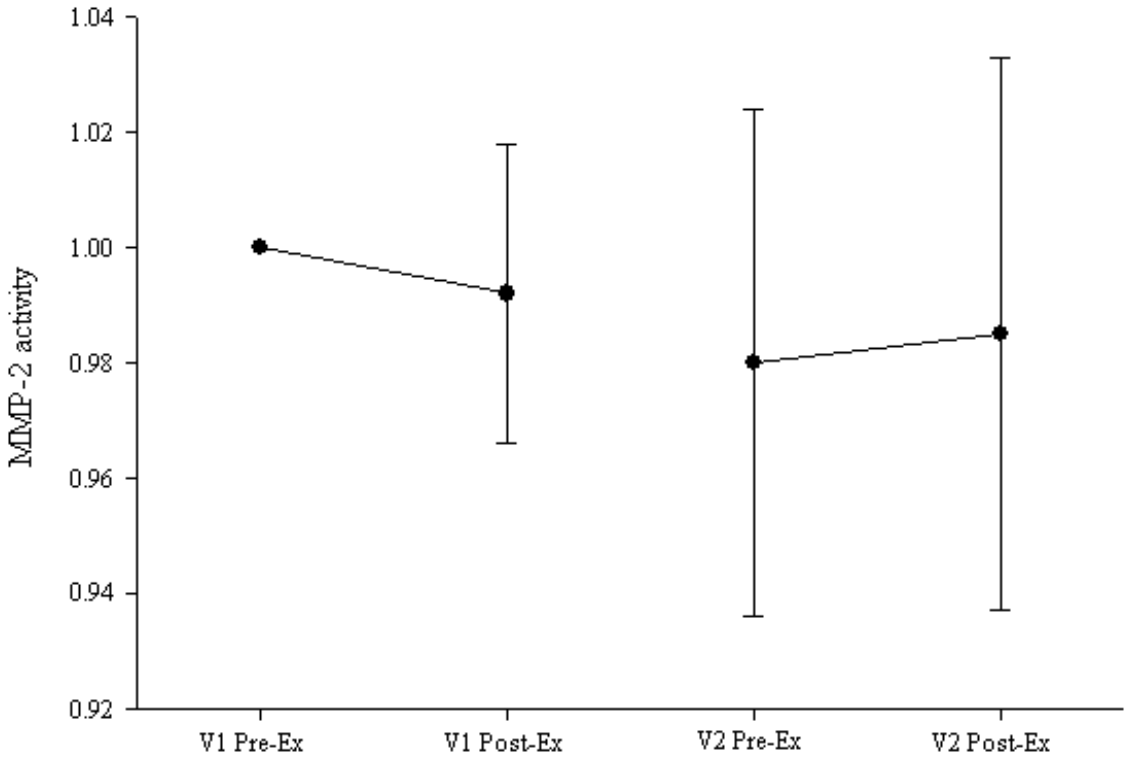

Figure 3-6. Effect of acute exercise, lifestyle intervention on MMP activity. Results from Table 3-5 in graphical form. Mean ± SD.

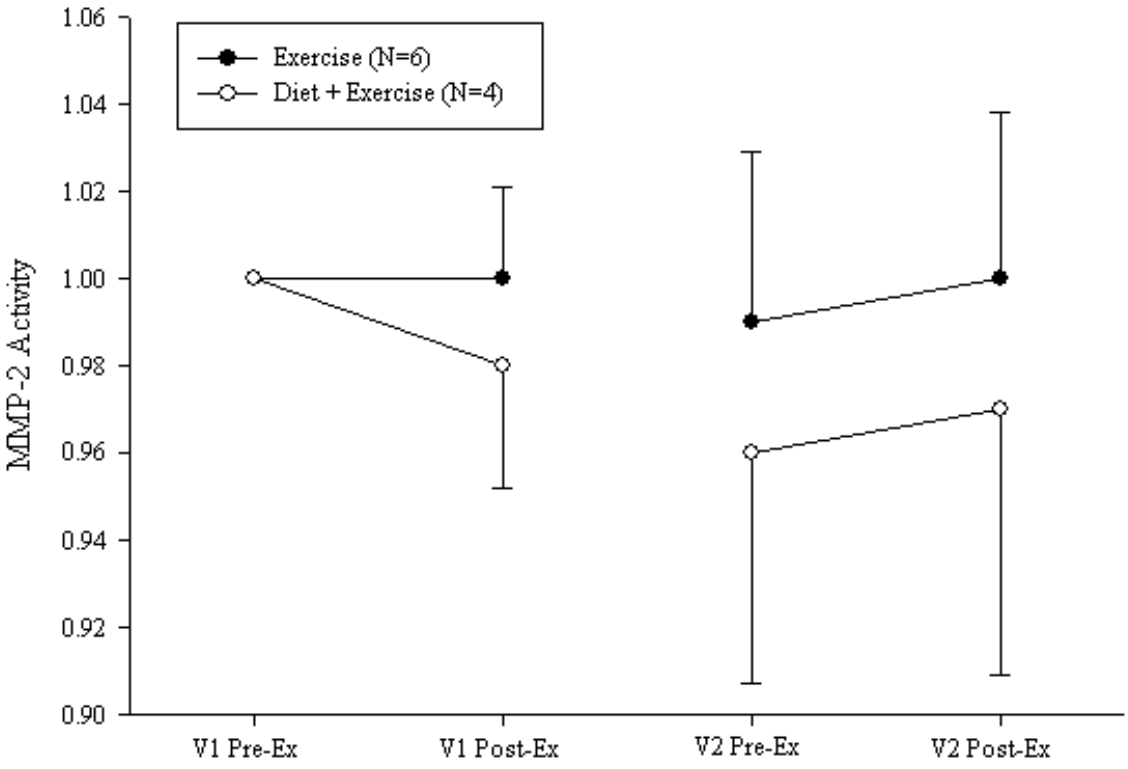

Figure 3-7. Acute, training response of exercise vs. diet and exercise group. Results from Figure 3-6 split into exercise and diet and exercise groups. Mean + SD.

3.3 Discussion

3.3.1 MMPs in Hypertension

MMP levels were not significantly different in the hypertensive and normotensive groups. This does not fall in line with our hypothesis that hypertensives would have increased proteolytic activity. Still, the smaller normotensive group (N = 10) was composed of sedentary participants in order to match the lifestyle of the hypertensive group. It is possible that a healthier control group may have decreased protease levels. It should also be noted that many of the samples used were frozen (-80°C) for over a year's time. There is a concern that MMP concentrations and activity may degrade over long periods of time in the freezer, though this has not been confirmed. If there is protein degradation taking place, it may affect the results obtained.

3.3.2 Correlations to MMP Concentrations

Partial correlations controlling for age showed an association between MMP-8 and MMPs -2 and -9. Many MMPs can cleave the pro-domains of other MMPs and one might expect their concentrations to correlate. Within the group of measured MMPs, however, only MMP-2 is known to cleave the pro-domain of MMP-9, and these two did not correlate. Previous data has shown correlations between MMP-2 and MMPs -1, -3, and -10 [78]. All of these correlations to MMP-2 implicate it in the regulation of other proteinases in the family. Indeed, correlations between MMPs generally suggest co-regulation, whether it is by mediators within or outside of the protein family.

Our data also showed MMP-9 and IL-6 correlated to CRP after the intervention, but not before. The correlation between CRP and IL-6 is not altogether surprising, since both of these proteins are known markers of inflammation. In fact, IL-6 and other cytokines promote the production of CRP by the liver. The correlation between CRP and MMP-9 further implicates the gelatinase in the inflammatory process. Finally, the negative correlation between MMP-2 and CRP is most interesting. It is possible that MMP-2 is degrading CRP, rendering it undetectable. This opens the door to a number of questions regarding the activity of proteases in plasma samples, and how they may affect the data gathered from them.

3.3.3 MMPs and acute exercise

A Repeated Measures Linear Model showed that all MMP concentrations increased following an acute exercise task, though MMP-2 was not significant. The MMP-9 increase is in line with previously published data [85, 91], though, to our knowledge, we are the first to report an increase in MMP-8 following acute exercise. Short-term exercise causes damage to the skeletal muscle and connective tissue, which can lead to acute inflammation and MMP activation [28, 91].

3.3.4 MMPs and long-term exercise

Contrary to our hypothesis, MMP levels did not decrease following a 12-week lifestyle intervention. This does not match data from Roberts et al [87, 88], which showed which found that exercise interventions decreased baseline MMP-9 levels, though their intervention only lasted 2-3 weeks. Again, it is possible that differences in

patient populations account for different findings. Our population was unique in that it was sedentary and unmedicated; other differences can be found.

3.3.5 MMP response to acute exercise after intervention

We hypothesized that the MMP response to acute exercise is attenuated by a lifestyle intervention. While this hypothesis is supported only as a trend by the MMP-8 measurements ($p = 0.076$), it is not significant for any of the MMPs tested. As is with our hypothesis that baseline levels of MMPs is reduced after training, there is no difference in MMP response to acute exercise and no support for this hypothesis from the current measurements. It should be noted that MMPs have both pro- and anti-inflammatory roles [92]. Therefore, unaffected MMP concentrations do not necessarily indicate that there was not an overall decrease of inflammation.

3.3.6 MMP Activity

One obvious limitation of measuring MMP concentrations only is that concentration does not necessarily translate to activity. For this reason, we carried out gelatin zymography experiments to detect gelatinase activity (MMP-2, -9). Still, due to the denaturing and subsequent renaturing of proteins in the gel, any inhibitory activity (TIMPs) were not detected in the assay.

Using the protein ladder and confirming with a gel run in the presence MMP inhibitor (20mM EDTA, Sigma), MMP-2 and -9 bands were identified, but only MMP-2 bands were strong enough to be analyzed. The MMP-2 standard run with each gel did

not produce a consistent band. This is in part due to small-volume pipetting error, but also due to degradation of MMP activity when stored at -20°C. In lieu of using the standard to normalize activity on different gels, bands were expressed as a fraction of the activity of the baseline sample. From this data, individual response to acute exercise pre- and post- training could be analyzed. As can be seen in Figure 3-6, response to acute exercise and training varied greatly, with some participants increasing activity and some decreasing. Still, given the small sample size, it should be noted that the training did seem to reduce MMP activity, though not significantly ($p = 0.232$). When separated into intervention groups, the diet and exercise group showed a greater decrease in activity than the exercise group. Additionally, the diet and exercise group decreased in activity after pre-training acute exercise, again, not significantly ($p = 0.209$, Figure 3-7). While none of these data are significant, with an increased sample size it is possible that these trends will become stronger. These results indicate that long-term exercise may decrease MMP activity, and that a diet and exercise intervention does this more effectively than just an exercise intervention. This suggests that a diet aimed at reducing blood pressure may have an inhibitory effect on MMP activation.

3.4 Conclusion

We have shown a number of correlations between MMPs and inflammatory markers, especially a negative correlation between MMP-2 and CRP. It has been suggested that MMP-2 and other proteases may degrade proteins such as CRP in plasma samples, possible skewing our reading of plasma protein levels. MMP-8 and-9 concentrations have been shown to increase in response to acute exercise. In response to a 12-week lifestyle intervention, there were no significant findings. MMP-2 activity data shows a possible decrease in activity in response to lifestyle intervention, though not significantly. A larger sample size is necessary.

Chapter 4: General Discussion

4.1 Summary of Results

This project has shed new light on physiological mechanisms in human hypertension and it's interaction with acute exercise, and lifestyle intervention. We have shown that naïve neutrophils stimulated with plasma from hypertensives form larger pseudopods than pseudopods formed after stimulation with plasma from normotensives. This data supports existing research reporting increased inflammation in human hypertension. In addition, these results fit with the proposed mechanical mechanism for pressure elevation in the microvasculature and increased total peripheral resistance. We also demonstrate a number of correlations found between MMPs and inflammatory markers, specifically a negative correlation between MMP-2 and CRP. It has been proposed that MMP-2 degrades CRP, rendering it undetectable. To the limited research on MMPs in exercise, we add that acute exercise significantly increases MMP-8, and confirm that it increases MMP-9 concentration.

4.2 Limitations

4.2.1 Naïve neutrophil pseudopod experiment

There are a number of limitations in the experiments conducted. The primary issue with the naïve neutrophil protocol is that it is an in-vitro experiment. It would be

preferable to search for pseudopods on homologous neutrophils from normotensives and hypertensives, however, this is problematic for a number of reasons. Most importantly, neutrophils drawn straight from a hypertensive patient comes from venous blood. A neutrophil that has successfully navigated through the microcirculation, venules and veins is probably not an activated cell, so is relatively unlikely to have pseudopods, or any other form of activation. The leukocytes that are suspected of being involved in inflammation and pressure elevation are cells that are likely trapped in the capillaries or bound to the endothelium. Since arterial blood cannot be drawn from human patients without heavily invasive techniques, the naïve experiment is a worthy starting point. Still, it is understood that the sort of activation seen in the naïve samples are much higher than what may be seen in the blood.

4.2.2 MMP Concentration Assay

For the MMP concentration assay, there were few limitations. Coefficient of variance values were within a reasonable range (Table 3-4). It is possible, however, that the variances observed were larger than the differences between hypertensive and normotensive groups. In other words, in human hypertension, the effect of elevated protease levels may be diminutive, but happen over a long period of time. Significantly increased protease concentrations in hypertensive rats help to show a possible mechanism for the disease, but are not representative of the condition in humans. Therefore, if a hypertensive has an 8% elevation in protease concentration, as compared to control, then the coefficients of variance from our experiment would not be acceptable. Still, this small increase may ultimately have consequences.

4.2.3 MMP Activity

The limitations of the MMP activity experiment have been discussed to some extent (Section 3.3.6). In essence, sample size must be increased before any conclusions can be made. The increase in sample size may diminish the variance, though it is possible that MMP activity is a trait that is particularly variable in human populations.

4.3 Future Studies

4.3.1 Homologous neutrophil pseudopod expression

While some questions have been answered by this project, others remain open to question. In the realm of the pseudopod experiment, it would be useful to conduct the experiment with homologous neutrophils. In spite of the limitations of this method previously discussed, if there are factors in the plasma that can induce pseudopod formation, these factors will be present in the venous circulation as well. Though activation of leukocytes from venous blood would likely be reduced compared to cells upstream, it is still possible that a difference between hypertensive and normotensive groups would be observed.

4.3.2 Receptor cleavage on naïve neutrophils

Another an interesting follow-up to the naïve neutrophil experiment would be to assess receptor density on naïve neutrophils after stimulation. Flow cytometery or other

techniques can be used to quantify insulin receptor, formyl peptide receptor, β_2 adrenergic receptor, or vascular endothelial growth factor receptor-2 density. If there is receptor cleavage through elevated protease levels / activity the incubation should result in greater reduction of receptor density when stimulated with plasma from hypertensives. This would shed light on the MMP aspect of this project as well. Though we did not observe increased MMP concentration in hypertensives, it is possible that other proteases are involved in receptor cleavage. Perhaps most interesting is the case of the formyl peptide receptor (FPR), since it functions in the retraction of pseudopods. Decreased extracellular FPR density on neutrophils exposed to hypertensive plasma would be a plausible explanation to the data reporting longer pseudopods on such cells.

4.3.3 General protease activity

MMP-2, -8 and -9 showed no relation to hypertension, though these are only three proteases among myriad of others in the body. General protease levels/activity assays should be run on hypertensive and control plasma. While colorimetric and chromogenic protease activity assays were attempted, with little success, it is possible that a fluorometric assay will produce smaller intra-sample variance.

4.3.4 MMP activity

The MMP activity assay must be run with a greater sample size. The gelatin zymography protocol is tedious and perhaps not best suited for the number of samples generated by clinical trials. For such large sample sizes there are assay kits that measure

MMP activity, though they are costly. Still, this type of large-scale analysis could better reveal trends in the patient population.

4.4 Conclusion

The current studies shed new light on the mechanisms by which hypertension progresses. The current experiments bring to light the possibility that there may be an abnormal activation of leukocytes in hypertension that may be affected by exercise and lifestyle choices. Future treatments of hypertension may have to focus not only on decreasing blood pressure but also on the accompanying inflammation, whether it be leukocyte activation, protease activity, or any other inflammatory mechanism. Once these processes are better understood, the possibility of new and more effective therapies can be explored.

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