ABSTRACT

Title of Document: THE EFFECT OF A 10 DAY CESSATION OF TRAINING IN OLDER ENDURANCE ATHLETES ON PATHOLOGICAL PRODUCTION OF NITRIC OXIDE AND REACTIVE OXYGEN SPECIES LEVELS IN CIRCULATING ANGIOGENIC CELLS

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Cardiovascular disease (CVD) is a serious disease, and is the leading cause of death in the United States. Aging and physical inactivity are two well-established risk factors for CVD. Previous research has identified circulating angiogenic cells (CACs) as a novel risk factor for CVD. CAC number and function are affected by aging and exercise. Nitric oxide (NO) and reactive oxygen species (ROS) are intracellular compounds which can affect the health of the vasculature and are also affected by exercise. Endothelial nitric oxide synthase (eNOS) is responsible for NO production within the endothelium, and eNOS "coupling" is a phenomenon that plays a role in the balance between production of ROS and NO. The literature also indicates that NO can be produced in either a pathological or physiological capacity depending on which isoform of NOS produces it. NO and ROS have been previously measured in CACs and have

been shown to affect *in vivo* and *in vitro* outcomes related to vascular function. Although NO, ROS, and CACs have all been studied in relation to exercise, no previous studies have examined how the cessation of training in older endurance-trained athletes affects these intra-cellular compounds through the eNOS-coupling pathway. The purpose of this study was to examine the effect of a 10-day cessation of exercise training in older endurance-trained athletes on ROS, NO and the eNOS-coupling pathway in CD34+ cells. NO and ROS were measured in isolated fresh CD34+ cells using fluorescent dye assays. The mRNA expression of genes involved in the eNOS-coupling pathway (endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), dihydrofolate reductase (DHFR), and guanosine triphosphate cyclohydrolase 1 (GTPCH1)) were measured using semi-quantitative Polymerase Chain Reaction (Semi-qt PCR). Flow mediated dilation was measured to gain information about endothelial function. No significant differences were detected after the cessation of training in CD34+ intracellular NO or ROS levels. Flow mediated dilation (FMD) also did not change significantly following cessation of training. eNOS mRNA expression was significantly lower following cessation of training but iNOS, DHFR, and GTPCH1 did not change. Taking into consideration the current literature, we expected to see changes in all of the above variables with the cessation of exercise training. There were several limitations present in our study, which could have affected our outcomes. Research in this area, specifically the eNOS coupling pathway, is still very new and this study shows that additional research is still needed to elucidate the underlying mechanisms in CACs.

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List of Abbreviations

ADMA	Asymmetric dimethylarginine
AMI	Acute myocardial infarction
BART	Brachial artery ultrasound test
BH2	Dihydrobiopterin
BH4	Tetrahydrobipterin
BMI	Body mass index
CAC	Circulating angiogenic cell
CAD	Coronary artery disease
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DEXA	Dual energy x-ray absorptiometry
DHFR	Dihydrofolate reductase
EDD	Endothelium dependent dilation
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
FACS	Fluorescent activated cell sorting
FMD	Flow mediated dilation
GTPCH1	GTP cyclohydrolase 1
HPLC	High performance liquid chromatography
iNOS	Inducible nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
ONOO-	Peroxynitrite
PBMC	Peripheral blood mononuclear cells
qt-PCR	Semi-quantitative polymerase chain reaction
RER	Respiratory exchange ratio
ROS	Reactive oxygen species
RT	Reverse transcriptase
SBP	Systolic blood pressure
SDF-1	Stromal derived factor-1
VEGF	Vascular endothelial growth factor
VO2MAX	Maximal oxygen uptake

Chapter 1: Introduction:

Cardiovascular disease (CVD) is the number one cause of death in the United States and accounted for nearly ¼ of American deaths in 2010 (Murphy et al 2013). Many Americans have adopted a sedentary lifestyle and as our population ages, the risk of developing CVD increases. Over 30% of the adult population in the U.S. report that they don't take part in any leisure time activity (Barnes 2012). This is especially troubling when it is well known and accepted among the scientific community that the incorporation of physical activity into a person's lifestyle can substantially reduce all-cause mortality (Blair et al 1995).

Researchers have established several major risk factors for developing CVD, including smoking, high cholesterol, sedentary lifestyle and age. This study will focus on the effects of aging and exercise (or lack thereof). While the academic community agrees that sedentary behavior and aging are risk factors for developing CVD, current research must focus on the underlying mechanisms contributing to risk. Understanding the underlying pathology can lead to important advances in therapeutic approaches. Mechanistic data have expanded over the past ten years and the vasculature continues to be an important area to investigate in relation to the risk factors of sedentary behavior and aging.

Optimally functioning blood vessels play a critical role in cardiovascular health. The risk factors discussed above can have a direct impact on important vascular functions such as vasodilation and vasoconstriction. When the vasculature is negatively affected by one of these risk factors, diseases such as hypertension can arise. One major risk factor which can adversely affect a person's blood pressure is sedentary behavior. The SUN

cohort study prospectively examined the association between sedentary lifestyle and hypertension in middle-aged participants (Beunza et al 2007). They concluded that interactive sedentary behaviors (driving or computer use), were positively associated with incidence of hypertension. The SUN cohort study results suggest that individuals who lead a sedentary lifestyle, are at increased risk for hypertension (Beunza et al 2007). Conversely, physical activity is known to reduce blood pressure, and can be an effective treatment for hypertension. A review by Hagberg et al (2000) stated that exercise training can reduce blood pressure in about ¾ of hypertensive patients. Additionally, low intensity training was found to be sufficient to elicit such changes, making this information very clinically relevant (Hagberg et al 2000). Thus, regular physical activity is essential to maintaining optimally functioning blood vessels.

The endothelium plays an important functional role within the vasculature. One of its primary roles is to control the tone of blood vessels through its release of relaxing and contracting factors (Vanhoutte 1996). The balance of these factors can be negatively affected in pathological states such as hypertension (Puddu et al 2000). Given its direct impact on hypertension, it is not surprising that exercise also plays a role in endothelial function. Endothelium-dependent dilation is often used as a measure of endothelial function, given its importance in maintaining blood pressure homeostasis and the flow of blood through the vessels. Clarkson et al (1999) examined the effects of a combined anaerobic and strength training program (10 weeks) on endothelial function in young healthy men. They found that endothelium dependent dilation was significantly improved in the men who took part in the 10-week exercise training, when compared to the control group. Hambrecht et al (2000) examined the effect of exercise training on

endothelial function in patients with coronary artery disease. They utilized acetylcholine-induced vasoconstriction to measure the effects of a 4-week exercise training program. Four weeks of exercise training caused a significant improvement in coronary endothelial function (Hambrecht et al 2000). If exercise can improve endothelium-dependent outcomes, which are typically negatively altered in cardiovascular disease states, delving deeper into the mechanisms at play could lead to important therapeutic information. Current research is heavily focused on molecular, biochemical and circulating factors, which could play an important role in this phenomenon.

Circulating angiogenic cells (CACs) are circulating adult stem cells, originating from the bone marrow, which have been shown to have potent angiogenic, proliferative, and vascular repair properties (Van Craenenbroeck et al 2010). Endothelial progenitor cells (EPCs) are a type of CAC, and the terms CAC and EPC are often used interchangeably. For our purposes, CACs and EPCs will both be viewed as circulating cells with angiogenic/progenitor properties. The number of CACs in circulation and their function has been identified as a novel risk factor for cardiovascular disease (Hill et al 2003). In populations free of disease, the level of circulating EPCs was shown to be a better predictor of functional vascular health than traditional risk factors (Hill et al 2003). CD 34+ cells are one subset of circulating angiogenic cells, which have been shown to have specific endothelial properties (Peichev 2000). Due to their direct relationship with the endothelial cell lineage, CD34+ cells are believed to have potent repair and angiogenic properties in the endothelium (Yang et al 2011). As a whole, circulating angiogenic cells have been studied extensively in relation to known cardiovascular disease factors such as age and sedentary behaviors.

Aging has been demonstrated to affect the function and number of CACs. In a study on 8 young men and 8 older men, it was found that older age was associated with a lower number of circulating EPCs (Thijssen 2006.) Schedubel et al (2003) also found lower numbers of EPCs mobilized to the blood in older patients, independent of risk factors such as hypertension, diabetes, male gender, or ventricular function. The number of CACs in circulation, while important to physiological outcomes, is not the only factor related to age. Research has also shown changes in CAC function with aging. Heiss et al (2005) examined age-related differences in the functional measure of flow mediated dilation (FMD) and it's association with the number and function of EPCs. They found that older men and women had significantly lower FMD, indicating underlying endothelial dysfunction. They also found that although the numbers of EPCs were not different between young and older men and women, the capacity of EPCs to migrate, proliferate and survive was impaired in the older group (Heiss et al 2005). This study illustrates that even if the number of cells in circulation is not reduced, their functional differences can still affect vascular health in an aged population. Hoetzer et al (2006) also found decreased migratory capacity in older vs. middle-aged and young men, despite comparable numbers of EPCs.

Lack of exercise or sedentary behavior is another commonly known cardiovascular disease risk factor, which, like aging, is associated with decreases in CAC number and function. Both acute and chronic exercise have been shown to result in changes in CAC number and function. Steiner et al (2005) investigated whether exercise training affected EPCs in patients with cardiovascular disease risk and coronary artery disease. In their twenty participants, they found that after 12 weeks of supervised running

they exhibited a significant increase in the number of EPCs. Their observed increase in EPCs was directly correlated with a change in flow-mediated dilation (Steiner et al 2005). Another group assessed the effects of exercise training on EPCs in patients with chronic heart failure. They found that after 8 weeks of supervised aerobic training, participants had increased levels of circulating EPCs. The participants also had an increase in the angiogenic cell mobilizing factors, vascular endothelial growth factor (VEGF), and stromal-derived factor 1 (SDF-1), present in their plasma (Sarto et al 2007). Training status can also affect the way that EPCs respond to an acute exercise stimulus. Jenkins et al (2009) found that after 30 minutes of endurance exercise at 75% of VO_2max (throughout), endurance-trained men saw an increase in EPCs while untrained men did not. This observation took place despite the two groups having similar levels of EPCs prior to the exercise bout.

Just as exercise training and acute exercise can increase CAC number and function, cessation of exercise in previously-trained individuals can also affect the CAC population. Witkowsi et al (2010) studied a group of men with a history of 30 or more years of moderate or greater intensity exercise. The men took a 10-day break in their training and changes in CD34+ cell population were measured. The CD34+ cell population decreased by 44% after 10 days without training and these changes were directly correlated with changes in forearm blood flow measures.

To summarize to this point, researchers have shown that both age and exercise can affect the number and behavior of circulating angiogenic cells and EPCs. Exercise increases the number of cells in circulation, while sedentary behavior is linked to a lesser number of cells in circulation (Thijssen et al 2009). While the number of cells in

important, the behavior of these cells has become a topic of great interest. Not only have older, diseased, and sedentary individuals been shown to have a lesser number of cells in circulation, but the cells from these populations also behave differently *in vitro*. Cells from these compromised populations exhibit decreased angiogenic properties and depressed function. The proper function of CACs is important because of their involvement in angiogenesis, repair, and adequate vascular function.

With the discovery of CACs and their role in healthy cardiovascular function, the biomedical community has begun to use these cells in therapeutic interventions. Autologous stem cell transplants present a viable option for therapy in individuals with vascular disease. Removing CACs, purifying them from the blood, and injecting the purified product into the same individual, as a bolus, is an excellent way to deliver angiogenic stem cells to injured areas without a resultant immune response. However, it soon became apparent that taking cells from an aged or diseased patient and re-injecting them in a bolus was not having the hypothesized beneficial effect. The demonstrated depressed function in CACs from older and diseased individuals presents a clear challenge for the development of autologous stem cell transplant as a therapy.

Heeschen et al (2004) found that bone marrow mononuclear cells, including CD34+ cells, from patients with chronic ischemic heart disease exhibited reduced neovascularization *in vivo* when compared to cells isolated from healthy controls. The Heeschen group also saw decreased migratory and colony-forming behaviors in bone marrow mononuclear cells from individuals with chronic ischemic heart disease. A recent study by Cogle et al (2014) examined bone marrow cells following acute myocardial infarction (AMI). Their group found that following an AMI, the percentage of CD34+

cells was decreased and a decreased capacity to form endothelial colonies was also present. The group performed bone marrow cell therapy and found a positive correlation between CD34+ percentage, and left ventricular ejection fraction (Cogle et al 2014). The experiments performed by the Cogle group demonstrate depressed CAC function in diseased patients, and the ability of CD34+ bone marrow cell therapy to partially rescue left ventricular ejection fraction. These results underline the need to understand the function of CACs in pathological conditions and mechanisms that can be targeted to increase function.

Previous to the Cogle study above, autologous stem cell transplantation in AMI patients was studied in clinical trials. The Cardiovascular Cell Therapy Research Network (CCRTN) of NIH funded a series of clinical trials on this topic. The TIME trials were designed to evaluate if the time elapsed between AMI and transplantation of bone marrow stem cells to be used in the transplant played a role in determining outcome. One of these clinical trials randomized patients to receive cell aspiration therapy 3 or 7 days following AMI. The researchers found that neither the 3 day nor 7 day post AMI treatment yielded positive results on left ventricular function (Traverse et al 2012). A second clinical trial within TIME, called LateTIME, examined autologous stem cell transplants 2-3 weeks following AMI to examine if a longer time elapsed following AMI before stem cell therapy would make a difference in outcome. Similar to the previous study, LateTIME also did not see any improvement in left ventricular function in patients with stem cell therapy 2-3 weeks following AMI when compared with those receiving placebo reperfusion (Traverse et al 2011). The results of recent studies on autologous stem cell transplants from diseased patients highlight CAC functional differences

between populations, and serve to illustrate the need for further research in this area to optimize potential therapeutic applications.

The differences in CAC behavior among different populations, which have been described above, have sparked new research in the pathways and mechanisms that could be contributing to such differences. Many researchers have looked to long-known factors contributing to vascular function to answer these questions. Nitric oxide (NO) is known as a potent vasodilator and contributor to vascular health. NO was first identified in 1989 by Furchgott et al, and was originally called "endothelium-derived relaxing factor." Furchgott and his group realized that when they were preparing rat aorta samples, if they rubbed the intimal surface, which removed endothelial cells, the relaxing response of the artery was lost (Furchgott and Vanhoutte 1989). Since its discovery, NO has been found to be involved in numerous aspects of vascular health. A review in Hypertension by Green et al (2013) concluded that flow-mediated dilation is, indeed, influenced by the presence of NO. This finding was important, as flow-mediated dilation is a functional measure of vascular health.

NO also plays an important role in vascular health by reducing platelet adherence and cell growth within the vessel wall (Fukai 2007). Myazaki et al (1999) examined the role of a NO synthase inhibitor (ADMA) in atherosclerosis. Their results showed that increased ADMA levels (indicating NO inhibition) were directly correlated with the thickness of the carotid intima-media, as well as increased arterial pressure and glucose intolerance (Myazaki et al 1999). Carotid intima-media thickness is often measured as an indication of the presence of atherosclerotic cardiovascular disease. As demonstrated above, if NO is not released from the endothelium, pathological conditions leading to

atherosclerosis can occur. Smooth muscle proliferation, leukocyte adhesion, and endothelial injury are some of the pathological conditions caused if the endothelium fails to release NO (Dusting et al 1998).

NO has also been shown to play a role in CACs. Ward et al (2011) conducted a series of experiments using endothelial NO synthase (eNOS) gene transfer experiments in CACs from coronary artery disease (CAD) patients and healthy controls to determine the role of NO in CAC function. eNOS gene transfer successfully increased nitric oxide levels in CACs. Their findings indicated that eNOS gene transfer in CACs from the CAD patients, due to increased NO levels, increased chemotactic migration and angiogenic tube formation *in vitro*. They also performed *in vivo* experiments to measure changes in neovascularization and ischemic hind-limb perfusion. *In vivo*, CACs from the CAD patients with eNOS gene transfer exhibited higher levels of NO, a greater ability to neovascularize, and improved ischemic hind-limb reperfusion. With eNOS gene transfer, *in vivo* neovascularization and ischemic hind limb reperfusion was almost completely rescued in the cells from CAD patients when compared to CACs from healthy controls. (Ward et al 2011). Mangialardi et al (2011) examined the effects of NO-donating atorvastatin on CACs. CACs from healthy volunteers and type-2 diabetic patients were examined. They cultured the CACs under both high glucose and low glucose conditions. The high glucose condition impaired migration, proliferation and outgrowth. Impaired function from high glucose conditions was rescued by NO-donating atorvastatin. They also saw improvement in function of CACs from type-2 diabetic patients with the addition of NO-donating atorvastatin (Mangialardi et al 2011).

NO is produced by NO synthase (NOS), of which several isoforms have been identified. Each isoform of NOS plays a different role in the balance of vascular health. NO can be measured globally, which is an overall measure of NO produced by all of the isoforms of NOS. However, which NOS isoform the NO is being produced by can provide insight into the pathway the NO will follow and what mechanisms it is involved in (Wilcox et al 1997). In this study we will focus on two forms of NOS: endothelial NO/NOS III (eNOS) and inducible NO/ NOS II (iNOS).

Endothelial NO synthase (eNOS), as indicated by its name, is responsible for the production of NO in endothelial cells. Aicher et al (2003) also demonstrated that eNOS plays a role in the mobilization of EPCs. They found that eNOS knockout mice had a diminished ability to mobilize EPCs from the bone marrow (Aicher et al 2003). Recently, it was discovered that the production of NO within endothelial cells by eNOS is the result of an important balance in vascular homeostasis called "eNOS coupling". When eNOS is "coupled", it produces NO to maintain vascular health. When eNOS becomes "uncoupled" endothelial dysfunction occurs due to an increased production of reactive oxygen species (ROS) and decreased production of bioavailable NO (Yang et al 2009). While low levels of ROS may be important to activate protective pathways, ROS in excess can lead to impaired vessel repair and angiogenesis (Fleisner and Thum 2011). High levels of endothelial ROS are present in many disease conditions, such as hypertension and cardiovascular disease (Schiffrin 2010). As discussed previously, aging and exercise are two risk factors that can affect vascular health. These risk factors also play a role in eNOS expression and function. Tanabe et al (2003) examined this

phenomenon in the rat aorta and found that exercise training can partially rescue the decreased expression of eNOS in aged rats.

Since the coupling of eNOS is such a delicate balance, cofactors are thought to play an important role. Tetrahydrobiopterin (BH4) is a cofactor largely responsible for the coupling of eNOS. When levels of BH4 are reduced, eNOS becomes uncoupled and begins to produce ROS in excess (Xie et al 2010). BH4 also plays an important role in endothelium dependent dilation (EDD), due to its role in endothelial NO bioavailability. Eskurza et al (2005) showed that BH4 is directly involved in age-impaired EDD. They demonstrated that BH4 could be a key factor in age-impaired EDD and that it could also be involved in the protective effect of exercise training on EDD. As Eskurza et al suggest, BH4 has been shown to be affected by age and exercise training status. With age, levels of BH4 decrease; however, habitual exercise can prevent this decrease and preserve NO bioavailability (Rush 2009). BH4 can also be oxidized to BH2 by ROS, also contributing to eNOS uncoupling and reduced NO bioavailability. This can lead to a vicious cycle of BH4 oxidation to BH2, eNOS uncoupling with increased ROS production, and further oxidation of BH4 due to peroxynitrite formation from eNOS uncoupling (Fuaki 2007).

GTP Cyclohydrolase I (GTPCH1) is the rate-limiting enzyme in the *de novo* production of BH4. Dihydrofolate reductase (DHFR) catalyzes the recycling pathway responsible for the conversion of BH2 back to BH4. Both of these enzymes are essential for the production and function of BH4 and, therefore, the coupled function of eNOS. Shear stress increases GTPCH1 activity and, as a result, BH4 levels (Pall 2009). Widder et al (2007) demonstrated this *in vitro* by inducing laminar shear stress on human

endothelial cells. They saw increases in both BH4 and GTPCH1 with laminar shear stress induction. DHFR is directly involved in the maintenance of the BH4 to BH2 ratio. This ratio can provide insights into vascular health. In fact, the ratio between BH4 and BH2, when tipped in the direction of BH2, can indicate eNOS uncoupling (Crabtree et al 2008). Since DHFR is responsible for the salvaging pathway that can reduce BH2 back to BH4, understanding how risk factors affect DHFR is critical.

Inducible NO is believed to be responsible for the pathological production of NO rather than the physiological production of NO by its counterparts, such at eNOS. Unlike eNOS, which produces NO for short periods of time when activated, iNOS produces NO for an extended period of time (Kroncke et al 1998). iNOS has been found to be active during cardiac events, inflammatory disease states, and other pathologic conditions (Kroncke et al 1998). iNOS is also believed to be involved in the production of peroxynitrite, a harmful reactive nitrogen species (Buttery et al 1996).

When NO is measured globally, it is difficult to discern if the NO present is being produced pathologically or physiologically. In some studies higher levels of NO can be found in higher risk groups, and cause confusion among researchers as to the exact role nitric oxide is playing. It is important to make a distinction between pathological vs. physiological production of NO in order to fully understand the mechanisms at play. Jenkins et al (2011) measured NO, iNOS, and eNOS in PBMCs from both sedentary and exercise trained men. Surprisingly they found higher levels of NO in the PBMCs from the sedentary men when compared to the exercise trained men. However, they also found higher levels of superoxide in the sedentary men. Upon further examination, they found that the sedentary group's iNOS mRNA levels were two-fold higher than the men in the

trained group. They also found that eNOS mRNA levels in the sedentary group were 50% lower than the trained men (Jenkins et al 2011). Examining the levels of eNOS and iNOS gave them extremely important insights into pathological vs. physiological NO production in their participants and elucidated mechanistic information that otherwise might have been overlooked.

In summary, exercise and aging are commonly accepted as important risk factors for cardiovascular disease. As science advances, research is beginning to shed light on the pathways and mechanisms involved in these risk factors at a cellular level. Research has identified circulating angiogenic cells (CACs) as playing an important role in these pathways at the cellular level. CACs are affected by age and exercise in a variety of ways, including their production of NO and ROS. The phenomenon of eNOS coupling/uncoupling presents an important mechanism in the homeostatic functioning of CACs. Studies have been conducted relative to age, exercise, NO, and eNOS coupling in a variety of contexts, but we propose to combine these factors in a single study on habitual exercise training and the cessation of training. We hypothesize the following

Hypothesis: In a group of long-term endurance-trained men and women athletes, a ten-day cessation of training will lead to an increase in pathological production of NO and ROS levels in CD34+ CACs, through the eNOS-uncoupling pathway.

Specific Aim 1: Measurement of NO and ROS in CD34+ cells at baseline and following cessation of training, using fluorescent assays.

Specific Aim 2: Flow-mediated dilation measurements in participants at baseline and following cessation of training to be used as a functional measure of NO bioavailability.

Specific Aim 3: mRNA expression measurement of eNOS, iNOS, DHFR, and GTPCH1 to examine the role of the eNOS uncoupling pathway

Chapter 2: Methods

Participants:

Participants were endurance-trained "Master athletes." Master Athlete was defined as taking part in exercise for at least four hours a week at high intensity, for at least 15 continuous years, and being between the ages of 50 and 80 years old. They were recruited from the local area including local running clubs and organizations. They had participated in exercise for greater than or equal to 4 hours a week, and greater than or equal to 4 days a week, at a high intensity. They were between the ages of 50 and 80 years old. Their BMI was required to be less than 30 and they had to be a non-smoker (for greater than 5 years). All participants were free of history of heart attack, stroke, lung disease, chronic obstructive lung disease, peripheral vascular disease, heart disease, anemia, liver disease, kidney disease and diabetes. Additionally participants were not controlling high blood pressure with the use of a prohibited medication (see attached list.) Participants were not taking any of the prohibited medications on the attached list at the time of the study. Women participants were post-menopausal for greater than or equal to 2 years, and had not used hormone therapy in the last year. All participants had to be able to complete a vigorous treadmill test without orthopedic limitations. Initial phone screenings were conducted to verify that participants fit the above criteria. Once a participant was determined to fit the criteria, we scheduled baseline testing.

Experimental Protocol:

The following experimental protocol was approved by the Internal Review Boards of both University of Maryland College Park and University of Maryland Baltimore.

Participants completed two days of on-site testing at University of Maryland College Park and the Baltimore VA Medical Center. The first testing day was for obtaining baseline measurements. The second testing day occurred after the participant had completed a ten-day complete cessation of training. In most cases, the subject came for baseline testing on a Wednesday, continued their regular training until Saturday, then stopped training Sunday through the following Wednesday, when the follow up testing took place. Throughout the period of training cessation, the subject kept a log of their weight and attempted to keep it constant.

Testing Day Procedures:

On both testing days, the procedures were the same. The participant arrived fasted at the University of Maryland College Park in the morning. Before testing commenced, the informed consent was explained and reviewed with the participant. Following obtaining the signed written consent of the participant, baseline questionnaires were completed. Each participant completed a health history and physical activity history questionnaire at the baseline visit. At both the baseline and follow-up visit, a screening visit data sheet was completed to confirm that the participant had not exercised in the past 24 hours, had not had any alcohol or vitamins in the past 24 hours, had not had caffeine overnight, and had not taken NSAIDs in the past 48 hours. Once the questionnaires were completed, the participant had their height and weight measured. Following height and weight measurements, the researcher performed a venous blood draw on the participant, using aseptic technique, to obtain a 50 mL blood sample.

Once the questionnaires and blood draw were completed, the subject was transported to the Baltimore VA Hospital for the assessment of flow-mediated dilation measurement, VO_2 max, and body composition.

Body Composition:

Body composition was measured by radiology technicians, using dual energy x-ray absorptiometry (DEXA) (DXA; Prodigy, LUNAR Radiation Corp). Percent body fat was measured and recorded.

Maximal Oxygen Consumption Testing (VO_2 Max)

VO_2 Max testing was conducted using indirect calorimetry (Quark, Cosmed USA), during a graded exercise test on a treadmill. Participants walked or ran at a constant velocity with grade increasing 2% every two minutes (starting at 0%). The test continued until maximal effort or exhaustion was reached. Standard criteria for reaching VO_2 max were used to determine the endpoint of the test. These criteria included a plateau in VO_2, with increasing workload and respiratory exchange ratio (RER) > 1.1. The highest oxygen consumption value for a 30 second increment was recorded as VO_2 max. Values are presented in both absolute (L/min) and relative (ml/kg/min) units. Standard ECG monitoring was used throughout the test, and a cardiologist was present.

Brachial Artery Ultrasound Test (BART)

BART is a non-invasive test to measure endothelial function by quantifying flow-mediated dilation (FMD). The subject lay supine during the testing process. Blood pressure cuffs were placed on both of the subject's arms. The cuff on the right arm was

used to take heart rate and blood pressure intermittently throughout the test. The cuff on the left arm was used for the actual testing. A one lead ECG was also used during the test. Once the subject was set up, 2D ultrasound and Doppler images were taken of the brachial artery for a baseline measure. The technician located the position on the brachial artery which provided the clearest picture and marked that spot. That marked spot was then used for all subsequent measures to ensure consistency. Then the blood pressure cuff on the right arm was inflated to 200 mmHg for 5 minutes. After 5 minutes, the cuff was released and images and Doppler data were taken to measure the response of the brachial artery to the hyperemic stimulus. Although maximum dilation occurs about one minute after the release of the cuff, images were taken for fifteen minutes, at one-minute intervals, following cuff release FMD was measured in percentage of change in brachial artery dilation from baseline to post-cuff release.

When testing in Baltimore was complete, the subject was transported back to College Park and released. On the second visit, the participant was compensated $200 at the completion of their participation.

PBMC Isolation:

Peripheral blood mononuclear cells (PBMCs), and $CD34^{+}$ cells were isolated from the 50 mL venous blood sample on each visit. PBMCs were isolated using Ficoll-based density gradient centrifugation. Briefly, the 50 mL blood sample was divided into 4, 50 mL conical tubes and diluted at a 1:1 ratio with PBS without Ca^{+2} and Mg^{+2}. Diluted blood was carefully overlaid onto 15 mL of Ficoll. Tubes were centrifuged at 300g for 30 min with slow acceleration and no brake. Following centrifugation, the

plasma was discarded, and PBMCs moved to new tubes. PBS+2%FBS+EDTA was added to the PBMCs and this solution was centrifuged for another 7 minutes at 300g. The supernatant was then removed and the PBMC pellet from each tube was brought up to ~2mL with PBS+2%FBS and combined into a single round-bottom tube. The sample was then centrifuged for 7 mins at 500g. The supernatant was removed and the pellet was brought up to 2mL with PBS+2% FBS. A 1:20 dilution of PBMCs and acetic acid/3% methylene blue was made and a PBMC count was performed using a hemocytometer.

CD 34+ Isolation:

CD 34+ cells were isolated from the PBMC sample using immunomagnetic selection (Stemcell Technologies) according to manufacturer's instructions. Briefly, the PBMC solution was brought up to a concentration of 2 x10 ^{8} cell/ ml. $CD34^{+}$ antibodies were added at 10% of the measured concentration of cells. The sample was then incubated with the added antibodies for fifteen minutes at room temperature. Following incubation, $CD34^{+}$ nanoparticles were added to the sample at 10% of the measured concentration of cells, and this solution was incubated at room temperature for ten minutes. Following incubation, the sample was brought up to a volume of 2500 µl with PBS+2%FBS and placed into the selection magnet for 5 minutes. After 5 minutes, the supernatant was poured off of the sample, while the tube was still in the magnet. Then the sample was removed from the magnet and brought back up to a volume of 2500 µl with PBS+2%FBS. The magnet selection procedure was then repeated a second time. After 2 magnet incubations and pours, the sample was brought up to 2mL with PBS+2% FBS, and a count of $CD34^{+}$ cells was performed using a hemocytometer. Purity of the proposed isolation protocol was measured during optimization by fluorescent activated

cell sorting (FACS) to ensure purity consistent with the literature. Each researcher involved in the project had their purity tested using FACS before they began testing experimental samples. 50% purity of CD34+ isolation was expected, utilizing comparable methods (Jenkins et al 2011).

ROS/NO Assay

Following isolation and count of the $CD34^+$ cells, samples were centrifuged at 500 x g for seven minutes. The supernatant was poured off, and the sample was brought up to 250 µl with PBS without Ca^{+2} and Mg^{+2}.

Phase I:

Master mixes were created containing 1.5 x 10^5 CD34+ cells per reaction and PBS without Ca^{+2} and Mg^{+2} to bring the mix up to 150 µl per reaction. A clear 96 well plate was used to plate 12 wells of $CD34^+$ master mix. The plate was then allowed to incubate at 37°C, 5% CO_2, for 6 hours.

Phase II:

During the last half hour of the incubation, master mixes were created for phase II. One master mix contained 2 µM H2DCFDA (for measurement of ROS) and the other contained 10 µM DAF-FM (for the measurement of NO). Both master mixes contained PBS without Ca^{+2} and Mg^{+2} to achieve the specified concentration of dyes. Following 6 hours of incubation, the plate was covered in foil and transported to the centrifuge. The plate was spun at 500g for 5 mins to pellet the cells. After the spin, the supernatant was removed. Half of the cells were treated with H2DCFDA master mix, and half were

treated with DAF master mix. All wells were thoroughly mixed. A row of control wells was also present including PBS without Ca^{+2} and Mg^{+2} only, H2DCFDA master mix without cells, and DAF master mix without cells. The plate was covered with foil and dyes were allowed to incubate on the cells for 30 minutes. Following incubation, the plate was spun again at 500g for 5 mins to pellet the cells, and the supernatant was removed. All wells were re-suspended with 150 µl of PBS without Ca^{+2} and Mg^{+2} and the plate was covered with foil and transported to the plate reader.

Measurement of Fluorescent Dyes:

The ROS/NO assay 96 well plate was read using a Multi-mode microplate reader (Biotek Synergy H2). The plates were read using the auto-gain set up at 535nm (appropriate for both H2DCFDA and DAF-FM). Measurements of fluorescence were saved for analysis.

mRNA Expression:

RNA Isolation:

Any $CD34^{+}$ cell sample that was not used for the ROS/NO assay was spun down to form a pellet, then dissolved in Trizol, and frozen at -80° C for RNA isolation at a later date. When the RNA was isolated, the samples were thawed, transferred to 2 mL Eppendorf tubes, and vortexed briefly. 200 µl of chloroform was added to each sample, the tubes were inverted, and then placed in the rotator for ten minutes. After 10 minutes, samples were centrifuged for 12 minutes at 4°C at 12,000g. After centrifugation, we carefully removed 50-75% of the upper clear layer and placed it in a second set of 2 ml Eppendorf tubes. 500 µl isopropyl alcohol and 5 µl glycogen (coprecipitant) were added

to each sample and placed in the rotator for 10 minutes. After ten minutes, the samples were spun for 8 minutes at 4°C and 12,000g. After centrifugation, the supernatant was removed and 2 mL of ethanol was added to each sample. Samples were vortexed briefly to dislodge the pellet, and then incubated at room temperature for 5 minutes. After incubation, the samples were centrifuged at 7500g for 5 mins at 4°C. Following the spin, the supernatant was removed and the pellet was allowed to air dry for ten to fifteen minutes. Once the pellet was clear, TE buffer and the sample were placed on ice and 25 µl of TE buffer was added to each sample. Samples were then frozen at -80°C until reverse transcription was ready to be performed.

Reverse Transcriptase (RT):

Samples were thawed and RNA concentration was determined for each sample using the spectrophotometer function of the multi-mode microplate reader (Biotek Synergy H2). Once RNA concentration was determined, calculations were made to determine how much sample was needed for 1 ug of RNA. A master mix was created using the RT kit (Applied Biosystems) for each sample, using 2 µl of RT buffer, 0.8 µl of 1.25 mM dNTP, 2 µl 10x random hexamers, 1 ul of reverse transcriptase. 5.8 µl of master mix was added to each well of the reaction plate, and then the required amount of RNA solution was added. DH_20 was added to bring each well up to 20ul. The reaction plate was gently mixed and then spun down before being loaded into the thermocycler (BioRad T100). Conditions for RT were 25°C for 10 mins, 37°C for 120 mins, 85°C for 5 seconds and 4°C until removed from thermocycler.

qt-PCR:

Semi-quantitative PCR was performed following the RT protocol. qt-PCR was performed to quantify mRNA levels of inducible nitric oxide (iNOS), endothelial nitric oxide (eNOS), dihydrofolate reductase (DHFR), and GTP cyclohydrolase 1 (GTPCH1). mRNA levels were measured for all subjects both before and after the cessation of training.

Gel electrophoresis was performed on each sample using agarose gel. 18s was used as the housekeeping gene for this analysis. Following gel electrophoresis, bands were visualized under UV light (Kodak) and images were captured for analysis. ImageJ was used to quantify the band intensities and to compare mRNA expression between samples.

Statistics:

This study design was an intervention design where cessation of training served as the intervention. Subjects were studied at baseline and following the intervention period. Data were analyzed using a repeated measures ANOVA. The significance level was set at $p < 0.05$. Data for subject demographics are presented as mean $\pm$ standard deviation and data for all other results are presented as means $\pm$ standard error. Power calculations were performed with preliminary data from fresh cell measures to determine the number of participants needed. With preliminary data (n=12), NO and ROS levels yielded an estimated power of 53% and 73%, respectively. We aimed to test 20 participants, which would give us an estimated power of 71% (NO) and 78% (ROS). We tested 19 participants, but 2 were eliminated due to positive graded exercise test results, one could

not be completed due to equipment malfunction, and one subject was eliminated because it was subsequently discovered that she did not fit the subject profile.

Chapter 3: Results:

Subject Characteristics:

Sixteen "Master Athletes" were studied; 11 men and 5 women. The women participants were all post-menopausal for at least two years and had not taken hormone replacement therapy for at least a year.

Most of the participants were from local running and triathlon clubs. They were healthy and highly fit for their ages, as can be seen in Table 1. Average relative Vo2 max (ml/kg/min) for both the men and women exceeded the "superior" range for their ages (Heyward 1998). Average body fat percentage, for both men and women, are in the healthy range. All participants were able to complete the treadmill test without orthopedic limitations.

Table 1. Subject Characteristics: Age, Maximal oxygen uptake (VO_2 max) , Body Fat, Body Mass Index (BMI), Systolic Blood Pressure (SBP), and Diastolic Blood Pressure (DBP). Mean ± standard deviation.

	Men (n = 11)	**Women (n = 5)**	**Combined Group (n = 16)**
Age (years)	62.3±8.5	61.3±4.5	62.3±6.9
VO_2 Max (ml/kg/min)	47.8 ± 6.4	46.9±4.1	47.3±5.5
Body Fat (%)	22.7 ±5.0	25.3±3.1	23.4±5.5
BMI (kg/m^2)	24.2±2.9	21.1 ± 1.3	23.3 ± 2.8
SBP (mmHg)	124.2 ±17.1	110 ±13.1	119±16
DBP (mmHg)	70.7±8.6	67.6 ± 5.9	70±8

Flow-Mediated Dilation (FMD):

FMD was measured at the Baltimore VA Medical Center during both visits. There was no change in FMD (p= 0.12), with the cessation of training. At baseline, FMD was 11.1% ± 1.4%, and following the ten-day cessation of training, FMD was 8.8% ± 1.3% (Figure 1).

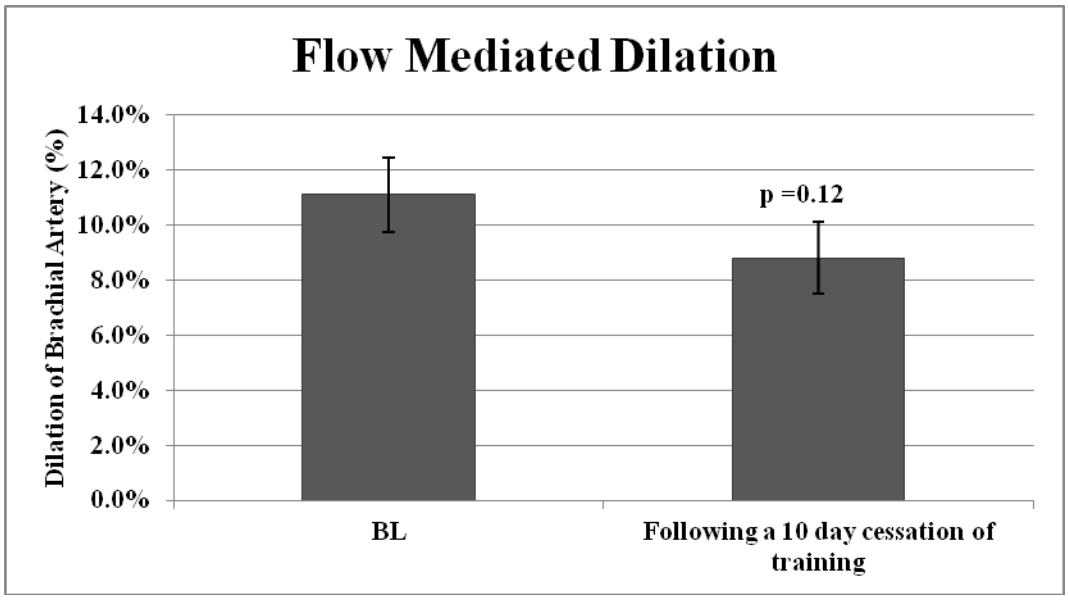

Figure 1. Average Flow-Mediated Dilation ± SEM of the brachial artery at baseline and after the cessation of training.

CD34+ Cell Nitric Oxide Levels

CD34+ intracellular Nitric Oxide (NO) levels from freshly-isolated cells were studied in 15 subjects at baseline and following the ten-day cessation of training. NO was measured using DAF and relative fluorescence was analyzed. At baseline, average DAF fluorescence (indicating NO) was 27,067 RFUs/5x10^4 ± 3712. Following the ten-day cessation of training, average DAF fluorescence was 30,419 RFUs/5x10^4 ± 3228. The increase in CD34+ intracellular NO levels was not significant (p= 0.169). (Figure 2)

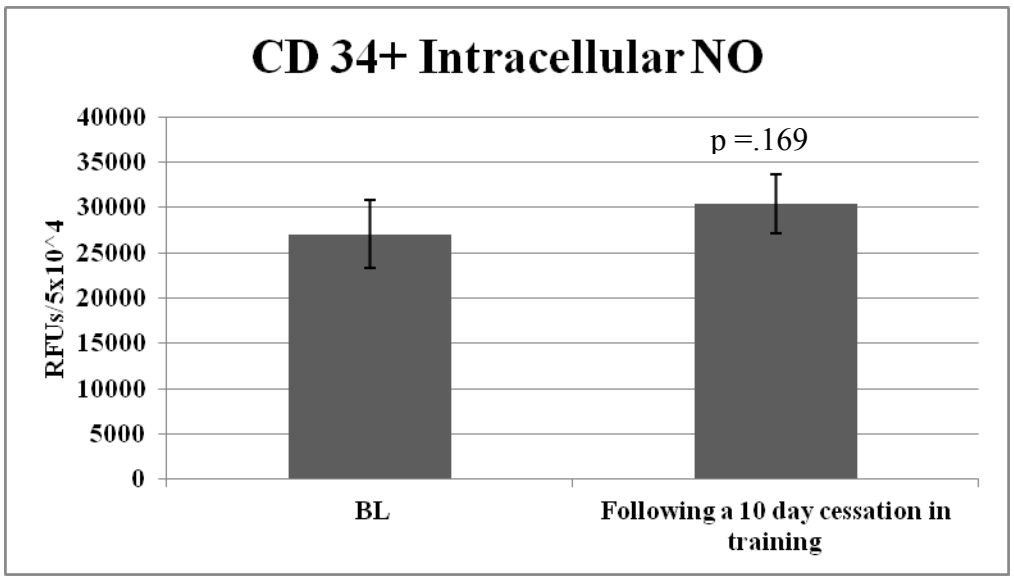

Figure 2. Intracellular Nitric Oxide in CD34+ cells at baseline and following the ten-day cessation in training.

ROS

Differences in CD34+ intracellular ROS from baseline to following the ten-day cessation of training were measured using H2DCFDA and relative fluorescence was analyzed. At baseline, average H2 fluorescence was 27,699 RFUs/5x10^4 $\pm$ 3552. Following the ten-day cessation of training, average H2 fluorescence was 32,850 RFUs/5x10^4 $\pm$ 3819. The increase in CD34+ intracellular ROS levels was not significant ($p= 0.107$) (Figure 3).

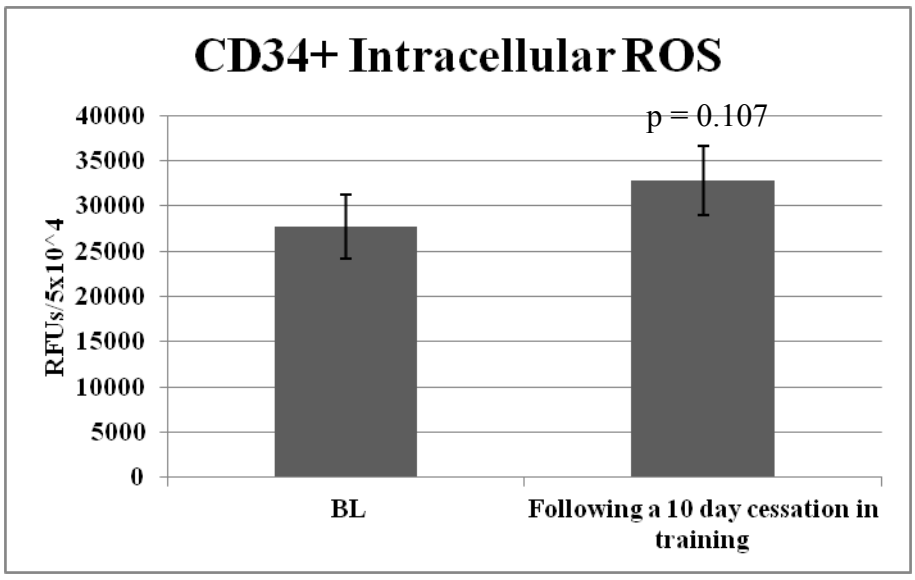

Figure 3. Intracellular Reactive Oxygen Species in CD34+ cells at baseline and following a ten-day cessation in training.

mRNA Expression:

Subjects' (n=13) CD34+ cells were frozen the day of isolation and later analyzed for mRNA expression. eNOS, iNOS, GCH1, and DHFR were quantified using qt-PCR and gel electrophoresis. All mRNA band intensities were normalized to the 18s house-keeping gene signal.

eNOS:

At baseline, eNOS mRNA, normalized to 18s signal, was 0.328 $\pm$ .06. Following the 10 day cessation of training, eNOS mRNA, normalized to 18s signal was 0.148 $\pm$ .03. The observed decrease in eNOS mRNA expression was significant ($p = 0.008$). (Figure 4).

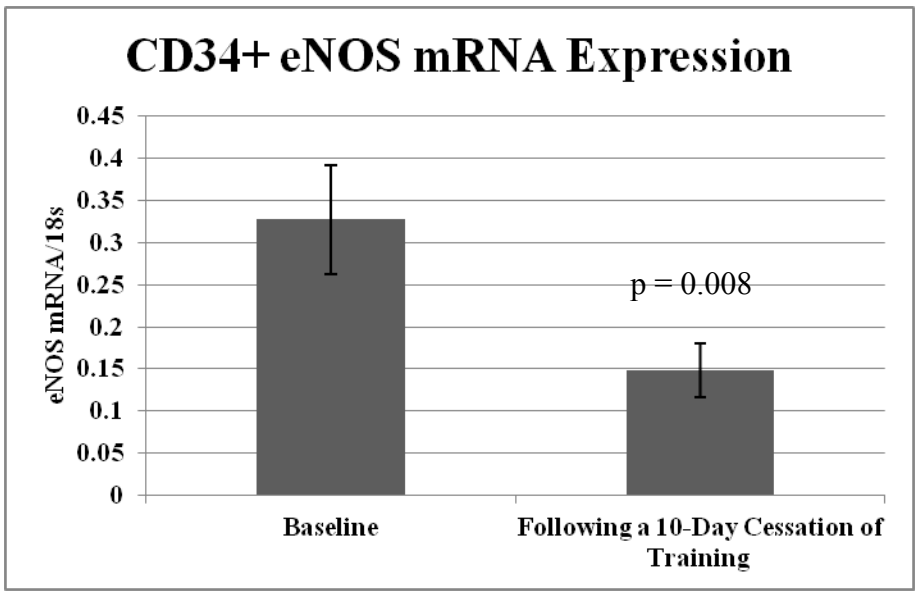

Figure 4. eNOS mRNA expression at baseline and following a 10 day cessation in training

iNOS

There was no change in iNOS mRNA expression from baseline to after the ten-day cessation of training ($p = 0.32$). At baseline, average iNOS mRNA expression was 0.7731 ± 0.130, and following a ten-day cessation of training, iNOS mRNA was 0.6475 ± 0.10. (Figure 5).

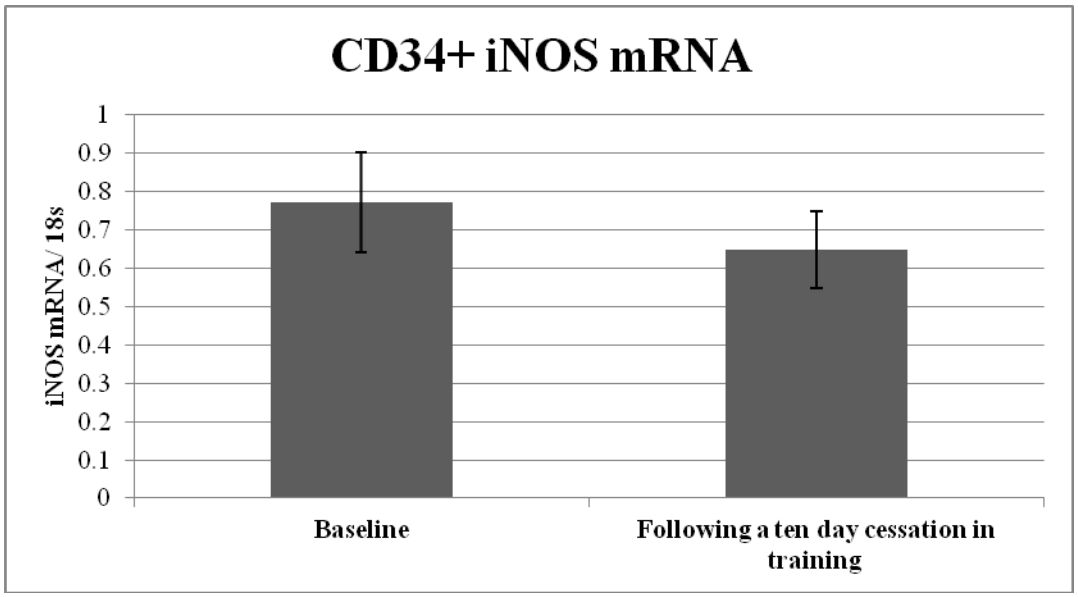

Figure 5. CD34+ iNOS mRNA expression at baseline and following a ten-day cessation in training

DHFR

There was no change in DHFR mRNA expression with a ten-day cessation of training ($p = 0.915$). At baseline, normalized DHFR mRNA expression was 0.567 ± 0.04, and following a ten-day cessation of training normalized DHFR mRNA was 0.561 ± 0.04. (Figure 6).

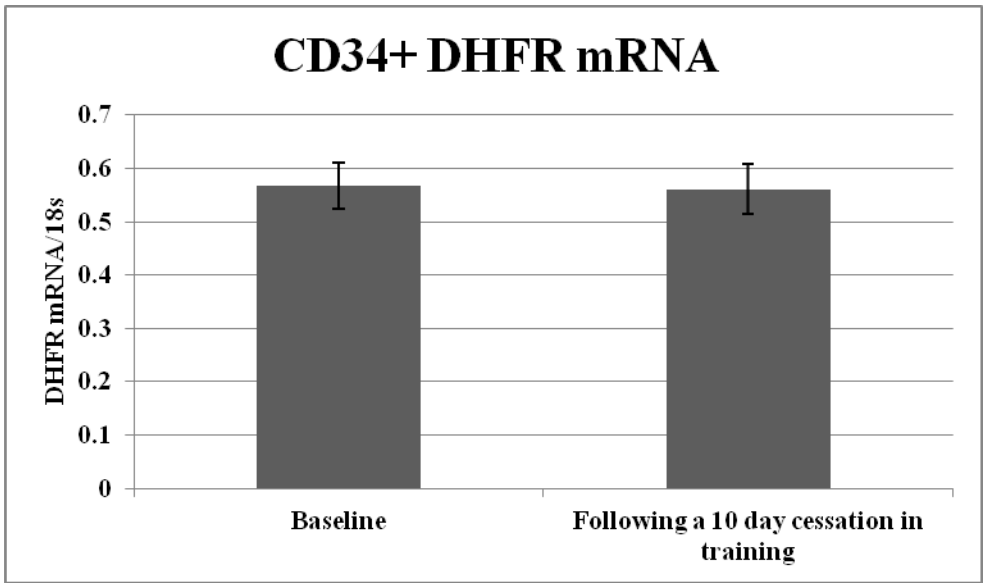

Figure 6. DHFR mRNA expression at baseline and following a ten-day cessation of training

GCH1

There was no change in GCH1 mRNA expression following the 10-day cessation in training ($p = 0.21$). At baseline, the average normalized GCH1 mRNA expression was $0.8665 \pm .06$. Following a 10-day cessation of training GCH1 normalized mRNA expression was $0.8281 \pm .05$. (Figure 7).

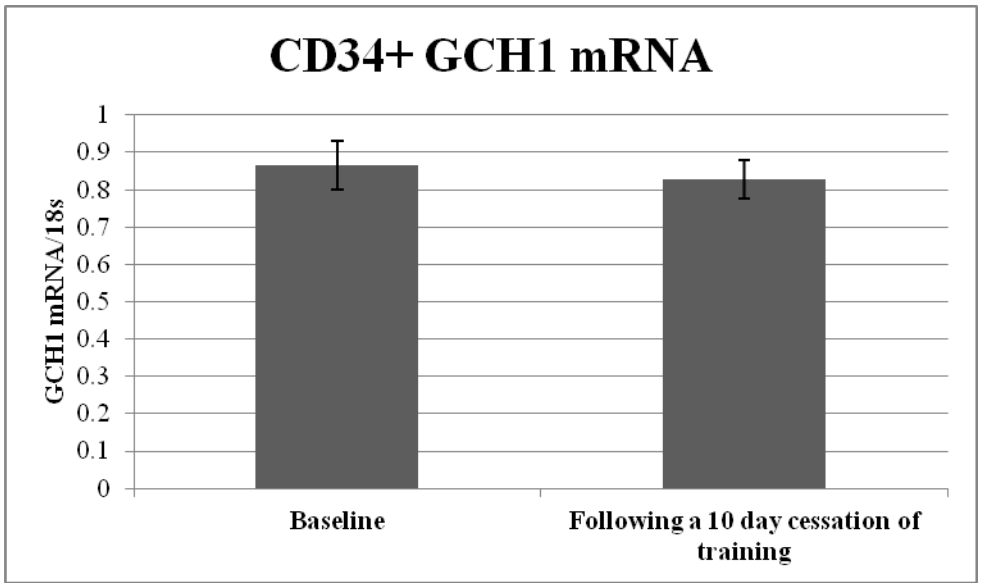

Figure 7. CD34+ GCH1 mRNA expression at baseline and following a 10-day cessation of training.

Chapter 4: Discussion:

Overall, the results of our study did not support our initial hypotheses. We hypothesized that a ten-day cessation of training would increase ROS and pathological production of NO in CD34+ CACs. However, we observed no changes in ROS and NO levels in circulating CD34+ cells following the cessation of training. We also hypothesized that a cessation in training would elicit negative changes in the eNOS coupling pathway. mRNA expression for genes related to the eNOS coupling pathway, including DHFR, GTPCH1, eNOS and iNOS, were examined. However, there were no observed changes in the expression of DHFR, GTPCH1, or iNOS in CD34+ cells with the cessation of training. However, there was a significant decrease in CD34+ eNOS mRNA expression following the 10-day cessation in training. Finally, we also expected to see changes in FMD after the cessation of training, but no significant changes were observed. These findings indicate that a 10-day cessation in exercise training following over 20 years of continuous exercise was not sufficient to elicit significant changes in the overall nitro-oxidative state of CD34+ cells and functional measures of endothelial function.

Flow Mediated Dilation

Following the 10-day cessation of training, FMD was numerically lower by 24%, however, this change was not statistically significant. FMD is a measure of endothelial function and is closely associated with cardiovascular health. FMD has been shown to be a valuable tool for analyzing the impact of interventions on the endothelium and equal in reliability to comparable invasive measures. (Raitakari 2000) While there is a known age-

related decline in FMD, exercise training appears to prevent this effect (Black et al 2009). When compared to the literature, our master athletes had relatively high FMD values at baseline. In an epidemiological study conducted by Yeboah et al (2007), FMD was measured in over 2,000 older adults. The subjects were ages 72-98, so they were generally slightly older than our subjects, but representative of the older population as a whole. The median FMD measured in these subjects was 3% in the women, and 2.4% in the men. As stated above, our subjects' average FMD was 11.1% at baseline, which is much higher than the average older adult. Our results support the theory that exercise training helps to prevent age-related decrease in FMD, and that a relatively short-term cessation of training may begin to reverse the positive effects of training. Future research should examine whether a longer break in training would cause FMD to continue to decrease.

Fresh Cell Fluorescence Measures: NO and ROS

In contrast to our hypothesis, fresh CD34+ cell measurements of ROS and NO did not change significantly with a ten-day cessation of training. Although the changes were not significant, some trends were observed. Intracellular NO and ROS were numerically higher by 25% and 26%, respectively following the cessation of training, although these changes were again not statistically significant. Intracellular NO is generally believed to be beneficial to the vasculature and overall health. NO plays an important and multi-faceted role within the vasculature as a potent regulator of vascular tone and directly impacting endothelial function (Maiorana et al 2003). In CACs, NO also serves an equally important regulatory role. NO bioavailability affects the migration and proliferation of CACs (Heiss et al, 2010).

Exercise training has been shown to positively affect circulating NO bioavailability (Maiorana et al 2003) However, our lab previously examined differences in intracellular NO and superoxide in CD34+ cells between sedentary and endurance trained men. They found 26% higher intracellular NO and 10% higher intracellular superoxide at baseline in the CD34+ cells of the sedentary men (Jenkins et al 2011). These findings were surprising, but upon further investigation into possible causal pathways, it was determined that the combination of high ROS and high NO levels can indicate the pathological production of NO. These findings of increased nitro-oxidative stress in C34+ cells in sedentary men provided the rationale for our initial hypotheses.

eNOS/iNOS

In the study by Jenkins et al (2011) discussed above, mRNA expression of eNOS and iNOS played a large role in explaining their unique findings. Previously, increased NO was viewed as generally beneficial, and the higher NO in their sedentary subjects was surprising. However, there are several isoforms of NOS and the different isoforms produce NO in very different ways. eNOS produces NO within the endothelium and is known for its beneficial effects on the vasculature (Forsetermann and Li 2011). eNOS also plays an important role in the migration and function of CACs (Ward 2011). iNOS has been found to be responsible for the pathological production of NO, which causes inflammation and oxidative damage (Sedoris, 2009). These differences between NOS isoforms are especially important when examining intracellular NO to provide context about how the NO is produced and whether it was a pathological or physiological production.

Our findings regarding eNOS and iNOS mRNA expression align somewhat with the concept of increased nitro-oxidative stress with less physical activity. Our findings are novel because of the population studied and the short duration of training cessation. The decrease in eNOS mRNA expression after the cessation of training was highly significant and underscores the idea that even short breaks in training can negatively impact the nitro-oxidative profile of CACs.

The eNOS "Coupling" Pathway:

As discussed above, eNOS is responsible for the beneficial production of NO and is linked to endothelial and CAC function. Recently, much of the literature relative to eNOS has been focused on the eNOS "coupling" pathway. Several recent studies have demonstrated that the eNOS coupling pathway is responsible for maintaining nitro-oxidative homeostasis and facilitates positive benefits for cardiovascular health (Bendall et al 2014). When eNOS is coupled, NO is produced and low levels of ROS are produced. If eNOS becomes uncoupled, the balance tips in the favor of ROS production and pathological levels of NO are produced. In the "uncoupled" state ROS and NO can combine to form peroxynitrite ($ONOO^-$), which has a negative impact on the coupling pathway and causes eNOS to remain uncoupled. This pathway can be seen in Figure 8 below (Crabtree et al 2011). Cofactors in the eNOS coupling pathway have recently been discovered to serve a critical role in maintaining nitro-oxidative homeostasis. Specifically, BH4 has been identified as the central cofactor involved in eNOS coupling (Benson et al 2013). BH4 bioavailability and function has been repeatedly linked to endothelial function and protection from cardiovascular disease (Chen et al 2014).

Given the current literature on the critical role of eNOS coupling in maintaining nitro-oxidative balance, we hypothesized that an increase in CD34+ intracellular NO and ROS following the cessation of training would be mediated via the eNOS "uncoupling" pathway. We utilized mRNA expression to examine this hypothesis. As discussed previously, eNOS mRNA expression in CD34+ CACs was significantly decreased after the ten-day cessation in training. We also examined cofactors intimately involved in the critical "coupling" of eNOS to elucidate possible mechanisms involved in the changes in eNOS expression, i.e. - the mRNA expression of GCH1 and DHFR.

GCH1 is the rate-limiting enzyme for the *de novo* synthesis of BH4 (Du et al 2009). Since BH4 has been shown to be critically important to the coupling of eNOS, we hypothesized that GCH1 would decrease following the cessation of training. Despite our significant decrease in eNOS observed following the cessation of training, we did not observe a significant change in GCH1 mRNA expression in CD34+ cells. Grijalva et al (2008) studied the eNOS-coupling pathway and exercise training in Goto-Kasizaki rats. In the exercise trained rats there was no significant difference in GCH1 expression, despite an increase in eNOS protein. Alp et al (2003) studied the BH4/eNOS coupling pathway in GCH1 transgenic mice. The GCH1 transgenic mice had an over expression of GCH1 and exhibited increased endothelial BH4 levels and decreased ROS production compared to the wild-type mice (Alp et al 2003). Bendall et al (2005) also used a transgenic mouse model to examine the eNOS coupling pathway and the role of GCH1. Their GCH1 transgenic mice exhibited greater NO production and preserved eNOS coupling (Bendall et al 2005). These studies show that results of research on GCH1 and

its role in the eNOS coupling pathway are varied and that its role in the eNOS coupling pathway is not fully understood at this point.

While our results did not support our hypothesis, ours is the first study, to our knowledge, to examine GCH1 expression in CACs, specifically CD34+ cells. Additionally, there is very little literature on GCH1 expression and its relationship to exercise training, and most of the existing research has been done in animal models. We formulated our hypotheses based (a) on literature on the eNOS coupling pathway (Figure 8.) and (b) the mechanism of GCH1 biosynthesis of BH4. However, given the mixed results in the literature and our findings in the present study, it is clear that more research in humans is needed in this area.

To further examine the eNOS coupling pathway relative to exercise training, we also assessed DHFR mRNA expression in CD34+ CACs. However, DHFR mRNA expression was not significantly altered by the ten-day cessation of training. DHFR is responsible for mediating the "salvaging pathway" to reduce BH2 back to BH4. Higher levels of BH2, in relation to lower levels of BH4, have been linked to decreased endothelial function. Takeda et al (2009) studied 163 patients with cardiovascular disease and found that higher levels of BH2 in the plasma negatively affected measures of flow-mediated dilation. They also found that treatment with statins reversed the ratio in favor of BH4 and improved FMD (Takeda et al 2009). Crabtree et al (2011) stated that DHFR serves a "key role" in coupling eNOS (figure 8). Due to DHFR's important involvement in the balance between BH2 and BH4, we believed that examining DHFR expression levels could provide some insight into the function of the eNOS-coupling pathway in CD34+ CACs. Despite a trend towards a decrease in FMD with the cessation in training,

we did not see a concurrent decrease in DHFR. A decrease in DHFR would indicate decreased activity of the salvaging pathway. Our results do not indicate a clear link between the cessation of exercise training and a reduction in DHFR and GCH1 mRNA expression in CACs of older endurance athletes.

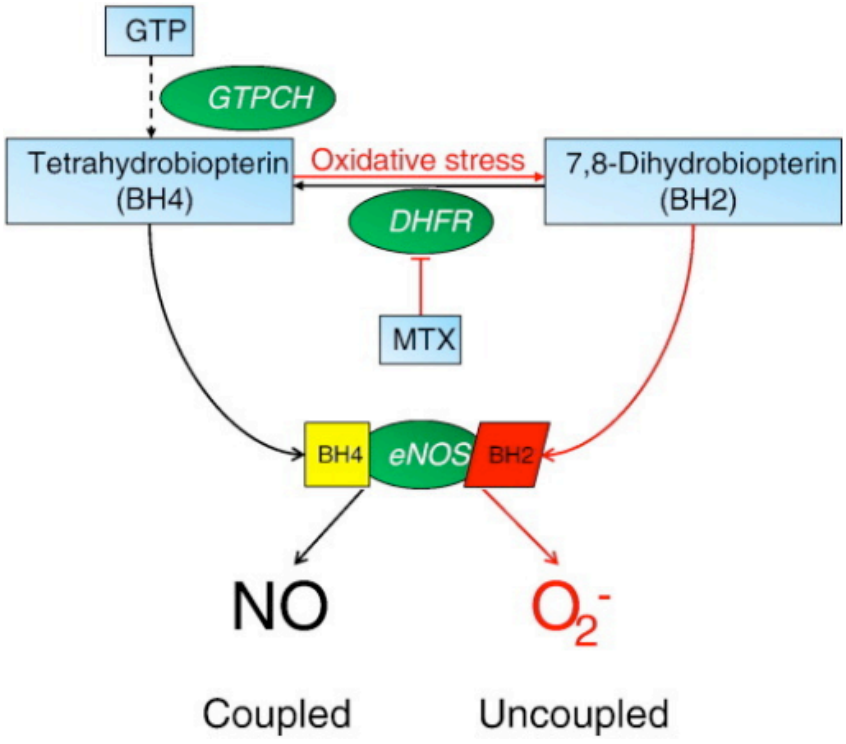

Figure 8. eNOS coupling pathway with cofactors DHFR and GCH1 (Crabtree et al 2011).

Chapter 5: Limitations:

There were several limitations to our study which could have contributed to results inconsistent with the literature and our hypotheses. First, we were unable to directly measure BH4, BH2, GCH1, or DHFR levels in fresh cells. These compounds are typically measured using high performance liquid chromatography (HPLC) in fresh cells. The ability to measure BH_4, BH_2, GCH1 and DHFR in the freshly isolated CD34+ cells could have given us better insight into their involvement in our observed changes in eNOS. In this circumstance, the samples we had to work with were already frozen, so mRNA expression measurement was the best option. The ability to analyze BH4 and BH2 levels directly, rather than upstream mRNA expression, could have allowed us to better determine the effects of exercise cessation on the eNOS coupling pathway and what mechanistic role BH4 is serving in CD34+ cells.

There were also some limitations in regards to our fresh cell measures of ROS and NO. About halfway through our study, there was some discussion about how the 96-well plates that we were using might be affecting our results. We had been using clear plates since the beginning of the study, and were then made aware that there may be significant cross talk between wells when using clear plates, which could be impacting our fluorescence measurements. In an attempt to mitigate this cross talk, we spread out the samples on the plate as best we could.

Another limitation, which is common in human research, was a low number of participants. Additionally, since CD34+ cells are already such a small fraction of the total CAC population, at times there were not enough cells available after the fresh cell

measures for mRNA expression to be measured. Several of our results were approaching significance and an increased sample size may have increased our ability to detect significant differences with the cessation of training.

Chapter 6: Further Research:

Further research into the eNOS coupling pathway in CACs should seek to measure BH4, BH2, GCH1, and DHFR levels in fresh cells. Direct measurement of the ratio of BH4 and BH2 at baseline and following a period of the cessation of training could give clear insight into how the eNOS-coupling pathway is functioning. The salvaging pathway has been shown to play an important role in vascular health, so being able to directly study this pathway in relation to exercise in older adults would be beneficial.

Chapter 7: Conclusion:

Our results showed that a ten-day cessation in training in older endurance athletes was not sufficient to elicit overall changes in flow-mediated dilation or nitro-oxidative stress in CD34+ CACs. However, the cessation of training was sufficient to significantly decrease eNOS mRNA expression and there was also a trend toward an uncoupled oxidative state in circulating CD34+ cells. Our study was the first to examine GCH1 and DHFR in CD34+ CACs, and while the results were not significant, there is still much research to be done in this area to examine changes in the eNOS-coupling pathway in CACs with exercise training. The results of this study illustrate that breaks in training, even for relatively short periods of time can impact eNOS levels in CACs and causes a trend towards negative changes in endothelial function. Aging, exercise, CAC function, and endothelial function are all important factors in cardiovascular disease. Therefore, the results of this study contribute to an important and growing body of literature on exercise and cardiovascular function in the older population.

University of Maryland at College Park

Department of Kinesiology

Project: Life-Long exercise and Novel Cardiovascular Disease Risk Factors

Unacceptable Medications List

Name	***Class***
Acebutolol	β-*Blocker*
Acetohexamide	*Anitdiabetes Drug*
Aldactone	*Diuretic (Thiazide)*
Amaryl	*Anitdiabetes Drug*
Atenolol	β -*Blocker*
Aquatensen	*Diuretic (Thiazide)*
Bendroflumethizaice	*Diuretic (Thiazide)*
Benzthiazide	*Diuretic (Thiazide)*
Betaxolal	β -*Blocker*
Bisoprolol	β -*Blocker*
Blocadren (Timoptic...)	β -*Blocker*
Carteolol	β -*Blocker*
Cartrol	β -*Blocker*
Carvediolol	β -*Blocker*
Chlorothiazide	*Diuretic (Thiazide)*
Chlorpropamide	*Antidiabetes Drug*
Chlorthalidone	*Diuretic (Thiazide)*
Cholestyramine	*Cholesterol Lowering Drug*
Colestid	*Cholesterol Lowering Drug*

Colestipol	*Cholesterol Lowering Drug*
Coreg	β *-Blocker*
Corgard	β *-Blocker*
Diabinese	*Antidiabetes Drug*
DiaBeta	*Antidiabetes Drug*
Diovan HCT	*Angiotensin II Receptor Antagonist*
plus HCTZ (thiazide diuretic)	
Diucardin	*Diuretic (Thiazide)*
Diurese	*Diuretic (Thiazide)*
Diuril	*Diuretic (Thiazide)*
Dymelor	*Antidiabetes Drug*
Enduron	*Diuretic (Thiazide)*
Esidrix	*Diuretic (Thiazide)*
Exna	*Diuretic (Thiazide)*
Ezide	*Diuretic (Thiazide)*
Fluvastatin	*Cholesterol Lowering Drug*
Furosemide	*Diuretic (Thiazide)*
Gemfibrozil	*Cholesterol Lowering Drug*
Glimepiride	*Antidiabetes Drug*
Glipizide	*Antidiabetes Drug*
Glucophage	*Glucose Lowering Drug*
Glucotrol	*Antidiabetes Drug*
Glynase	*Antidiabetes Drug*
Humulin	*Glucose Lowering Drug*
Hydrochlorothiazide (HCTZ)	*Diuretic (Thiazide)*
HydroDIURIL	*Diuretic (Thiazide)*
Hydroflumethiazide	*Diuretic (Thiazide)*

Hydromox	*Diuretic (Thiazide)*
Hydro-Par	*Diuretic (Thiazide)*
Hygroton	*Diuretic (Thiazide)*
Hyzaar	*Angiotensin II Receptor Antagonist*
plus HCTZ (thiazide diuretic)	
Iletin	*Glucose Lowering Drug*
Indapamide	*Diuretic (Thiazide)*
Inderal (Inderide, Inderide LA)	β *-Blocker*
Insulin ...	*Glucose Lowering Drug*
Kerlone	β *-Blocker*
Labetalol	*α and* β *-Blocker*
Lasix	*Diuretic (Thiazide)*
Lescol	*Cholesterol Lowering Drug*
Lente	*Glucose Lowering Drug*
Levatol	β *-Blocker*
Lipitor	*Cholesterol Lowering Drug*
Lopid	*Cholesterol Lowering Drug*
Lopressor	β *-Blocker*
Lorelco	*Cholesterol Lowering Drug*
Lovastatin	*Cholesterol Lowering Drug*
Lozol	*Diuretic (Thiazide)*
Maxide	*Diuretic (Thiazide)*
Metahydrin	*Diuretic (Thiazide)*
Metformin	*Glucose Lowering Drug*
Methyclothiazide	*Diuretic (Thiazide)*
Metolazone	*Diuretic (Thiazide)*
Metoprolol	β *-Blocker*

Mevacor	*Cholesterol Lowering Drug*
Micronase	*Antidiabetes Drug*
Minipress	*α -Blocker*
Mykrox.	*Diuretic (Thiazide)*
Nadolol	β *-Blocker*
Naturetin	*Diuretic (Thiazide)*
Naqua	*Diuretic (Thiazide)*
Niacin (vitamin B3 nicotinic acid)	*Cholesterol Lowering Drug*
Nicobid, Nicolar	*Cholesterol Lowering Drug*
Normodyne	*α and* β *-Blocker*
Novolin	*Glucose Lowering Drug*
Oretic	*Diuretic (Thiazide)*
Orinase	*Antidiabetes Drug*
Penbutolol	β *-Blocker*
Pindolol	β *-Blocker*
Polythiazide	*Diuretic (Thiazide)*
Pravastatin	*Cholesterol Lowering Drug*
Pravachol	*Cholesterol Lowering Drug*
Prazosin	*α -Blocker*
PressTab	*Antidiabetes Drug*
Probucol	*Cholesterol Lowering Drug*
Propranolol	β *-Blocker*
Questran	*Cholesterol Lowering Drug*
Quinethazone	*Diuretic (Thiazide)*
Renese	*Diuretic (Thiazide)*
Saluron	*Diuretic (Thiazide*)
Slo-Niacin	*Cholesterol Lowering Drug*

Simvastatin	*Cholesterol Lowering Drug*
Spironolactone	*Diuretic (Thiazide)*
Sectral	β *-Blocker*
Tenormin, (Tenoretic)	β *-Blocker*
Timolol	β *-Blocker*
Thalitone	*Diuretic (Thiazide)*
Tolazimide	*Antidiabetes Drug*
Tolbutamide	*Antidiabetes Drug*
Tolinase	*Antidiabetes Drug*
Toprol	β *-Blocker*
Trandate	*α and* β *-Blocker*
Tricholormethiazide	*Diuretic (Thiazide)*
Valsartan plus HCTZ	*Angiotensin II Receptor Antagonist*
plus HCTZ (thiazide diuretic)	
Velosulin	*Glucose Lowering Drug*
Visken	β *-Blocker*
Zaroxolyn	*Diuretic (Thiazide)*
Zebeta	β *-Blocker*
Zocor	*Cholesterol Lowering Drug*

Important Note: If the subject is taking a drug that is not on this list, please refer to a reference regarding prescription drugs (e.g., The Pill Book ,10th Edition or on the web at PDR.net) to insure that the drug does **not** affect lipid or glucose metabolism.

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