DOES ADMINISTRATION OF VITAMIN C ATTENUATE AN ACUTE MENTAL-STRESS INDUCED IMPAIRMENT IN ENDOTHELIAL FUNCTION?

by

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Abstract

Acute mental stress has been shown to elicit a transitory impairment in the function of the endothelial cells that line the arteries. While this effect of mental stress on endothelial function is generally accepted, the mechanisms by which this occurs remain unclear. One putative mechanism is the generation of reactive oxygen species (ROS). The objective of the study performed for this thesis was to examine whether orally administered vitamin C (an antioxidant which can combat the negative effects of ROS) can attenuate reductions in brachial artery endothelial function due to mental stress. Fifteen men (21 ± 2 yrs) were given 1000mg of vitamin C or placebo over two experimental days (one per visit) in a randomized, double-blinded, within-subject study. Acute mental stress was induced using the Trier Social Stress Task (TSST), which consists of both a speech and mental arithmetic task. Flow-mediated dilation (FMD), an index of endothelial function, was assessed using ultrasound at baseline, prior to the acute mental stress task, and 30, and 90 minutes post-stress. Saliva samples were taken to measure the stress hormone cortisol at all FMD time points as well as immediately post-stress. Stress reactivity was also characterized by changes in heart rate (HR) and mean arterial pressure (MAP). A significant stress response was elicited by the TSST in both conditions as MAP, HR and salivary cortisol all increased significantly. Contrary to the hypothesis, endothelial function was unaffected by time ($p = 0.631$) or condition ($p = 0.792$). However, a correlation was found between Δ%FMD at 30 minutes and ΔMAP ($p = 0.015$) and Δsalivary cortisol ($p < 0.001$) in the placebo condition but not the vitamin C condition ($p = 0.480$, $p = 0.461$). These findings indicate that acute mental stress may not impair endothelial function in healthy, young adults. The observation that relationships between stress reactivity parameters and changes in FMD were abolished in the vitamin C condition may indicate that ROS signaling influences FMD post-

stress. Further research is required to elucidate the role of ROS in modulating stress-induced changes in endothelial function.

Co-Authorship

Meghan Plotnick was responsible for writing all of the chapters of this thesis with guidance, comments, and revisions provided by Dr. Kyra Pyke.

The manuscript in Chapter 3 entitled “Does administration of vitamin C attenuate an acute mental stress induced impairment in endothelial function?” was prepared for future submission and is the work of Meghan Plotnick in collaboration with her co-authors Dr. Kyra Pyke, Dr. Brendan Gurd, Trisha Scribbans and Katrina D’Urzo (School of Kinesiology and Health Studies, Queen’s University).

Meghan Plotnick was responsible for conception and design of the research question, examining the relevant literature, designing the study, acquisition of data, analyzing the data, running statistical analyses, interpreting the results, and writing the manuscript. Dr. Kyra Pyke provided critical revision of the manuscript for important intellectual content, direction in all components of the study and was the principle investigator on the research grant, which funded this study. Dr. Brendan Gurd and Trisha Scribbans provided guidance, resources, and access to Dr. Gurd’s laboratory for salivary cortisol analysis. Katrina D’Urzo aided in data collection and analysis of blood velocity.

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absFMD – absolute FMD

ACTH – adrenocorticotropic hormone

AUC – area under the curve

BH2 – 7,8 dihydrobiopterin

BH4 - tetrahydrobiopterin

BMI – body mass index

C – condition

CAD – coronary artery disease

CRH – corticotropin releasing hormone

CVD – cardiovascular disease

E – epinephrine

EDHF – endothelium derived hyperpolarizing factor

ELISA – enzyme-linked immunoassay

eNOS – endothelial nitric oxide synthase

F – female

FMD – flow-mediated dilation

GTN – glyceryl trinitrate

GTPCH1 – guanylate cyclohydrolase 1

H_2O_2 – hydrogen peroxide

HPA – hypothalamic-pituitary adrenal

HR – heart rate

L-NMMA – eNOS inhibitor NG monomethyl-L-arginine

M – male

MAP – mean arterial pressure

NADPH – nicotinamide adenine dinucleotide phosphate

NE – norepinephrine

NO – nitric oxide

NS – not specified

O_2^- - superoxide

$ONOO^-$ - peroxynitrite

PAR – physical activity recall

RH – reactive hyperemia

ROS – reactive oxygen species

SR – shear rate

T – time

TBARS – thiobarbituric acid reactive substances

TSST – Trier Social Stress Task

TxC – time by condition

Chapter 1

Introduction

The vascular endothelium is a single layer of cells that lines the inner wall of the vasculature. The endothelium plays an important role in regulating vascular homeostasis and health (Poredos and Jezovnik 2013). The endothelial cells are sensitive to hemodynamic shear stress; a frictional force caused by blood flow through the vessel (Celermajer et al. 1992). In response to this stimulus, the endothelial cells produce vasoactive substances such as nitric oxide (NO; Furchgott and Zawadski 1980, Mitchell et al. 2008, Osanai et al. 2000), which allow for vasodilation of the artery. Flow-mediated dilation (FMD) characterizes the sequence of events by which the endothelial cells sense blood flow induced shear stress, respond by releasing vasodilator molecules, and ultimately elicit an increase in vessel diameter.

The most commonly used test of endothelial function in a laboratory setting is the reactive hyperemia technique (Celermajer et al. 1992). In this technique, a cuff is wrapped around the forearm and inflated for 5 minutes to induce vascular occlusion. Upon deflation, a large increase in forearm blood flow is elicited causing an increase in blood flow associated shear stress in the upstream brachial artery (Celermajer et al. 1992). The resulting changes in diameter are measured using high-resolution ultrasound (Celermajer et al. 1992) and FMD is quantified as the percent change in arterial diameter from baseline to peak diameter post-reactive hyperemia. Poor FMD responses have been observed in populations with cardiovascular disease (CVD) risk factors (Celermajer et al. 1992) and in individuals suffering from diseases that impact the cardiovascular system (Johnstone et al. 1993, Lteif et al. 2005, Hamburg et al. 2008, Recio-Mayoral et al. 2011, Banerjee et al. 2011). Furthermore, it has been shown that individuals

who exhibit FMD impairments also have low NO bioavailability (Seals et al. 2009, Tang et al. 2013). Indeed, NO has been shown to be a major vasodilator in the conduit artery FMD response (Green et al. 2014) and has various antiatherogenic properties (Cooke et al. 1997). A reduction in NO bioavailability results in a loss of arterial vasoprotection (Taddei et al. 2000, Seals et al. 2009, Spier et al. 2004), which can compromise proper arterial function. Acute stimuli such as a high fat meal (Vogel et al. 1997, Gaenzer et al. 2001) and pain (Jambrik et al. 2005a, King et al. 2015) can also cause a transient decline in endothelial function. It has been suggested that if such temporary impairments of endothelial function are frequent and prolonged, the cumulative effect may be a significant attenuation of vasoprotection (Black and Garbutt 2002).

Many studies have shown that acute bouts of mental stress transiently impair vascular endothelial function in healthy individuals (Ghiadoni et al. 2000, Spieker et al. 2002, Broadley et al. 2005). The mechanism(s) by which acute mental stress negatively impacts endothelial function, however, remains incompletely understood. One potential mechanism through which mental stress may impair endothelial function is via a stress-induced production of reactive oxygen species (ROS; Iuchi et al. 2003, Poitras and Pyke 2013). ROS are defined as highly reactive molecules containing oxygen (Luschak et al. 2014) and these molecules are produced both as byproducts of normal cellular metabolism and for cell regulation purposes. While ROS in low concentrations are essential to regulation of vascular tone and homeostasis within the endothelium (Chatterjee and Fisher 2014, Brandes et al. 2014), high concentrations of ROS or oxidative stress can be harmful to the vasculature. This notion is supported by evidence of improved endothelial function following the supplementation of vitamin C, a known antioxidant and ROS scavenger, in individuals with CVD (Levine et al. 1996, Gokce et al. 1999, Tousoulis et al. 1999, Duffy and Vita 1999, Kugiyama et al. 1998). *In vitro* studies provide further

evidence that ROS may impair endothelial function through reducing NO bioavailability through: (1) oxidizing NO to peroxynitrite (Laursen et al. 2001, Milistien and Katusic 1999) and (2) uncoupling endothelial nitric oxide synthase (eNOS) through decreasing the bioavailability of eNOS essential cofactor tetrahydrobiopterin (BH4; Zheng et al. 2003, Wever et al. 1997, Förstermann and Munzel 2006, Förstermann 2006, Kinoshita et al. 1997).

Although a role for elevated oxidative stress in acute mental stress induced endothelial dysfunction has yet to be examined, there is evidence to support the involvement of ROS. Oxidative stress is elevated in individuals with significant life stress such as caregiving for a chronically ill child (Epel et al. 2004) and psychiatric disorders (Black et al. 2014, Ben-Shachar & Laifenfeld 2004, Whatley et al. 1998, Andreazza et al. 2008). Furthermore, other acute stimuli, such as an acute bout of oscillatory shear stress, are capable of causing physiologically relevant increases in oxidative stress that impair FMD (Johnson et al. 2013) and elevated oxidative stress has been observed in association with acute mental stress (Morimoto et al. 2008, Sivonová et al. 2004). This is supported by *in vitro* evidence that cortisol, a well-known factor of the stress response, has been shown to stimulate ROS generation (Iuchi et al. 2003). The presented evidence suggests that stress-induced endothelial dysfunction may be mediated by an elevation in ROS. The purpose of the study performed for this thesis was to determine whether vitamin C supplementation can attenuate a decline in endothelial function as measured by FMD following mental stress in healthy, young men. Improvement in mental-stress induced impairment in FMD through vitamin C administration would suggest a mechanistic role for ROS in stress-induced endothelial dysfunction.

Objective: The primary objective of the study performed for this thesis was to determine whether orally administered vitamin C can attenuate reductions in brachial artery FMD due to mental stress at both 30 and 90 minutes post-stress.

Specific Hypothesis: An increase in reactive oxygen species (ROS) contributes to acute mental stress induced impairment in FMD, such that oral provision of vitamin C will attenuate reductions in brachial artery FMD due to mental stress at both 30 minutes and 90 minutes post-stress.

Chapter 2

Literature Review

2.1 The endothelium

The endothelium is a single layer of cells that line the inner surface of all vessels in the vascular system. Endothelial cells are responsible for sensing and responding to a parallel frictional drag force called shear stress which increases as a result of an increase in blood flow (Cunningham and Gotlieb 2005). In response to shear stress, the endothelial cells modulate vascular tone of the surrounding smooth muscle through the production of vasoactive substances such as prostacyclin (Mitchell et al. 2008, Osanai et al. 2000), endothelium derived hyperpolarizing factor (EDHF; Bellien et al. 2006, Bellien et al. 2008), endothelin-1 (Berger et al. 2001, Thijssen et al. 2008) and nitric oxide (NO; Joannides et al. 1995, Mullen et al. 2001). The phenomenon by which the endothelial cells sense blood flow-induced shear stress and respond by releasing molecules which vasodilate the blood vessel is known as flow-mediated dilation (FMD).

NO is the most widely studied molecule that contributes to FMD. Endothelial nitric oxide synthase (eNOS) is responsible for the production of NO through the conversion of the substrate L-arginine into L-citrulline and NO (Alderton et al. 2001). Newly made NO quickly diffuses from the endothelium to the smooth muscle cells of the blood vessel and activates guanylate cyclases that allow the smooth muscle to relax (Hofman et al. 2005). NO has various anti-atherogenic (vasoprotective) qualities such as inhibition of smooth muscle proliferation, leukocyte adhesion, and platelet aggregation and adhesion (Cooke et al. 1997). Moreover, low NO bioavailability is observed in individuals with endothelial dysfunction

such as the elderly (Taddei et al. 2009, Seals et al. 2009) and those with cardiac dysfunction (Tang et al. 2013). Specifically, Taddei et al. (2000) compared forearm responses to acetylcholine infusion (an endothelial dependent vasodilator) across the following four subgroups: old sedentary, old athletes, young sedentary, and young athletes. At baseline, endothelial responses to acetylcholine were significantly lower in the sedentary compared to the aerobically trained elderly group as well as significantly lower in both old subgroups compared to the young subgroups. Researchers found that co-infusion of the eNOS inhibiter NG monomethyl-L-arginine (L-NMMA) caused a significantly greater impairment in endothelial responses to acetylcholine in the aerobically trained elderly group than in sedentary elderly group. This evidence suggests that endothelial dysfunction in the sedentary elderly group was possibly due to a decline in NO bioavailability that was preserved by habitual exercise throughout the lifetime. It has been suggested that low NO bioavailability results in an impaired FMD response to an increase in shear stress and concomitantly reduced vasoprotection (Taddei et al. 2000, Seals et al. 2009, Spier et al. 2004). Vasoprotection refers to any substance that maintains necessary functions of the vascular wall such as vasodilation (Gewaltig et al. 2002). Furthermore, low NO bioavailability is associated with endothelial dysfunction, which appears to precede and be involved in the development of atherosclerosis (Nabel et al. 1990, Healy 1990, Celermajer et al. 1992, Drexler and Hornig 1999, Kawashima and Yokoyama 2004).

2.1.1 Assessment of Endothelial Function

Examination of coronary artery endothelial function can be performed invasively through vasodilator infusion (Vita et al. 1990, Schächinger et al. 2000, Suwaldi et al. 2000). However, the use of invasive techniques as a standard measure of coronary artery endothelial

function is a not feasible methodology. Celermajer et al. (1992) was the first to outline a non-invasive method to examine endothelial function using FMD. This group used high-resolution ultrasound to track changes in brachial arterial diameter during FMD and in response to glyceryl trinitrate (GTN) which acts directly on smooth muscle and allows measurement of endothelial independent dilation. In order to increase brachial artery blood flow and shear stress to elicit FMD, the reactive hyperemia (RH) method was employed in which a pneumatic cuff was placed around the forearm just downstream of the brachial artery measurement site. After a period of inflation, release of the cuff causes a large increase in blood flow and shear stress in the brachial artery (Celermajer et al. 1992) causing FMD (Buga et al. 1991, Joannides et al. 1995, Melkumyants et al. 1989). FMD is quantified as the percent change in arterial diameter from baseline to the peak diameter post RH. An impaired FMD with an intact GTN response allows for isolation of dysfunction to the endothelium.

In Celermajer et al.'s (1992) study, it was observed that RH induced FMD was impaired in populations at risk for cardiovascular disease (CVD) including children with symptom-free familial hypercholesterolemia, adult smokers, and in adults with coronary artery disease (CAD). A later study observed a strong correlation between coronary artery FMD and brachial artery FMD (Takase et al. 1998). This study solidified the use of brachial artery FMD as a surrogate measure of coronary artery endothelial function. Since these publications, associations between impaired brachial artery FMD and other conditions associated with CVD such as aging (Seals et al. 2009), diabetes mellitus (Johnstone et al. 1993), metabolic syndrome (Lteif et al. 2005, Hamburg et al. 2008), and chronic kidney disease (Recio-Mayorai et al. 2011, Banerjee et al. 2011) have been observed. Furthermore,

brachial artery FMD has been shown to be prognostic for cardiovascular risk (Widlansky et al. 2003, Lerman and Zeiher 2005, Shechter et al. 2014).

2.2 Nitric oxide and reactive hyperemia flow-mediated dilation.

The role of NO in FMD was established through studies in which the eNOS inhibiter NG monomethyl-L-arginine (L-NMMA) was infused. Post L-NMMA infusion, standard reactive hyperemia induced FMD was almost abolished such that no significant change in diameter was observed (Joannides et al. 1995, Mullen et al. 2001). This finding suggested that impaired FMD was indicative of reduced NO bioavailability (Pyke and Tschakovsky. 2005, Pyke et al. 2010). However, two well-designed studies comparing FMD with infusion of saline and infusion of eNOS inhibitor L-NMMA have shown that NO is not obligatory for RH-FMD in the brachial (Wray et al. 2013) and radial (Pyke et al. 2010) arteries. These findings support the notion that there may be compensatory mechanisms that stimulate vasodilation during eNOS inhibition (Bellien et al. 2007). A recent meta-analysis by Green and colleagues (2014), however, has shown that NO remains a major player in the FMD response of conduit arteries in the majority of studies reviewed.

2.3 Reactive Oxygen Species

Reactive oxygen species (ROS) are highly reactive molecules containing oxygen (Zhou et al. 2013). ROS can be sub-categorized into free radicals such as superoxide (O_2^-) and peroxynitrite ($ONOO^-$) and non-radicals such as hydrogen peroxide (H_2O_2). ROS are typically considered a harmful species generated as a byproduct of cellular metabolism (Craige et al. 2015), as these molecules can cause damage to lipids, membranes, proteins, and DNA due to free radical reactions (Zhou et al. 2013). The detrimental role of ROS prompted

the “free radical theory of aging” by Harman (1956) which suggested that the production of free radicals throughout the lifetime are ultimately responsible for aging the cell through attacking important cellular constituents. This notion was initially supported by studies in which antioxidant supplementation improved the lifespan of mice (Harman 1957).

Although traditionally considered damaging molecules, many important functions of ROS have been discovered over the past few decades. ROS are produced as a byproduct of normal cellular metabolism and also generated by oxidases within the cell like nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or xanthine oxidase to serve a function in signal transduction (Zhou et al. 2013). In the endothelium, ROS play an important role in regulating vascular tone and homeostasis (Chatterjee et al. 2014, Brandes et al. 2014). ROS become harmful, however, in high levels – a state called oxidative stress. Oxidative stress occurs when there is an imbalance between ROS and antioxidants such that a greater number of ROS are produced than antioxidants (Sies et al. 1985, Sies et al. 1986, Sies et al. 1991). In this state, antioxidants are unable to counterbalance the highly reactive nature of ROS. This amplified ROS production has been observed in various cardiovascular pathophysiologies such as hypertension and atherosclerosis (Touyz 2005, Grendling and FitzGerald 2003, Mazor et al. 2010).

2.3.1 Reactive oxygen species and the endothelium

A role for ROS in endothelial dysfunction is supported by the beneficial effects of vitamin C administration on FMD (Johnson et al. 2013, Hirai et al. 2000, Evans et al. 2003, Anderson et al. 2006, Hamabe et al. 2001, Takase et al. 2004b, Silvestro et al. 2002, Plantinga et al. 2007, Stramatelopoulous et al. 2003, Ellis et al. 2001, Gokce et al. 1999, Levine et al. 1996). Moreover, Heitzer and colleagues (2001) showed that the amount of

systemic oxidative stress might be a predictor of cardiovascular event risk in patients with CAD. In this study, coronary artery disease patients' forearm blood flow responses to acetylcholine with and without co-administration of vitamin C were performed. Subsequent 4.5 years of follow-up showed that those who had greater improvements in forearm vascular responses with vitamin C administration were more likely to experience a cardiovascular event than those with lower responses. This suggests that the amount of vascular oxidative stress may represent an important and clinically relevant mechanism of endothelial dysfunction (Heitzer et al. 2001).

As FMD and vasodilation in response to acetylcholine have both been shown to be primarily NO mediated responses, this suggests that ROS has detrimental effects on NO bioavailability. Indeed, in vitro studies have shown that ROS molecule O_2^- (superoxide) reacts rapidly with available NO to produce $ONOO^-$ (peroxynitrite; Laursen et al. 2001, Milistien and Katusic 1999). This negatively affects the endothelium by reducing vasodilator NO availability and furthering oxidative stress related damage to proteins, lipids, and DNA. The reaction of O_2^- with NO is ~3-4 times more rapid than the antioxidant enzyme superoxide dismutase is able to scavenge superoxide (Schulz et al. 2008) - suggesting that the production of peroxynitrite is a significant side effect of ROS generation. Furthermore, O_2^- is known to uncouple eNOS through interfering with eNOS essential cofactor tetrahydrobiopterin (BH4) availability by: (1) oxidizing BH4 to inactive dihydrobiopterin (BH2; Wever et al. 1997, Förstermann and Münzel 2006, Förstermann 2006, Kinoshita et al. 1997), (2) decreasing expression of the BH4 synthesizing enzyme guanylate cyclohydrolase 1 (GTPCH1; Zheng et al. 2003) and (3) by depleting NADPH, a molecule essential for BH4 synthesis (Nichol et al. 1985) as well as BH4 regeneration from BH2 (Scott-Burden 1995;

Fig. 1). Uncoupled eNOS produces superoxide instead of NO, which is capable of producing more $ONOO^-$ thereby initiating a vicious cycle in which ROS generation stimulates further ROS generation (Crabtree et al. 2009).

Interestingly, not all ROS are harmful to the endothelium. In vitro studies using isolated bovine arterial segments have shown that H_2O_2 is unable to react with NO and actually stimulates guanylate cyclase in the surrounding smooth muscle cells (Wolin and Burke 1987, Burke and Wolin 1987). Furthermore, other in vitro studies have shown that H_2O_2 activates and enhances expression of eNOS and increase NO bioavailability (Drummond et al. 2000, Thomas et al. 2002, Bretón-Romero et al. 2012, Bretón-Romero et al. 2013). While this in vitro evidence has provided insight into the potential role of H_2O_2 in regulating vascular tone, in vivo evidence provides a more physiologically relevant picture. An elegant supporting in vivo study was performed by Ray and colleagues (2011) in which rats with a genetic modification to elicit excessive production of H_2O_2 displayed improved endothelial function and a reduction in blood pressure. Endothelial function and blood pressure were normalized in these rats with antioxidant supplementation (Ray et al. 2011). This suggests that H_2O_2 has a physiologically relevant role in the ability of the endothelium to dilate. The vasoprotective functions of H_2O_2 compared to the detrimental effects of O_2^- suggest that the presence of ROS in the endothelium is somewhat complicated and perhaps dependent on the species formed (Craige et al. 2015).

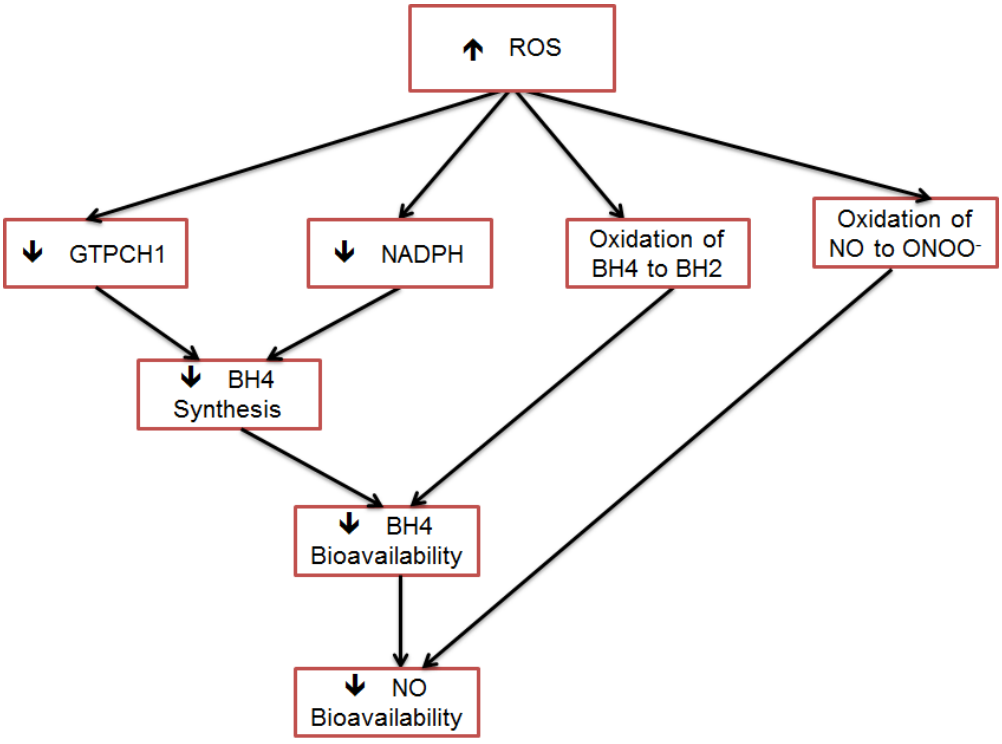

Figure 1. The harmful effects of reactive oxygen species (ROS) such as superoxide on BH4 and NO bioavailability. ROS decreases BH4 synthesis through both decreasing expression of guanulate cyclohydrolase 1 (GTPCH1; which catalyzes the rate-limiting reaction in BH4 synthesis) and by depleting nicotinamide adenine dinucleotide phosphate (NADPH; essential for the final reaction in BH4 synthesis). ROS also depletes BH4 through oxidizing BH4 to 7,8- dihydrobiopterin (BH2). A decline in BH4 bioavailability depletes NO bioavailability via eNOS uncoupling.

2.4 Mental Stress

Stress is defined as any disruption in homeostasis due to internal or external events that require adjustment by the hormonal, behavioural, and autonomic systems (Maier and Watkins, 1998). Psychosocial stress represents an imbalance between perceived demands and coping abilities (Cohen et al. 1995). Acute stress refers to exposure to an immediate stressor (minutes) such as some types of job stress and chronic stress refers to repeated exposure to acute episodes of stress (days; Black and Garbutt). Mental stress can be captured or quantified in a number of different ways including by self-report questionnaire such as the Job Content Questionnaire (Karasek et al. 1998), salivary cortisol and heart rate (Kirschbaum et al. 1993). The following sections will review the literature with respect to: (1) physiological responses to mental stress; (2) chronic mental stress and cardiovascular disease; (3) i. acute mental stress in the laboratory, ii. participant characteristics that influence laboratory induced stress responses, and iii. the effect of acute mental stress on endothelial function; as well as (4) reactive oxygen species as a putative mechanism linking acute mental stress and endothelial function.

2.4.1 Physiological responses to mental stress

Hans Selye (1950) first described the stress response as the general adaptation syndrome. This adaptive response is beneficial from an evolutionary standpoint, as its function is to prepare the body to respond in a manner that increases the likelihood of survival (Tsigos et al. 2002). This response is commonly referred to as the “fight or flight” response in which the body prepares to either combat (“fight”) or escape from (“flight”) the stressor (Cannon 1929). When faced with an anxiety-provoking situation, the thalamus and frontal lobes first integrate environmental stimuli and evaluate the stressor (Tsigos et al.

2002). Depending on appraisal of the stressor, an emotional response is generated in the limbic system, which is able to act on the hypothalamus and activate the hypothalamic pituitary adrenal (HPA) axis (Tsigos et al. 2002).

There are two major systems that are activated at the onset of psychosocial stress: the autonomic nervous system and the HPA axis (Fig. 2). The autonomic nervous system provides an initial rapid response to stressful stimuli, which is characterized by increases in heart rate and blood pressure (Tsigos et al. 2002). These cardiovascular responses are attributed in part to increased sympathetic drive and the resulting release of catecholamines (Black and Garbutt 2002) as well as parasympathetic withdrawal (Hatfield et al. 1998, Spalding et al. 2000, Smeets 2010). The onset of mental stress also stimulates hypothalamic release of corticotropin releasing hormone (CRH), which enters the hypothalamic-pituitary portal vessels and acts on the anterior pituitary gland to elicit the production of adrenocorticotropic hormone (ACTH; Tsigos et al. 2002). ACTH enters the circulation and acts on the adrenal cortex to increase production of the stress hormone cortisol (Tsigos et al. 2002). The HPA axis provides a "delayed" response to stress; this is evidenced by observations of peak cortisol as early as 20 (Kirschbaum et al. 1995b, Schommer et al. 2003, Rimmele et al. 2007) to 40 minutes (Ghiadoni et al. 2000) post-stress. It appears that salivary cortisol levels return to baseline sometime between 40 (Rimmele et al. 2003) and 80 (Schommer et al. 2003) minutes post-stress task.

Cortisol has a number of functions that are integral in preparing the body for action in acutely stressful situations; these include increasing glucose availability, inhibiting non-essential functions (e.g., bone growth and reproduction), and suppressing immune function (Brooker et al. 2006). Cortisol plays a key role in regulating the duration of the stress

response through inhibitory feedback at neural sites such as extra-hypothalamic centers, the hypothalamus, and the pituitary gland (de Kloet 1991). This is important as it minimizes the duration of the detrimental functions of cortisol such as catabolism and immunosuppression (Tsigos et al. 2002).

Cortisol responses were initially and typically described as a non-specific reaction to general stress or situations in which central goals such as physical preservation are endangered (Selye 1950). However, this does not necessarily explain why social situations like public speaking elicit an HPA axis response and are interpreted as stressful. Dickerson & Kemeny (2004) hypothesized that cortisol responses may be activated under situations in which preservation of the "social self" is compromised. This is based on the idea that the social self reflects self-esteem, social value, and others' perception of an individual's worth; an important characteristic of success and survival in a social group (deWaal 1989, Gilbert 1997).

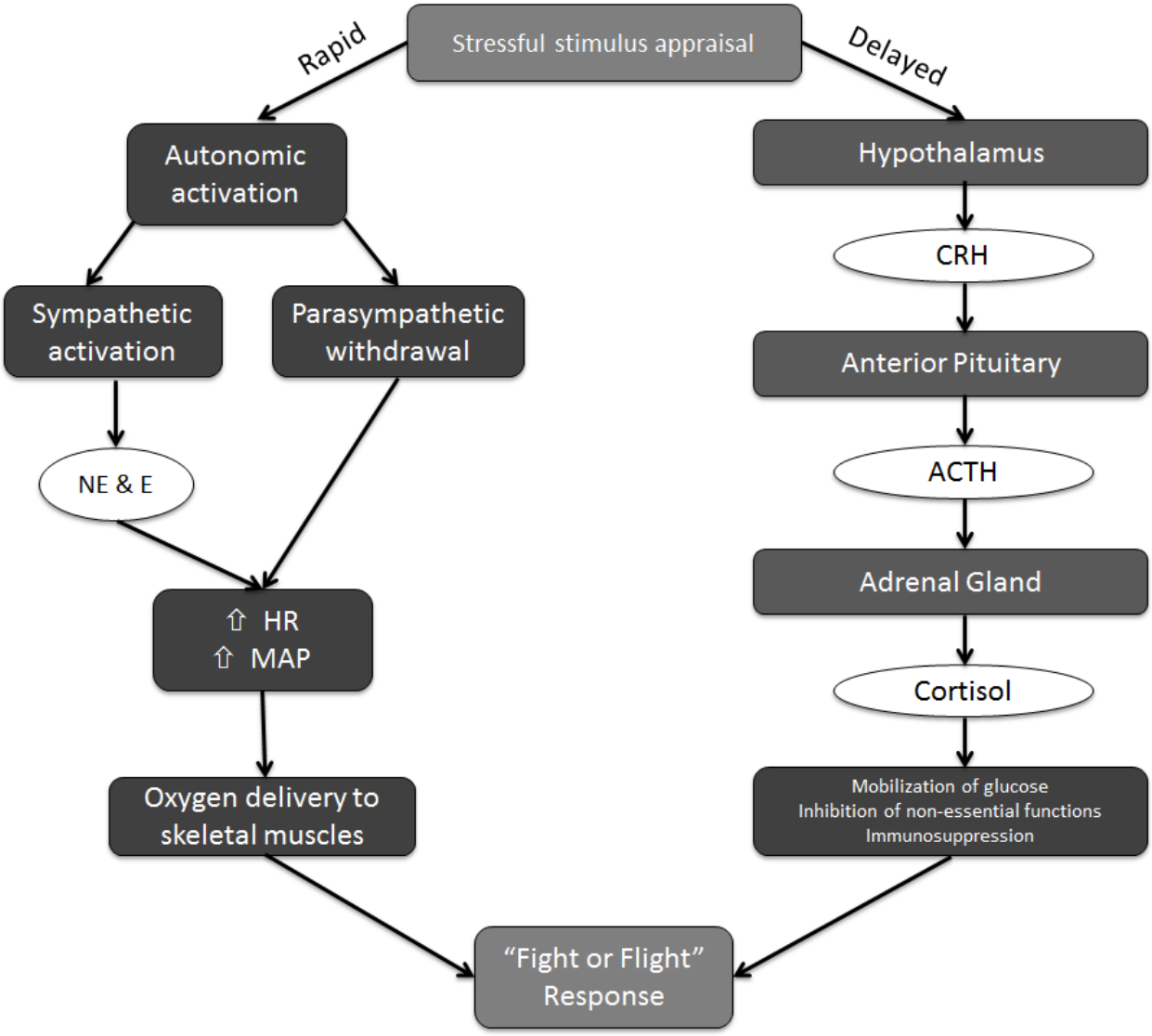

Figure 2. Physiological responses to mental stress. The ultimate purpose of the stress response is to prepare the body to elicit a "fight or flight" response to cope with the stressor. First, a stimulus is appraised by the brain as stressful, which launches a rapid autonomic response in which sympathetic activation and parasympathetic withdrawal lead to an increase in heart rate (HR) and mean arterial pressure (MAP). An increase in HR and MAP promote oxygen delivery to the skeletal muscles. A delayed hypothalamic-pituitary adrenal (HPA) axis response is also elicited which ultimately leads to cortisol production. Cortisol allows for the mobilization of glucose and inhibitions of non-essential functions and immunity in order to respond to the stressor. Abbreviations: ACTH, adrenocorticotropic hormone; CRH, corticotropin releasing hormone; E, epinephrine; NE, norepinephrine.

2.4.2 Chronic mental stress and cardiovascular disease

Many studies have shown that chronic mental stress is associated with an increased risk of development of type 2 diabetes (Huth et al. 2014, Cosgrove et al. 2012) and CVD (Bairey Merz et al. 2002, Peter and Siegrist 2000). Specifically, these studies have examined the role of job stress using questionnaires such as the Karasek Job Content Questionnaire, in order to characterize stress. The Karasek Job Content Questionairre has been validated as a tool to assess job strain internationally (Karasek et al. 1998). Furthermore, one study showed that high job strain as assessed by the Karasek Job Content Questionnaire was associated with salivary cortisol (Masilamani et al. 2012). Stress is often considered an independent risk factor for CVD (Iso et al. 2002, Yusuf et al. 2004, Richardson et al. 2012). Lifestyle associated psychosocial stress measures such as Type A behaviour personality (Williams et al. 1980), hostility (Julkunen et al. 1994), and job stress (Lynch et al. 1997, Bairey et al. 2002, Peter and Siegrist 2000) are all independently associated with atherosclerosis. Moreover, correlations between stress and myocardial infarction (Yusuf et al. 2003), stroke (Iso et al. 2002), and coronary heart disease (Richardson et al. 2012) have been observed.

Although the relationship between mental stress and impaired cardiovascular function is well established, the mechanisms that link the two remain incompletely understood. Evidence suggests that a decline in endothelial function may be involved. For instance, Takase et al. (2004a) examined the relationship between sleep deprivation and emotional stress on brachial artery FMD in university students after the final exam period. Immediately following this 4-week exam period, brachial artery FMD declined from 7.4 ± 3.0% to 3.7 ± 2.3%. Longer-term mental stress such as chronic caregiving to a spouse with Alzheimer's disease is also associated with a significantly poorer FMD compared to non-caregiving controls (Mausbach

et al. 2010). However, the mechanisms through which chronic mental stress leads to impaired endothelial function remain unclear.

2.4.3 Acute mental stress

Studies examining the effects of acute mental stress have recently been carried out, in part to better understand the mechanisms that underlie the relationship between chronic mental stress and CVD. A recent review by Poitras & Pyke (2013) provided two points that summarize current thinking on the importance of studying the impact of acute mental stress on vascular function. The first point being that acute mental stress may exacerbate a predisposition for cardiovascular incidents. This notion is supported by evidence that acute bouts of psychosocial stress provoke myocardial ischemia in patients with CAD (Deanfield et al. 1984). The second point is that repeated intervals of transient endothelial dysfunction stimulated by multiple bouts of acute mental stress may cumulatively result in a clinically important attenuation of endothelially-mediated vasoprotection.

2.4.3.1 Laboratory induced acute mental stress

The large variability in individual response to mental stress has been well documented (Kirschbaum and Helhammer 1989). This presents a challenge in studying psychosocial stress and thus, it is essential to develop and utilize a stress task that reproducibly activates the HPA axis. There are a number of factors that may influence an individual's response to laboratory stress tasks. These factors can be thought of as a) characteristics of the task itself and b) characteristics of the participants undergoing the task. Factors of the task itself such as: task difficulty (Pruessner et al. 1999), lack of control (Peters et al. 1998), and social support during the task (Kirschbaum et al. 1995a), appear to affect cortisol responses to the task. In order to best study the stress response, it is important to find

an intervention which will elicit activation of the autonomic system and HPA axis to the largest extent in the greatest amount of people. A number of stress tasks are cited in the literature including: mirror tracing (Collins & Frankenhauser 1978, Steptoe et al. 2002), computerized Stroop test (Muldoon et al. 1992), speech task (Bassett et al. 1987, Al'Absi et al. 1997), and mental arithmetic (Jørgensen et al. 1990, Suarez et al. 1991, Tersman et al. 1991). Physical stressors, such as the cold pressor, test also elicit increases in cortisol production (Bullinger et al. 1984) and have been used as a stress paradigm (Szijgyarto et al. 2014). The most widely used stress task is called the Trier Social Stress Task (TSST). Developed in 1993 by Kirschbaum, Pirke, and Helhammer, the TSST incorporates both a speech and mental arithmetic task and was developed to be a reproducible method of inducing mental stress. Kirschbaum and colleagues (1993) found profound increases in HPA axis hormones ACTH and serum and salivary cortisol post-task. However, it should be noted that there was still inter-individual variability; 70% responded with at least a 2.5 nmol/L increase in salivary cortisol suggesting that 30% had a smaller response or no response.

There are a number of reasons that may explain why the TSST elicits such a robust response. Uncontrollability of a task or a situation in which behaviour cannot elicit an outcome appears to be an important component of a stress task (Dickerson & Kemeny 2004). This context of "forced failure" can be incorporated into a stress task by, for instance, giving the participants headphones that play loud and unpleasant noise whilst performing a task (Peters et al. 1998). Uncontrollability is increased during the mental arithmetic section of the TSST in which the participant is told their answer is incorrect despite it being correct (Dickerson & Kemeny 2004). Another seemingly important characteristic of a stress task is social evaluative threat, which can be achieved by any motivated performance situation

where an individual's capabilities (intelligence, speed, etc.) are being tested and appraised by experimenters (Dickerson & Kemeny 2004). The TSST incorporates an element of social evaluative threat by video recording and having lab personnel or a "stress panel" administer and watch the entire task. Dickerson & Kemeny (2004) performed a meta-analysis and showed that stress tasks that were uncontrollable or incorporated social evaluative threat elicited the greatest cortisol responses. Furthermore, Dickerson & Kemeny (2004) found a synergistic effect of tasks, like the TSST, that were both uncontrollable and incorporated social evaluative threat on cortisol and ACTH responses compared to tasks with only one factor.

2.4.3.2 The effect of acute mental stress on endothelial function

The first study to examine the effect of acute mental stress on FMD was done by Ghiadoni and colleagues in 2000. In this study, 10 healthy middle-aged males performed a public speaking task. Brachial artery FMD declined significantly from a mean of 5.0 ± 2.1% pre-stress to 2.8 ± 2.3% at 30 minutes and 2.3 ± 2.4% at 90 minutes post- stress. Indeed, the decline post-mental stress lowered the RH-FMD such that it was comparable to the impaired baseline FMD of a separate group of non-insulin dependent diabetic participants. Impaired RH-FMD post-mental stress in healthy individuals has also been observed in more recent studies using a similar public speaking task (Broadley et al. 2005, Szjigyarto et al. 2013) and a rapid colour-identifying task (Spieker et al. 2002). Furthermore, these impairments have been observed immediately (Jambrik et al. 2004b, Jambrik et al. 2005) and at 10 minutes (Jambrik et al. 2004a, Jambrik et al. 2004b, Szijgyarto et al. 2013, Spieker et al. 2002), 15 minutes (Broadley et al. 2005), and between 30-90 minutes post-stress (Plotnick et al. 2015, Ghiadoni et al. 2000, Spieker et al. 2002, Table 1).

There appears to be variation in the magnitude of decline in FMD post-mental stress. Some variability was observed in Ghiadoni et al.'s (2000) study at 30 and 90 minutes post stress with some participants demonstrating no change or a modest decline in FMD, while others displayed a greater impairment. More variability, however, has been observed in studies that have examined FMD at 10 minutes post-stress. For example, Szjigyarto et al. (2013) found an overall modest decline that only became significant with the removal of an outlier. Poitras et al. (2014) found an improved post-prandial FMD measured 10 minutes following a stress task that was performed every hour for 4 hours after a meal compared to the post-prandial, no stress condition. It is possible that more time must elapse post-stress task for a consistent decline in FMD to develop. Indeed, pilot work performed in preparation for this thesis study showed a significant decline in FMD in 5 of 6 participants at both 30 (*unpublished data*) and 90 minutes (Plotnick et al. 2015) post-stress. This suggests that an overall average decline in FMD is most likely to occur at 30 and 90 minutes post-stress.

Table 1. A summary of studies that have examined FMD post-acute mental stress.

Reference	Subjects (n)	Subject age (years)	Subject gender	Type of stress intervention	FMD Time points	Pre-Stress FMD	Post-stress FMD
Broadley et al. 2005	36	40.8 ± 2.1	NS	Speech task	15 min	4.5 ± 0.7%	1.4 ± 1.1%*
Ghiadoni et al. 2000	10 (healthy)	50.4 ± 9.6	M	TSST	30, 90, 240 min	5.0 ± 2.1%	30 min; 2.8 ± 2.3%* 90 min; 2.3 ± 2.4%* 240 min; 4.1 ± 2.0%
Jambrik et al. 2004a	10 5HiHyp, 5LoHyp	27.7 ± 4.5	8 F / 2 M	Arithmetic task	8 min	LoHyp – 12.10 ± 2.59% HiHyp - ~12.5	LoHyp; 6.48 ± 1.72%* HiHyp; ~11%
Jambrik et al. 2004b	20 10HiHyp, 10LoHyp	19-35	12 F / 8M	Arithmetic task	4, 20 min	LoHyp - ~11% HiHyp – ~ 11.8%	LoHyp; ~6% at 4 min*, ~ 5% at 20 min* HiHyp; ~11.8% at 4 min, ~9% at 20 min
Jambrik et al. 2005b	17 8HiHyp, 9LoHyp	19 - 35	7 F / 10 M	Arithmetic task	Immediate, 12 min	LoHyp - ~10% HiHyp - ~13%	LoHyp; ~4.5% immediately*, ~5.5% at 12 min* HiHyp; ~9.5% immediately*, ~10.5% at 12 min*
Plotnick et al. 2015	6	22.2 ± 3.7	2F / 4M	TSST	90 min	9.3 ± 3.7 %	6.7 ± 5.5%*
Spieker et al. 2002	23	20 – 31	NS	Flashing light task	10 min	8.0 ± 1.1%	10 min; 4.1 ± 1.0%*
Szijgyarto et al. 2013	16	21 ± 2.3	M	TSST	10 min	5.2 ± 0.6%	4.1 ± 0.5%*

* indicates significantly different than respective pre-stress values. Abbreviations: F, female; FMD, flow-mediated dilation; M, male; NS, not specified; TSST, Trier Social Stress Task.

2.4.4 Reactive oxygen species: a putative mechanism linking acute mental stress and endothelial dysfunction

In healthy individuals, higher levels of perceived life stress such as caregiving for a chronically ill child are correlated with higher levels of oxidative stress (Epel et al. 2004). A small number of studies have shown that acute mental stress is associated with increases in oxidative stress in post-menopausal women and university-aged students (Sivonová et al. 2004). In addition, acute stimuli other than mental stress, such as exercise (Johnson et al. 2012), have been shown to impair brachial artery FMD in apparently healthy individuals in association with acutely elevated ROS. Together, the above evidence suggests that an increase in ROS post-acute mental stress may be responsible for the decline in FMD observed.

In concert with this notion, it is thought that cortisol may indirectly affect endothelial function through increasing ROS production (Iuchi et al. 2003, Poitras & Pyke 2013). For instance, a study done by Joergensen et al. (2011) found that in a random sample of elderly adults 65 years and older, 24-hour urinary excretion of cortisol was linked with elevated markers of DNA and RNA damage; a widely accepted biomarker of oxidative stress (Kasai et al. 1997, Valvanidis et al. 2009). It should be noted that this study did not examine other age cohorts and as such, it is unclear whether the same results would be observed in a young healthy population. Furthermore this evidence is correlational and does not provide conclusive evidence that cortisol elicits an increase in ROS. A study by Broadley et al. (2005) showed that blocking cortisol production using metyrapone resulted in no impairment in FMD post-mental stress (4.3 ± 0.9% to 5.1 ± 0.8%) compared to a significant impairment in the placebo condition (4.5 ± 0.7% to 1.4 ± 1.1%). Furthermore, administration of

antioxidant vitamin C to individuals undergoing glucocorticoid therapy has been shown to attenuate previously impaired FMD (Iuchi et al. 2003). These pieces of evidence lend support to the hypothesis that glucocorticoids such as cortisol may impair endothelial function via ROS production; a hypothesis that has yet to be investigated.

2.5 Summary and Conclusions

Laboratory induced acute mental stress is a useful tool in understanding how stress affects cardiovascular physiology and health. The TSST appears to elicit the greatest stress response and is the most commonly used laboratory stress task. Studies using the TSST and other stress tasks have shown that FMD is impaired post-acute mental stress. The mechanism(s) through which acute mental stress impairs endothelial function, however, remain incompletely understood. One putative mechanism of stress-induced endothelial dysfunction is elevated ROS which are highly reactive molecules containing oxygen. While low levels of ROS play a regulatory role in the endothelium, high levels of ROS or oxidative stress appear to be detrimental to proper endothelial function. The following chapter describes the results of a study designed to examine the mechanistic role of oxidative stress in mental-stress induced endothelial dysfunction as measured by reactive hyperemia FMD.

Chapter 3

Does administration of vitamin C attenuate an acute mental-stress induced impairment in endothelial function?

3.1 Introduction

Several authors have reported that acute bouts of mental stress transiently impair vascular endothelial function in healthy individuals (Ghiadoni et al. 2000, Spieker et al. 2002, Broadley et al. 2005). It has been hypothesized that the cumulative effect of brief, repeated, stress induced impairments in endothelial function, which would briefly attenuate the endothelium's vasoprotective function, may contribute to the connection between chronic psychosocial stress and the development and progression of cardiovascular disease (Poitras & Pyke 2013, Ghiadoni et al. 2000, Rozanski et al. 1999). The mechanism(s) by which acute mental stress negatively impacts endothelial function, however, remain incompletely understood.

One putative mechanism through which mental stress may impair endothelial function is via a stress-induced production of reactive oxygen species (ROS; Iuchi et al. 2003, Poitras and Pyke 2013). Low levels of ROS play an important role in normal cell signaling within the endothelium through regulating vascular tone and homeostasis (Chatterjee et al. 2014, Brandes et al. 2014). Elevated levels of ROS or oxidative stress, however, appear to be harmful to the vasculature. This is supported by evidence of improved endothelial function following the supplementation of vitamin C, a known antioxidant and ROS scavenger, in individuals with chronic diseases characterized by cardiovascular impairment (Ting et al. 1996, Levine et al. 1996, Gokce et al. 1999, Tousoulis et al. 1999,

Duffy and Vita 1999, Kugiyama et al. 1998). Furthermore, in vitro studies provide mechanistic evidence that oxidative stress impairs normal endothelial cell function by reducing NO bioavailability in the endothelium. Specifically, ROS have been shown to both oxidize NO to peroxynitrite (Laursen et al. 2001, Milistien and Katusic 1999) and uncouple endothelial nitric oxide (eNOS) through the oxidation of the eNOS essential cofactor tetrahydrobiopterin (BH4; Zheng et al. 2003, Wever et al. 1997, Förstermann and Münzel 2006, Förstermann 2006, Kinoshita et al. 1997).

Although a role for elevated oxidative stress in acute mental stress induced endothelial dysfunction has yet to be examined, there is evidence to support the involvement of ROS. A small number of studies have investigated and observed elevated oxidative stress in association with acute mental stress (Morimoto et al. 2008, Sivonová et al. 2004). Furthermore, cortisol, a key stress hormone, has been shown to induce the production of ROS (Iuchi et al. 2003). Finally, endothelial dysfunction caused by acute stimuli such as a bout of oscillatory shear stress (Johnson et al. 2013) can be attenuated by vitamin C supplementation, indicating that temporary shifts in homeostasis can elicit physiologically relevant increases in oxidative stress. Together, this evidence suggests that acute mental stress induced endothelial dysfunction may be mediated by an elevation in ROS. In light of this background, the purpose of this study was to determine whether vitamin C supplementation can attenuate a decline in endothelial function as measured by flow-mediated dilation (FMD) following mental stress in young healthy men. An attenuation of post-stress endothelial dysfunction with vitamin C supplementation would support a mechanistic role for ROS in the deleterious impact of acute mental stress.

3.2 Methods

3.2.1 Participants

The study protocol was approved by the Health Science Research Ethics Board (Appendix A) at Queen's University and all participants provided written consent to participate in this study (Appendix B). Fifteen healthy, male volunteers recruited from Queen's University and the community (Kingston, Ontario) participated in the study.

3.2.2 Screening Visit

All interested individuals initially came in to the laboratory for a screening visit in which their eligibility for the study was assessed via a medical screening questionnaire (Appendix C) and assessment of blood pressure (BpTRU BPM-100, BpTRU Medical Devices, Coquitlam, BC). Individuals who reported cardiovascular or metabolic disease, taking medications to treat cardiovascular risk factors, who were hypo or hypertensive, or who self-reported participation in greater than two structured sessions of physical activity each week were excluded. Participants were also introduced to the study protocol and screened to ensure a clear image of the artery and strong blood velocity signal could be obtained.

3.2.3 Experimental Design

Participants came into the lab for two experimental sessions after fasting (12 hours) and abstaining from exercise, caffeine, or alcohol consumption 24 hours prior. The entire experimental visit was completed between 1130 – 1700 hours in order to control for the circadian variation of cortisol (Weitzman et al. 1971). The experiment was done using a within-subjects design such that each participant underwent the same protocol on each experimental day, however, the condition (vitamin C or placebo) was double blinded and

both the condition and version of the stress task (see below) were counterbalanced. The experimental visits were separated by at least 48 hours.

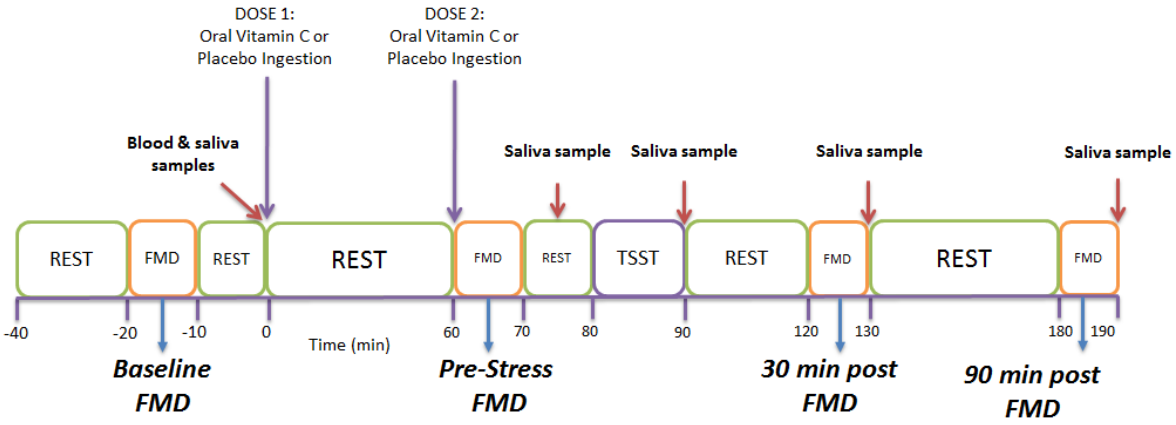

Figure 3. Protocol timeline for each experimental visit. Brachial artery flow-mediated dilation (FMD) was assessed at four time points: baseline, after vitamin C consumption and prior to the stress task (referred to as Pre-Stress FMD), 30 minutes and 90 minutes after the Trier Social Stress Task (TSST). Saliva samples were collected at each of the FMD time points as well as immediately post-TSST.

3.2.4 Experimental Procedures

3.2.4.1 Continuous Monitoring

Heart rate (HR) was measured continuously using three ECG electrodes on the chest and abdomen. Mean arterial pressure (MAP) was monitored using an automated blood pressure device (Finometer PRO, Finapres Medical Systems, Amsterdam, The Netherlands) and recorded in LabChart (AD Instruments, Colorado Springs, CO) for future analysis.

3.2.4.2 Brachial Artery Diameter Measurements

Brachial artery diameter was measured using echo ultrasound functioning at 12 MHz in B-mode (Vivid i2 GE Medical Systems). Parameters on the ultrasound were set to optimize the longitudinal images of the lumen-arterial wall interface. Data was collected at an insonation angle of 68° for reasons previously described (Pyke and Tschakovsky 2007) and remained constant between all trials. Ultrasound images were acquired from the ultrasound machine using VGA to USB frame grabber (Epiphan Systems Inc.) and recorded as avi files on an independent computer using commercially available software (Camtasia Studio 7, TechSmith Corporation, Okemos, MI).

3.2.4.3 Brachial Artery Velocity Measurements

Brachial blood velocity was measured using Doppler ultrasound operating at 4MHz (Vivid i2 GE Medical Systems). The Doppler shift frequency spectrum was analyzed using a Multigon 500P TCD spectral analyzer (Multigon Industries) from which the mean blood velocity was determined as a weighted mean of the spectrum of Doppler shift frequencies. Continuous sampling and recording of the resultant output from the Multigon was performed for further analysis.

3.2.4.4 Vitamin C and Placebo Supplements

Vitamin C, a potent antioxidant, was chosen to attenuate the possible oxidative response to mental stress. Participants were randomized to either ingest oral vitamin C or placebo depending on the visit. Vitamin C tablets (Jamieson 500 mg capsules, Toronto, ON) and placebos (Kingston General Hospital pharmacy 500 mg of lactose powder) were taken with water. Participants were given two 500mg doses separated by one hour as described in Figure 3. This total dose of 1000 mg of vitamin C was administered to saturate human plasma, neutrophils, monocytes, and lymphocytes (Levine et al. 1996). Furthermore, oral supplementary vitamin C increases plasma vitamin C levels, which peak between 90-120 minutes after ingestion (Levine et al. 1996, Padayatty et al. 2004). Two doses were given in order to keep plasma vitamin C levels high during the entire duration of the experiment after baseline measures.

3.2.4.5 Trier Social Stress Test (TSST)

Two variations of the TSST were used to prevent habituation over the two visits. An unfamiliar panel of two laboratory personnel read instructions to the participants. Participants were told they had 10 minutes to prepare a 5-minute speech. In one speech task, participants had to defend themselves against a false accusation of shoplifting (Kirschbaum et al. 1993). In the other speech task, participants had to explain why they deserved a particular job opportunity after being recently fired. The participant was told to continue talking, maintain eye contact, and stay still for the entire 5 minutes by way of standard prompts. Immediately following the speech task, the participants underwent a mental arithmetic task in which they were asked to serially subtract either 13 or 17 from a 4-digit prime number. Periodically, the participants were told their answer was incorrect (even if correct) to increase the uncontrollability of the task (Kirschbaum et al. 1993). Immediately following the TSST, the

participant was asked to rate their stress on a subjective scale of 0-10 (10 being the maximum stress).

3.2.4.6 Brachial Artery Reactive Hyperemia FMD (RH-FMD)

RH-FMD tests were performed at four time points (Fig. 3) on the left arm. The occlusion cuff was placed on the participant's left forearm at the antecubital fossa, which is located distally to the brachial artery ultrasound measurement. Brachial artery velocity and diameter were recorded as follows: (1) one minute of baseline prior to cuff occlusion, (2) the last minute of the 5-minute forearm cuff occlusion (cuff inflation to 250 mmHg), and (3) for three minutes post-release of cuff occlusion.

3.2.4.7 Saliva Sampling

Saliva samples were taken throughout the study (Fig. 3). Participants were asked to suck on a synthetic swab (Sarstdedt Salivette; Ghiadoni et al. 2000, Poll et al. 2007) for approximately 2 minutes or until saturation. The saliva samples were then stored in the fridge until the data collection session was completed. At the end of each data collection session, samples were centrifuged at 4°C for 2.5 minutes at 2500 rpm and 1070 relative centrifugal force (IEC-Centra MP4R; International Equipment Company, Mass, USA). Once the samples were spun, the swabs were removed and samples were stored in a -80°C freezer for future analysis (Garde and Hansen 2005).

3.2.4.8 Blood Sampling

Venous blood samples were taken via standard venous catheterization before the baseline FMD test (Fig. 3) for analysis of total cholesterol and glucose. Samples were collected in a heparin tube and analyzed in-house during the time course of the experiment using a Cholestech LDX system (Cholestech) from whole blood. Due to difficulties in obtaining

blood or other technical issues, there were incomplete measurements for total cholesterol (missing values from 4 participants) and glucose (missing values from 5 participants).

3.2.4.9 7-Day Physical Activity Recall (PAR)

During the second rest period (between 30 and 90 minutes post-stress FMD tests, Fig. 3), a 7-day physical activity recall (PAR) was obtained to quantify physical activity levels (Appendix D). Energy expenditure was determined using self-reported physical activity and sleep. Two participants had incomplete PAR information and as such were excluded from analysis.

3.2.5 Data Analysis

3.2.5.1 Stress Reactivity

HR, MAP, salivary cortisol, and subjective stress ratings were analyzed to characterize stress reactivity. MAP and HR were analyzed offline in Lab Chart (AD Instruments, Colorado Springs, CO) in 3-s average time bins. The HR data for all participants was analyzed (n = 15), however, due to equipment malfunction, we were unable to obtain MAP data for one participant (n = 14). Responses to the stress task were quantified as the peak 1-minute average of MAP and HR during the task. Salivary cortisol responses were determined as the peak post-stress cortisol time point (which may have been obtained immediately, 30, or 90 minutes post-stress task). A sample immediately post-stress was missing for one participant, and as such peak cortisol was determined based on the highest cortisol value obtained at 30 or 90 minutes post-stress. Saliva samples stored at -80°C were thawed to room temperature, vortexed, centrifuged at 1500g for 15 minutes, and assayed in duplicate using enzyme-linked immunoassay (ELISA) according to the kit instructions (No. 1-3002;

Salimetrics, State College, PA, USA). Subjective stress ratings were obtained for eleven participants; four participants were missing baseline ratings and were excluded from analysis (n = 11). Change scores for HR, MAP and cortisol (peak response to the stress task – baseline) were used to express stress reactivity for correlation analysis.

3.2.5.2 Brachial Artery Velocity and Diameter

Blood velocity was analyzed offline in 3-second average time bins using acquisition software (LabChart, AD Instruments, Colorado Springs, CO), as previously described by Pyke et al. (2008). Vessel diameter was measured offline using automated edge detection and wall tracking software (Woodman et al. 2001; Encoder Analysis FMD, Reed Electronics) as previously described (Pyke and Jazuli 2011). The investigator was blinded to the trial (baseline, pre-stress, 30 min post, 90 min post) and condition (vitamin C vs. placebo) during FMD analysis. Diameter data were compiled into 3-s time bins and aligned with the 3-s average velocity data for the calculation of shear rate.

3.2.5.3 Shear rate

The shear rate was determined using the following equation: shear rate = blood velocity / brachial artery diameter. The shear rate stimulus was quantified as the shear rate area-under-the-curve (SR-AUC) until the time of peak diameter measurement. Shear rates for all trials were collected in 9 participants. In 6 participants, velocity signals were lost in one or more trials and as such, an individual average shear rate was determined from the available baseline, 30 and 90 minute trials and substituted for the missing trials. Since the pre-stress trial was significantly different from baseline in the 9 participants with complete data sets, it was not included in the average. Statistical outcomes were the same when run with only the 9

complete data sets compared to when run with the additional 6 data sets filled in with averages for missing trials.

3.2.5.4 Flow-mediated Dilation

The same investigator carried out all RH-FMD analyses. RH-FMD is reported as both absolute change (absFMD) and peak change (%FMD). %FMD was calculated as the percent change in arterial diameter from baseline prior to cuff occlusion to the peak 3-s average diameter time bin following release of cuff occlusion (Equation 1). absFMD was calculated as the peak 3-s average diameter minus the baseline diameter (mm). The diameter in the last minute of occlusion was used in place of baseline diameter in 14 of the 120 trials due to superior wall tracking. Δ%FMD was calculated as the difference in FMD from the pooled baseline (the average of the two pre-TSST time points: baseline and pre-stress (these were not significantly different) and either 30 or 90 minutes post-stress. One 30-minute FMD trial was not useable due to poor image quality and the average of the other trials for this participant within the condition was used as the 30-minute %FMD value. No differences in statistical outcomes were observed with the inclusion and removal of this participant.

$$\%FMD = \left(\frac{Diameter_{Peak} - Diameter_{Baseline}}{Diameter_{Baseline}} \right) \times 100\% \quad \text{(Equation1)}$$

3.2.5.5 Statistical Analysis

All statistical analyses were performed using SigmaPlot 11 software. All values are expressed as the mean ± SD. Statistical significance was set at $p < 0.05$. A paired t-test was used to compare participant characteristics (energy expenditure, glucose, and total

cholesterol). The variables %FMD, absFMD, baseline diameter, SR-AUC, baseline SR, were compared using a two-way, repeated measures ANOVA. The factors were condition (vitamin C vs. placebo) and time (FMD variables: baseline, pre-stress, and 30 and 90 minutes post-stress). The stress reactivity variables HR, MAP, salivary cortisol, and stress ratings were compared using a two-way, repeated measures ANOVA using factors condition (vitamin C v. placebo) and time (prior to the stress task vs. peak). Linear regression analysis was used to assess associations between Δ%FMD at 30 and 90 minutes with ΔMAP, ΔHR, and Δsalivary cortisol. Regression analysis was only performed in participants who had complete data sets in both the vitamin C and placebo conditions.

3.3 Results

3.3.1 Subject characteristics

Fifteen participants, age 21 ± 2, BMI of 22.1 ± 2.0 kg m^{-2} took part in this study. There were no significant differences in energy expenditure, fasting glucose, or total cholesterol across condition (Table 2).

Table 2. Participant characteristics

	Placebo	Vitamin C	P value
Energy expenditure (kcal/kg/week)	234 ± 9	233 ± 11	0.200
Total Cholesterol (mg/dl)	141 ± 29	139 ± 26	0.754
Glucose (mg/dl)	80 ± 5	74 ± 7	0.070

All data are expressed as mean ± SD. Energy expenditure, n = 13; total cholesterol, n = 11; glucose, n = 10.

3.3.2 Stress Reactivity

For all stress reactivity variables (HR, MAP, salivary cortisol, and stress ratings) there was a main effect of time ($p < 0.001$) and no effect of condition or time by condition interaction (Fig. 5 A-D).

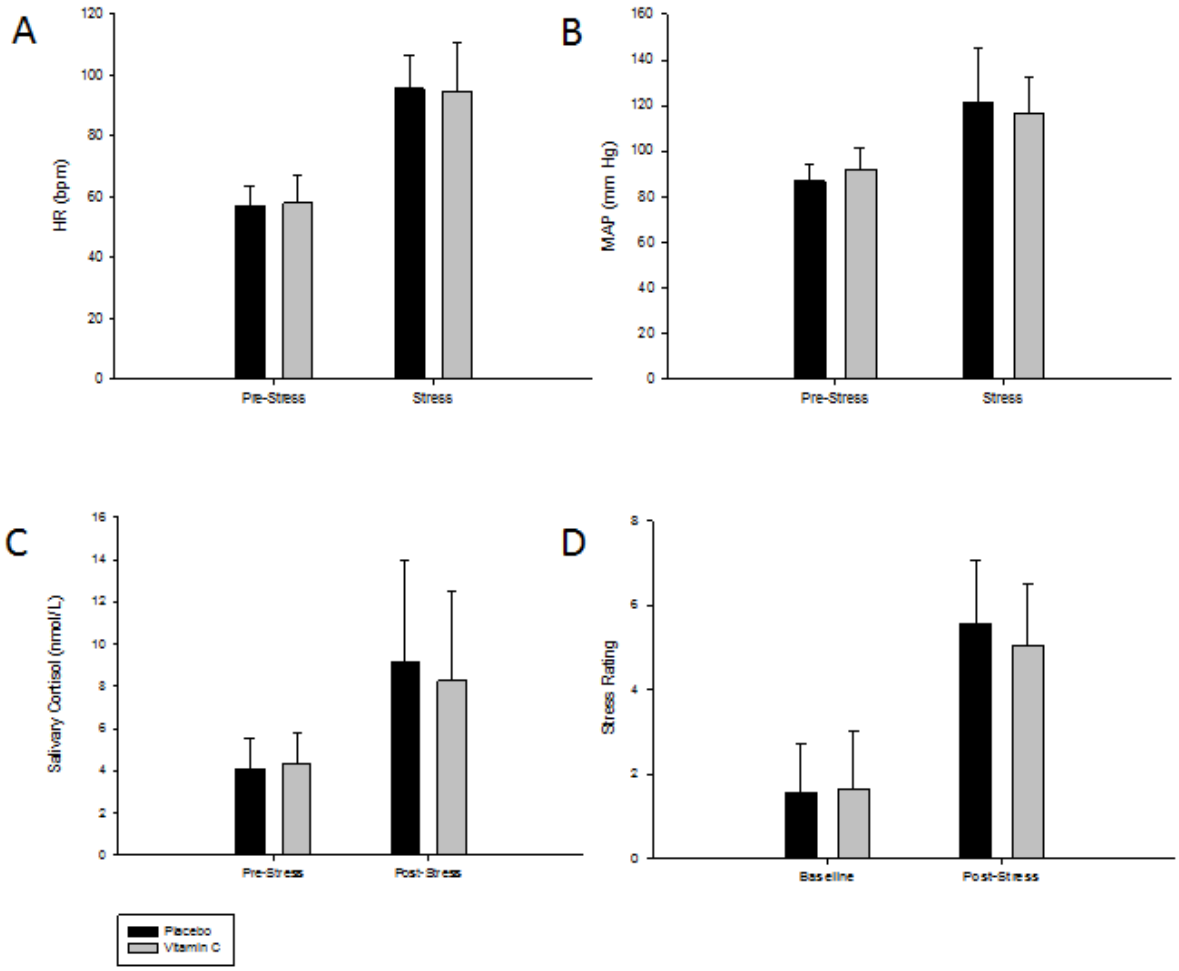

Figure 4. Stress Responses

Stress responses in placebo and vitamin C conditions as measured by (A) heart rate (HR) T, $p < 0.001$; C, $p = 0.858$; TxC, $p = 0.818$, (B) mean arterial pressure (MAP) T, $p < 0.001$; C, $p = 0.963$; TxC, $p = 0.105$, (C) salivary cortisol T, $p < 0.001$; C, $p = 0.237$; TxC, $p = 0.608$, and (D) subjective stress rating on a scale of 0-10 T, $p < 0.001$; C, $p = 0.543$; TxC, $p = 0.268$. HR and MAP are compared from pre-stress to peak values during stress. Salivary cortisol is compared from pre-stress to the peak value measured post-stress. Abbreviations: T, time; C, condition; TxC, time by condition interaction. All data are mean ± SD. HR, n = 15; MAP, n = 14; salivary cortisol, n = 15; stress ratings, n= 11.

3.3.3 Shear rate (SR)

No main effect of, time ($p = 0.052$), condition ($p = 0.330$), or time by condition interaction ($p = 0.289$) was found for baseline SR (Table 3). For the shear rate stimulus (AUC to time of peak diameter) there was a main effect of time ($p = 0.007$) which post-hoc analysis showed was due to a higher value of SR-AUC at baseline (6128 ± 2974 a.u.) compared to pre-stress (4707 ± 2413 a.u., $p = 0.003$; Fig. 5A). No main effect of condition ($p = 0.792$) or time by condition interaction ($p = 0.314$) was observed.

3.3.4 Brachial artery flow-mediated dilation (FMD) and brachial artery diameter

A significant effect of time on baseline diameter was observed ($p = 0.007$; Table 3). Post hoc analyses revealed that baseline diameter was significantly higher at the baseline FMD trial (0.342 ± 0.043 cm) compared to both pre-stress FMD (0.333 ± 0.043 cm; $p = 0.011$) and 30 minutes post-stress (0.336 ± 0.044; $p = 0.047$) trials. No main effect of condition ($p = 0.310$) or time by condition interaction ($p = 0.527$) was found for baseline diameter. For %FMD, no significant effect of condition ($p = 0.792$), time ($p = 0.631$) or time by condition ($p = 0.573$) was observed (Fig. 5B; Figure 6 shows individual %FMD responses at baseline vs. 30 minutes (panels A & C) and 90 minutes (panels B & D) post-stress). Similar to %FMD, analysis of absFMD revealed no significant effect of condition ($p = 0.994$), time ($p = 0.651$), or time by condition interaction ($p = 0.648$; Table 3). No main effect of time ($p = 0.226$), condition ($p = 0.454$), or time by condition interaction ($p = 0.917$) was observed for time to peak diameter (Table 3).

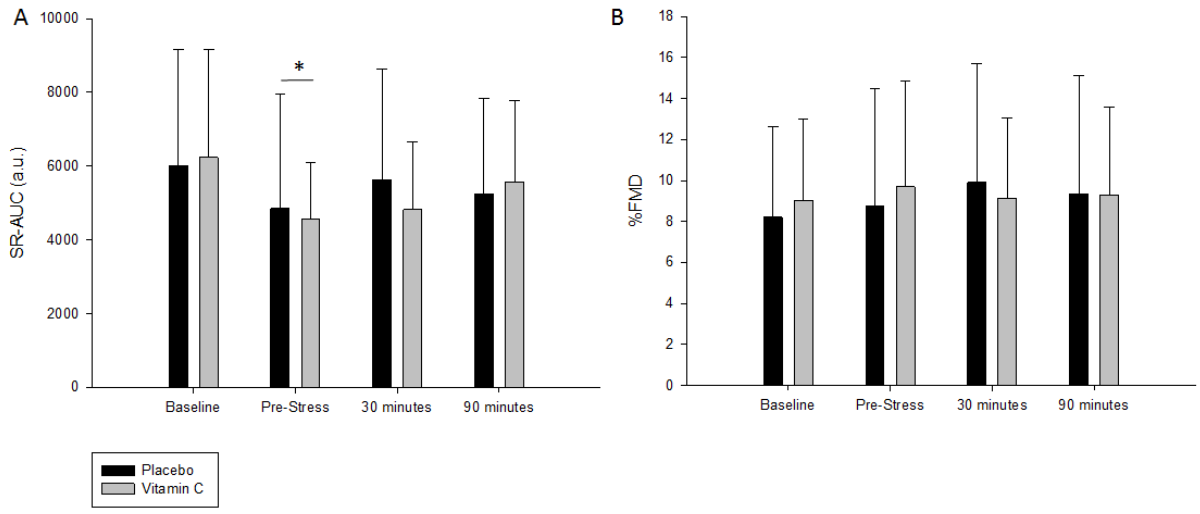

Figure 5. Reactive hyperemia flow-mediated dilation and shear rate stimulus

(A) The shear rate stimulus elicited by reactive hyperemia obtained as shear rate area under the curve (SR-AUC) until peak diameter. (B) Flow-mediated dilation quantified as a percent change from baseline diameter (%FMD). * Indicates significant difference compared to baseline. All data are mean ± SD.

Table 3. FMD Characteristics

	Baseline	Pre-Stress	30 minutes	90 minutes	P Value
absFMD (mm)					Time: p = 0.994
Placebo	0.30 ± 0.16	0.29 ± 0.18	0.33 ± 0.18	0.32 ± 0.19	Condition: p = 0.651
Vitamin C	0.30 ± 0.13	0.31 ± 0.15	0.31 ± 0.12	0.30 ± 0.12	Time x Condition: p = 0.648
Baseline Diameter (cm)					Time: p = 0.007
Placebo	0.344± 0.042	0.339 ± 0.042	0.338 ± 0.046	0.343 ± 0.045	Condition: p = 0.310
Vitamin C	0.339 ± 0.045	0.329 ± 0.046	0.333 ± 0.043	0.335 ± 0.045	Time x Condition: p = 0.527
				Post hoc (Time)	*Baseline v. Pre-Stress:* p = 0.008* *Baseline v. 30 minutes:* p = 0.046*
Baseline shear rate (1 s^{-1})					Time: p = 0.052
Placebo	20.1 ± 12.0	14.4 ± 7.4	17.7 ± 9.8	16.2 ± 9.3	Condition: p = 0.330
Vitamin C	20.7 ± 16.1	13.9 ± 4.7	13.1 ± 4.5	14.2 ± 4.0	Time x Condition: p = 0.289
Time to Peak Dilation (s)					Time: p = 0.226
Placebo	58 ± 32	48 ± 20	58 ± 28	53 ± 26	Condition: p = 0.454
Vitamin C	57 ± 28	46 ± 12	52 ± 29	53 ± 24	Time x Condition: p = 0.917

All data are mean ± SD. * Indicates significance compared to baseline, $p < 0.05$

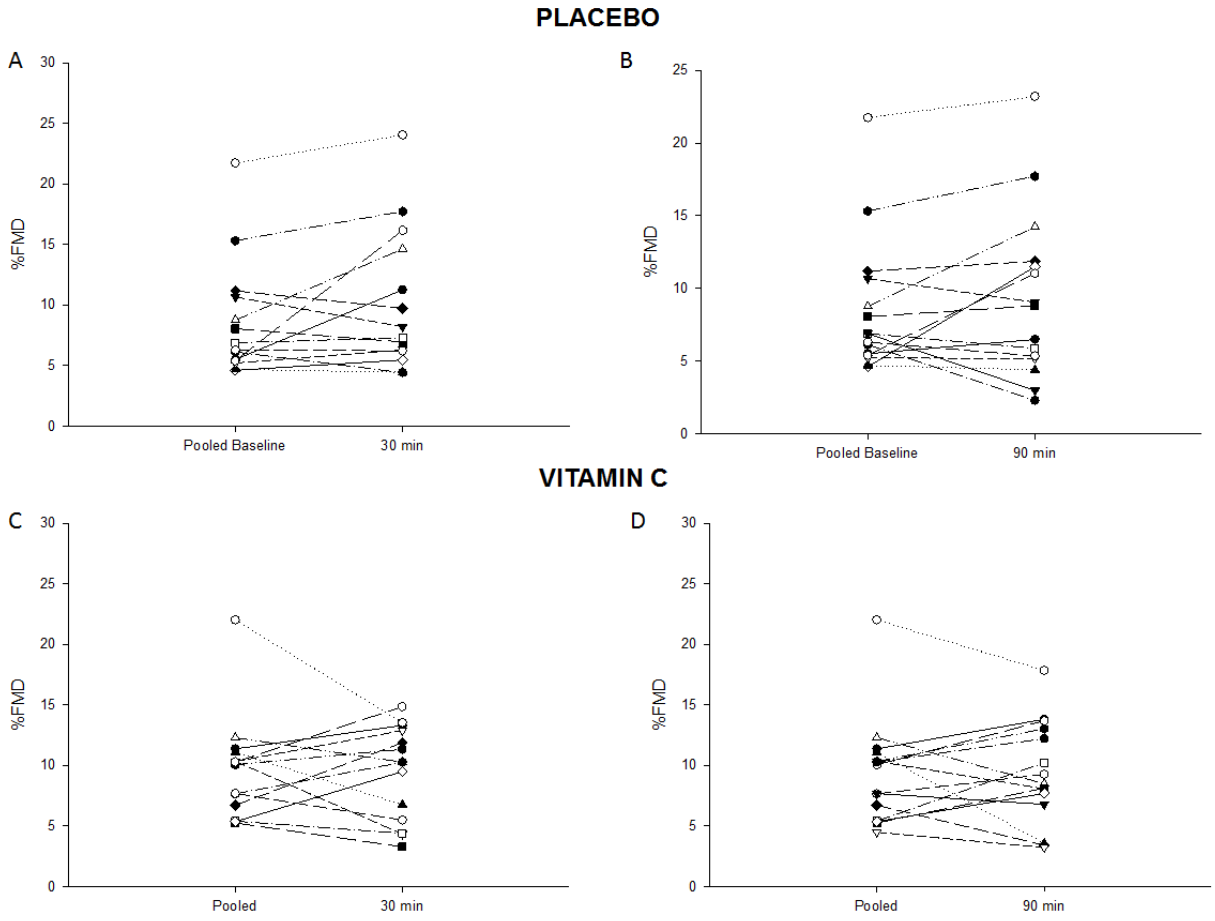

Figure 6. Individual %FMD changes from pooled baseline

Individual changes in percent change in flow-mediated dilation (%FMD) responses over time for all participants from pooled baseline (the average %FMD of baseline and pre-stress) to 30 minutes (panel A and C) and 90 minutes (panel C and D) post-stress. Panels A and B, placebo condition; panels C and D, vitamin C condition. Each symbol type represents the same participant in all four panels.

3.3.5 Association between Δ%FMD and MAP and salivary cortisol reactivity

A linear regression was performed to explore the potential relationship between Δ%FMD and changes in HR, MAP, and salivary cortisol. In the placebo condition a significant correlation was found between Δ%FMD at 30 minutes and both MAP reactivity (Fig. 7A, solid circles) and salivary cortisol reactivity (Fig. 7C, solid circles). No significant association was observed between the Δ%FMD at 90 minutes and MAP reactivity (r^2 = 0.151, p = 0.189), however there remained a significant association between Δ%FMD at 90 minutes and salivary cortisol reactivity (r^2 = 0.375, p = 0.020). In contrast, in the vitamin C condition no associations between the Δ%FMD and reactivity were found (30 min post: Fig 7B and D, open circles; 90 min post: Δ%FMD and MAP- r^2 = 0.0292, p = 0.577; Δ%FMD and salivary cortisol- r^2 = 0.0461, p = 0.461). No associations between Δ%FMD and HR were found for either condition at either time point (data not shown). Cortisol reactivity was significantly correlated between conditions (r^2 = 0.421, p = 0.012), however, Δ%FMD at 30 minutes was not (r^2 = 0.0654, p = 0.377) nor was MAP reactivity (r^2 = 0.211, p = 0.114).

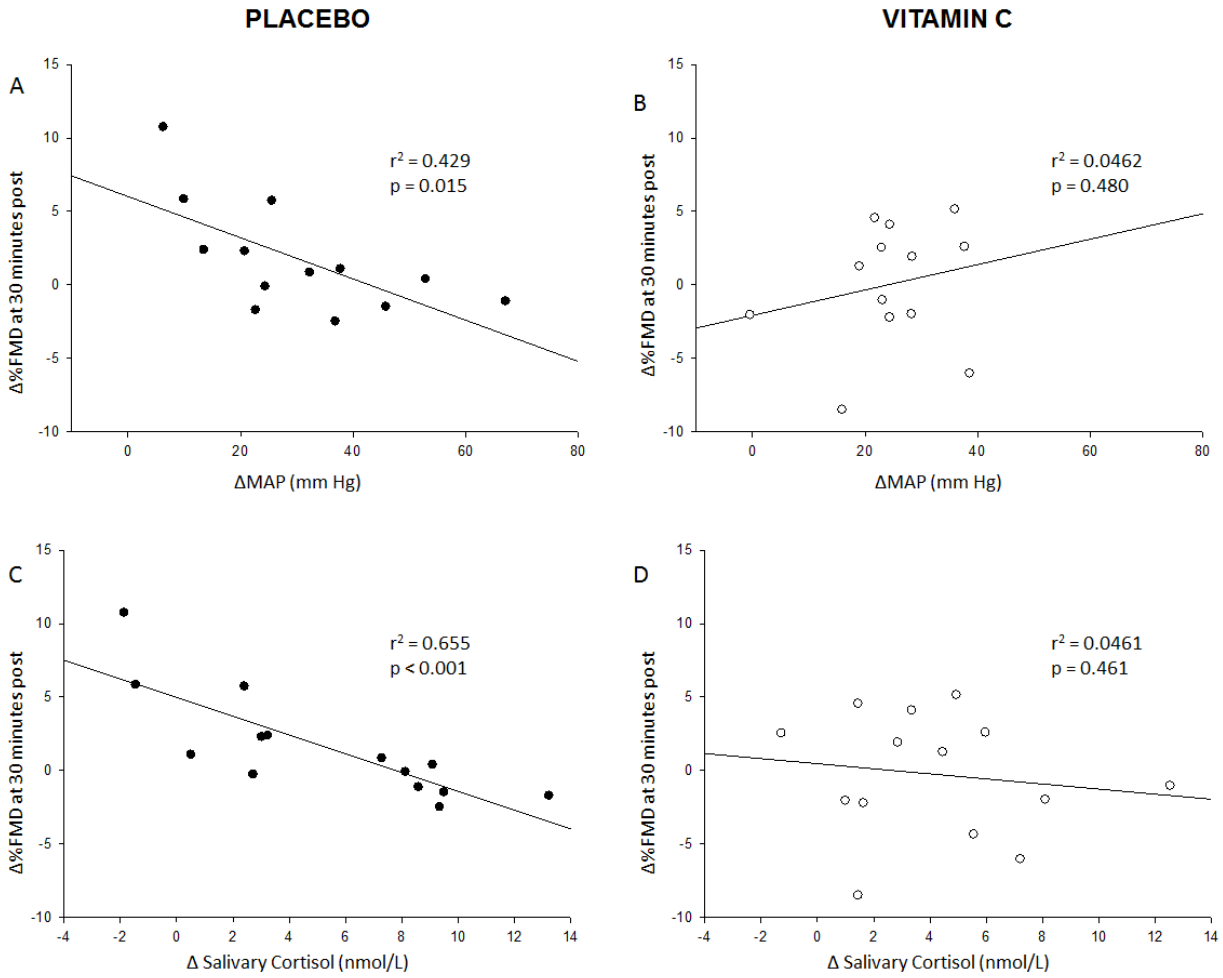

Figure 7. Relationship between Δ%FMD and stress reactivity

Change in MAP from pre-stress to peak during the stress task in the (A) placebo condition (n = 13) and (B) vitamin C condition (n = 13); change in salivary cortisol from pre-stress to peak cortisol obtained post-stress in the (C) placebo condition (n = 14) and (D) vitamin C condition (n = 14). Solid circles indicate placebo condition and open circles indicate vitamin C condition. Of the 15 participants, 14 exhibited peak cortisol immediately or at 30 minutes post-stress. Removal of the one individual that had a peak cortisol at 90 min from the analysis did not change the significance of the correlation (data not shown).

3.1 Discussion

The purpose of this study was to investigate whether acute mental-stress induced impairments in brachial artery FMD could be attenuated by administration of the antioxidant vitamin C. The primary finding of this study, however, is that there was no impairment of FMD following acute mental stress in either the placebo or vitamin C conditions, despite robust stress reactivity measured by salivary cortisol, HR and MAP. These findings indicate that, in contrast to several previous studies; acute mental stress may not impair endothelial function in a population of young, healthy males. As the mean FMD response was not reduced following the mental-stress intervention, this study was unable to address the role of ROS in stress-related impairments in endothelial function as intended. Secondary exploratory analysis revealed a significant correlation between Δ%FMD and cortisol as well as MAP reactivity in the placebo condition but not in the vitamin C condition. This finding suggests a potential role of ROS in modulating the impact of acute stress on endothelial function.

3.1.1 Impact of mental stress on reactive hyperemia flow-mediated dilation

Many studies have reported that reactive hyperemia FMD is compromised between 10 and 90 min following a bout of acute mental stress in healthy individuals (Broadley et al. 2005, Ghiadoni et al. 2000, Spieker et al. 2002, Szijgyarto et al. 2013, Plotnick et al. 2015). Despite comparable stress response magnitude to other studies (~Δ4.5 nmol/L in salivary cortisol, ~Δ35 bpm in HR, and ~Δ30 mm Hg in MAP; Ghiadoni et al. 2000, Broadley et al. 2005, Spieker et al. 2002) a post-stress decline in FMD was not observed. However, in agreement with the current findings, previous reports have identified some variability in the

impact of acute mental stress on FMD (Szijgyarto et al. 2013, Poitras et al. 2014). For example, although Szijgyarto et al. (2013) found a modest mean decline in reactive hyperemia FMD post-stress, some participants experienced an increase, others a decrease, and some had virtually no change in FMD. Furthermore, Poitras et al. (2014) found that following either a low or high fat meal, the group average FMD did not decrease but actually increased relative to a post-prandial, stress-free condition. In contrast to the present study, the investigations by Szijgyarto et al. (2013) and Poitras et al. (2014) assessed FMD at 10 minutes post-stress. Since the previous work that examined FMD at 30 and 90 minutes post-stress showed less variability in FMD impairments (Ghiadoni et al. 2000, Plotnick et al. 2015), it was thought that the impact of stress on endothelial function may take longer than 10 minutes post-stress to develop consistently across participants. However, the findings of the current study suggest that variability in FMD post-stress exists for an even greater period post-stress. These conflicting findings suggest that the relationship between FMD and acute mental stress may be quite complex and dependent on individual physiology.

There are many factors that may influence the FMD response to stress in any given individual. Characteristics such as hypnotizability (Jambrik et al. 2004a, Jambrik et al. 2004b, Jambrik et al. 2005b) and hostility (Gottdiener et al. 2003) have been shown to mediate the FMD response to mental stress. Specifically, those who exhibit low hypnotizability (Jambrik et al. 2004a, Jambrik et al. 2004b, Jambrik et al. 2005b) and high hostility (Gottdiener et al. 2003) are more susceptible to FMD declines post-stress. It is possible that because screening for susceptibility to stress induced impairments in FMD was

not performed in this study, a wide range of characteristics created a wide range of responses to stress contributing to a failure to observe a mean decline in FMD. Age may also play a role in determining susceptibility to declines in FMD due to mental stress (Kudielka et al. 2004a, Kudielka et al. 2004b, Kudielka et al. 1999); however in middle aged participants, Ghiadoni et al. (2000) and Broadley et al. (2005) found similar stress reactivity and impairments in FMD as others who have examined acute mental stress induced declines in FMD in a their twenties (Spieker et al. 2002, Szijgyarto et al. 2013, Plotnick et al. 2015).

3.1.2 **Associations between Δ%FMD and stress reactivity**

An interesting finding from this study was that there was an association between Δ%FMD and stress reactivity parameters in the placebo condition but not in the vitamin C condition. This correlation existed across a wide range of Δ%FMD responses in the placebo condition such that those who had the lowest reactivity to the stress task exhibited an enhanced %FMD post-stress task and those who exhibited the greatest reactivity to the stress task exhibited declines in %FMD. This is the first time that an effect of vitamin C on a stress reactivity- endothelial function association has been observed; previous studies that have examined correlations between stress reactivity variables and FMD post-stress have found mixed results. Ghiadoni et al (2000) found a significant correlation between reductions in %FMD post-mental stress and increases in HR but not MAP or salivary cortisol; and Spieker et al. (2002) found no correlation between the change in MAP or HR and %FMD in the radial artery. The present finding suggests that there is a component(s) of the normal stress

response that may either enhance or impair endothelial function and that this component is disturbed by the presence or effects of vitamin C.

Vitamin C could interfere or interact with factors elicited in the stress response either through its function as an antioxidant, or through other direct functions. As some ROS molecules have been shown to be beneficial to endothelial function (Wolin and Burke 1987, Burke and Wolin 1987, Drummond et al. 2000, Thomas et al. 2002, Capettini et al. 2008, Bretón-Romero et al. 2012, Bretón-Romero and Lamas 2013, Ray et al. 2011) and others have been shown to be detrimental (Wever et al. 1997, Förstermann and Münzel 2006, Kinoshita et al. 1997, Zheng et al. 2003), it is possible that the type and amount of ROS generated could influence the effect of mental stress on %FMD. Stress reactivity may reflect ROS levels, for example, cortisol production has been shown to be associated with ROS production (Iuchi et al. 2003, Joergensen et al. 2011). As such, a small amount of ROS production (elicited by a low stress response) may enhance endothelial function (i.e., an increase in %FMD) and a large ROS response to mental stress (elicited with large stress response) may be detrimental to endothelial function (i.e., a decline in %FMD). If vitamin C, in its antioxidant capacity, interfered with ROS, this may explain the disrupted Δ%FMD - reactivity relationship. However, there remained substantial variability in Δ%FMD post-stress in the vitamin C condition. Therefore, a putative attenuation of a ROS influence on other stress response parameters (inflammation cytokines, endothelin-1) may have had a dominant influence on FMD (Steptoe et al. 2001, Steptoe et al. 2002, Steptoe et al. 2007, Edwards et al. 2006, von Kanel et al. 2006, Brydon et al. 2005, Veldhuijzen van Zanten et al.

2005, Spieker et al. 2002). Further studies examining the relationship between vitamin C, stress, and FMD are required to gain more insight into this preliminary observation.

3.1.3 Variation in baseline diameter and the shear stress stimulus

In this study, baseline diameter was significantly higher at the baseline FMD test compared to both pre-stress and 30 minutes post-stress. With a smaller baseline diameter, a smaller absolute change in diameter could yield the same %FMD potentially masking evidence of a post-stress impairment. However, absFMD did not differ over time suggesting that this is unlikely. Pyke and Jazuli (2011) performed four closely spaced FMD trials and found a similar decline in baseline diameter from the first trial. The decline in baseline diameter may be explained by a declining baseline shear stress (just shy of reaching significance in the current study) brought about by continued forearm resistance vessel constriction with continued supine rest after the 30 min pre-assessment rest period.

SR-AUC to peak diameter was significantly higher at baseline compared to pre-stress, with no impact of vitamin C. Again, Pyke & Jazuli (2011) showed that SR-AUC to peak dilation was largest in the first of four trials with no inter-trial intervention, which suggests time spent resting in the laboratory accounts for this finding. Neither the pre-stress SR-AUC nor FMD differed from the post-stress values. Moreover, the statistical outcomes of the regression analysis yielded the same pattern of results when Δ%FMD was calculated from the pre-stress value alone rather than the pooled baseline (average of the first two trials). Thus, the higher stimulus in the first trial does not influence any conclusions regarding the impact of stress or vitamin C in the current study. In the future, a longer rest period would be

beneficial to ensure baseline diameter and forearm resistance vessel dilation are stable at resting levels prior to the application of an intervention.

3.1.4 Limitations

Some measurements were not available for the entire subject pool due to technical difficulties. However, statistical outcomes with and without strategies to replace missing data yielded similar results and therefore this was unlikely to have impacted our conclusions. The subject pool was homogenous (young, healthy males) and therefore the results of this study cannot be generalized to females and other populations. In this study, FMD was only measured at 30 and 90 minutes post-acute mental stress; a methodology that was influenced by previous findings of quite consistently impaired endothelial function at these time points (Ghiadoni et al 2000, Plotnick et al. 2015). However, other studies have found declines in FMD in young healthy males as early as 10 minutes following stress (Spieker et al. 2002, Szijgyarto et al. 2013) thus it is possible that an overall impairment in FMD was present but missed due to the study timeline. An initial hypothesis for this study was that an elevation in cortisol might be involved in an increase in ROS leading to post-stress impairment of FMD; however, participants were not screened based on cortisol responses to the stress task. It is possible that including individuals with a wide range of cortisol responses in this study limited our ability to detect a mean decline in FMD compared to a design in which only the highest cortisol responders were included. Additionally, since peak cortisol occurs at different time points in different individuals, more frequent post-stress salivary cortisol samples may have more accurately quantified peak cortisol, potentially altering the

relationship between reactivity and FMD changes. Finally, although a measure of oxidative stress to confirm an increase in ROS post TSST was attempted, it was difficult to interpret due to high assay variability (data not shown). As such, this study was not able to establish whether oxidative stress increased systemically with mental stress.

3.1.5 Summary and Conclusions

In contrast to several other studies, no overall decline in FMD measured 30 and 90 minutes post-acute mental stress was observed. This suggests that acute mental stress does not result in a universal prolonged impairment in endothelial function in healthy, young males. This finding is discrepant to other studies the literature and a necessary future direction in the field of mental stress and endothelial function is to better understand what factors cause the endothelium to be vulnerable to the impact of acute mental stress. A secondary, exploratory analysis found correlations between Δ%FMD and MAP as well as salivary cortisol reactivity in the placebo condition only. These preliminary findings suggest that the presence of vitamin C may interact with factors of the stress response and modulate their effect on endothelial function.

Chapter 4

General Discussion

One in 5 people in Canada suffer from mental health issues (Smetanin et al. 2011). Given that stress is considered an independent risk factor for CVD (Iso et al. 2002, Yusuf et al. 2004, Richards et al. 2012) and chronic exposure to negative life events such as unemployment or caregiver burden predict the development of psychological disorders, physical illness, and life expectancy (Cohen et al. 2007), it is important to better understand the physiological mechanisms linking psychosocial stress and CVD. Furthermore, identifying the mechanisms of stress-induced endothelial dysfunction may be helpful in assessing the efficacy of treatments to combat the negative effects of mental stress. The study presented in Chapter 3 aimed to gain more insight into the mechanisms of stress-induced endothelial dysfunction.

4.1 Strengths and weaknesses of the thesis study

The strengths of the study described in Chapter 3 include its double-blinded, randomized and placebo-controlled design. The use of salivary cortisol as opposed to serum cortisol to quantify stress reactivity is also a strength of this study as it is the preferable method of assessing cortisol responses during and post-stress (Godzansky et al. 2005). This is because salivary cortisol measures free cortisol, which is cortisol that is not bound to cortisol-binding globulin or other proteins and is thus biologically active. Furthermore, since other studies that have examined acute mental stress and FMD also used salivary cortisol to

measure stress reactivity (Ghiadoni et al. 2000, Szjigyarto et al. 2013), this allowed comparison of the cortisol results in this manuscript to previous studies. In addition, previous studies have characterized hemodynamic stress responses using single measurements of BP and HR at various time points throughout the stress task (Carroll et al. 2001, Moseley & Linden 2006); which may underestimate the peak stress response. This study used continuous HR and BP measurements, which allowed for more accurate determination of the true peak stress response.

As described in Chapter 3, due to technical difficulties, we were unable to obtain data for certain variables (e.g., MAP, glucose, total cholesterol) in all participants. However, this likely did not have an effect on the outcomes. Additionally, it should be noted that shear rate was used to estimate the reactive hyperemia shear stress stimulus. Shear rate has been shown to be an appropriate and acceptable surrogate for the shear stress stimulus; this is evidenced by similar results observed when using shear rate to those obtained calculating shear stress using viscosity measurements (Padilla et al. 2008, Parkhurst et al. 2012). Although analysis to determine whether systemic ROS generation increased with mental stress was attempted, this objective was not met. Plasma samples were obtained to estimate ROS production using thiobarbituric acid reactive substances (TBARS) analysis, which have been used previously to assess acute changes in ROS in humans (Johnson et al. 2012). However, this assay suffered from high variability and was technically difficult to carry out. As such, the results were not included. The unexpected lack of an overall decline in FMD post-mental stress made it difficult to test the hypothesis of whether oxidative stress contributes to impairments

in FMD post-stress. This was likely due to great variability in FMD responses to stress which may be due to individual differences that affect susceptibility to stress induced endothelial dysfunction (to be discussed in section 4.2).

A physical activity inquiry was performed during screening visits with the aim of including a group with low fitness. Screening involved self-report and those who reported engaging in greater than two structured physical activity sessions each week were screened out and unable to participate in the study. However, self-report is limited (Klesges et al. 1990) and in addition, while physical activity and fitness are related, physical activity does not perfectly predict fitness (Emaus et al. 2010). Physical fitness was not assessed due to the added time commitment in an already lengthy and complex study. Fitness has been shown to have an effect on stress responses (i.e., higher fitness is associated with lower cortisol and heart rate responses to stress; Klaperski et al. 2014, Rimmele et al. 2007) and screening for fitness could have prevented potential varying influence of physical fitness on stress reactivity and FMD. However, other studies that have observed average declines in FMD of ~50% did not perform participant screening based on fitness or other individual factors (Ghiadoni et al. 2000, Broadley et al. 2005, Spieker et al. 2002). This previous work suggested that this screening was not necessary to observe an effect on FMD.

4.2 Variability in FMD responses to acute mental stress

Pilot work performed prior to completion of the thesis study supported the expectation that mental stress would cause a significant impairment in FMD at 30 minutes (*unpublished data*) and 90 minutes (Plotnick et al. 2015) post-stress in healthy, young

individuals. This finding, along with evidence from the literature (Ghiadoni et al. 2000), was used as a basis for the timing of post-stress measurements. Although the majority of studies have shown a clear negative impact of acute stress on endothelial function, there is some evidence of individual variability (Szijgyarto et al. 2013, Ghiadoni et al. 2000, Poitras et al. 2013). There are several factors that might influence individual responses to stress such as personality traits (Oswald et al. 2006), age (Kudielka et al. 2007), gender (Kirschbaum et al. 1992, Therrien et al. 2010), and physical fitness (Klaperski et al. 2014, Rimmele et al. 2007). Perception of the task itself may also influence FMD responses; there is some evidence to suggest that positive emotions evoke changes in physiological variables that are also elevated due to stress (e.g., MAP, HR) and are associated with increases in FMD (Miller et al. 2006, Miller et al. 2010, Sugawara et al. 2010). It is possible that in this educated population, the task was not perceived as stressful but as a challenge and a possibly positive experience, which may explain why FMD increased in some participants. Evidence suggests that the impact of acute mental stress created by laboratory stress tasks is unclear and possibly bi-directional depending on a number of individual factors. However, the majority of evidence appears to favor a negative effect of more prolonged or chronic stress on endothelial function (Mausbach et al. 2010, Takase et al. 2004a, Cooper et al. 2010, Toda & Nakanishi-Toda 2011).

4.3 Future directions

4.3.1 The potential role of ROS in stress-induced endothelial dysfunction

As mentioned previously, although an assay to quantify ROS production post-mental stress was attempted, the results could not be interpreted confidently due to high assay variability. In future research, it would be helpful to further explore the relationship between mental stress and systemic oxidative stress. If ROS does play a role in stress-induced endothelial dysfunction, it would be interesting to determine which type of ROS is involved given that some ROS have been shown to be beneficial (Wolin and Burke 1987, Burke and Wolin 1987, Thomas et al. 2000, Drummond et al. 2000, Ray et al. 2011) and others have been shown to be detrimental (Johnson et al. 2013, Hirai et al. 2000, Evans et al. 2003, Anderson et al. 2005, Hamabe et al. 2001, Takase et al. 2004b, Silvestro et al. 2002, Plantinga et al. 2007, Stramatelopoulous et al. 2003, Ellis et al. 2001, Gokce et al. 1999, Levine et al. 1996) to endothelial function. This may prove to be difficult in human participants as most assays of systemic oxidative stress are non-specific to the type of ROS molecule. However, it is possible in cells or in animal models, which could be a useful way to gain some initial insight.

Another interesting future direction would be to examine antioxidant status and whether baseline activity or expression of natural antioxidant producing enzymes, such as superoxide dismutase, is altered by mental stress. As the definition of oxidative stress is a situation in which the generation of ROS exceeds the generation or presence of antioxidants able to combat the ROS, it would be of interest to see whether mental stress affects both the antioxidant and ROS generation sides of this balance. There is also a need to further understand the role that oxidative stress plays in individuals with chronic life stress. Research

has shown that individuals with chronic stress such as caregiver burden have elevated levels of oxidative stress biomarkers (Aschbacher et al. 2013). Therefore, another future direction might be to examine the effect of vitamin C on FMD in individuals with chronic stress.

Another mechanistic future direction regarding oxidative stress would be to examine the importance of eNOS cofactor BH4 bioavailability post-mental stress on stress-induced endothelial dysfunction. As ROS seems to impair NO bioavailability through a variety of mechanisms that ultimately reduce BH4 bioavailability, it would be interesting to see whether this mechanism plays a significant role in stress-induced endothelial dysfunction.

4.3.2 Other mechanisms of stress-induced endothelial dysfunction

There is much to learn about how mental stress affects the cardiovascular system and what mechanisms link stress and CVD. While the study presented in Chapter 3 focused on psychosocial stress and ROS, it is possible that stress affects other processes that contribute to susceptibility to CVD. For instance, some evidence suggests that inflammation increases as a result of mental stress (Steptoe et al. 2001, Steptoe et al. 2002, Steptoe et al. 2007, Edwards et al. 2006, von Kanel et al. 2006, Brydon et al. 2005, Veldhuijzen et al. 2005) and that acute systemic inflammation impairs FMD (Hingorani et al. 2000, Erzen et al. 2006). Examining whether the extent of inflammation generated due to mental stress affects susceptibility to FMD impairments would provide insight into the role of inflammation on stress induced endothelial dysfunction. The type of inflammatory factor generated may complicate the role of inflammation in FMD responses to stress; certain factors such as IL-6 have been shown to be both detrimental (Erzen et al. 2006, Hingorani et al. 2000, Hung et al.

2010) and beneficial (Katusic et al. 1998, Huang et al. 2005, Antoniades et al. 2011) to eNOS activity and endothelial function. There is also some evidence that mental-stress induced endothelial dysfunction is mediated by endothelin-1 (ET-1; Spieker et al. 2002) and sympathetic nervous system activity (Hijmering et al. 2002, Eriksson et al. 2007), however, there is some controversy regarding the role of sympathetic nervous system activity (Dyson et al. 2006, Spieker et al. 2002). Future studies that clarify the relative importance of these factors that become elevated during mental stress would be helpful in understanding their role in mental stress induced endothelial dysfunction.

Finally, a number of individual characteristics have been shown to have an effect on FMD responses to mental stress (e.g., hostility, hypnotizability, fitness). The mechanisms through which these individual factors influence or mediate the FMD response to mental stress remain unclear. A greater understanding of these mechanisms may aid in determining effective interventions to target the effects of these specific factors. Furthermore, some research has shown that positive emotions evoke the opposite response to mental stress in that FMD is actually improved (Miller et al. 2006, Miller et al. 2010, Sugawara et al. 2010). Specifically, Miller et al. (2010) showed that joyful music was associated with a significant improvement in FMD and in contrast, anxiety provoking music was associated with a trending negative (but not statistically significant) decline in FMD. Furthermore, Sugawara et al. (2010) found improvements in FMD after 15-30 minute bouts of laughter. This suggests that positive and negative emotions may elicit opposing mechanisms to promote and attenuate endothelial function, respectively. It would be interesting to examine the effects of

acute stress on FMD after a bout of positive emotions (e.g., laughter, joyful music); if positivity does elicit opposing mechanisms to stress, it may have a protective effect on FMD and attenuate declines post-stress. Furthermore, it would be interesting to see if affect or perception of the stress task has an impact on FMD responses post-stress. As previously mentioned, positive emotions are associated with increases in FMD (Miller et al. 2006, Miller et al. 2010, Sugawara et al. 2010). As such, perhaps the use of a scale or questionnaire post-stress task in order to get a sense of individuals' perception of the task would reveal that opposite FMD responses are dependent on positive or negative perceptions of the task.

4.3.3 Additional factors that may play a role in stress-induced endothelial dysfunction

The results of the study presented in Chapter 3 suggest a need for further research to better understand the effect of individual factors on the susceptibility to stress-induced endothelial dysfunction. As mentioned previously, individual characteristics such as low hypnotizability (Jambrik et al. 2004a, Jambrik et al. 2004b, Jambrik et al. 2005b) and high hostility (Gottdiener et al. 2003) have been shown to play a role in susceptibility to FMD impairments post-stress. This suggests that pre-existing circumstances or characteristics can determine FMD responses to acute stress. As such, it would be interesting to examine whether the extent of chronic life stress experienced by individuals influences the FMD response to an acute laboratory induced bout of mental stress. For instance, as the majority of this population was undertaking university studies, determining whether perceived academic stress affects the FMD response to acute mental stress is an intriguing future direction.

Another interesting future direction would be to examine endothelial function at more time intervals post-stress. This study only assessed FMD at 30 and 90 minutes post-stress and it is possible that more FMD time points would add clarity to the potential impairment time course. Furthermore, there is evidence to suggest that females have lower salivary cortisol responses to the TSST and that cortisol responses may be dependent on phase of the menstrual cycle (Kirschbaum et al. 1999). As phase of the menstrual cycle appears to have an effect on FMD, phase may also dictate the magnitude of female FMD responses to stress (Adkisson et al. 2010). Studies investigating FMD responses to mental stress according to sex or by phase of the menstrual cycle would greatly add to this knowledge base.

4.4 Conclusion

Studying the impact of mental stress on endothelial function is important because it provides insight into the negative impacts of chronic stress. It is thought that repeated bouts of acute mental stress may cumulatively have a negative effect on the vascular system (Poitras and Pyke 2013, Black and Garbutt 2002). As mentioned, understanding some of the factors that influence susceptibility to acute stress induced impairments in FMD may provide insight into interventions to prevent these negative long-term consequences on the vascular system. Examination of interventions to reduce or control, for instance, hostility or negative perceptions of a task, may attenuate the endothelial impairments due to a bout of stress and potentially protect the vasculature from the effects of chronic stress.

It was initially hypothesized that vitamin C would attenuate an acute mental stress induced impairment in FMD compared to the placebo condition. Contrary to the hypothesis, the study described in this thesis did not find a mental-stress induced impairment in endothelial function. This was observed in the presence of a significant stress response as measured by salivary cortisol, MAP, HR, and subjective stress ratings. Despite finding no overall negative effect on endothelial function, an interesting association between Δ%FMD and salivary cortisol and MAP was observed in the placebo condition but not in the vitamin C condition. This suggests that vitamin C somehow disrupts the relationship between the stress response and its effect on endothelial function. The findings of this study further contribute to a small but growing body of knowledge of acute mental stress and endothelial function and beg for further research to determine what factors are responsible for susceptibility to stress-induced endothelial dysfunction.

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Appendix A

Research Ethics Board Approval

Amendment Acknowledgment/Approval Letter

November 26, 2014

Dr. Kyra Pyke
School of Kinesiology & Health Studies
Queen's University

RE: File #6013154 PHE-148-14 NSERC title: Shear stress and the regulation of vascular function and structure; Project title: Can BH4 protect against mental stress induced impairments in endothelial function?

Dear Dr. Pyke:

I am writing to acknowledge receipt of the following:

- Request to perform a separate branch of the study - recruiting an additional 20 young healthy males to examine the impact of vitamin C supplementation.
- A copy of the protocol timeline
- Addition of saliva sampling for determination of salivary cortisol
- A copy of the revised consent form (clean and tracked changes copy) (Version 3: 06/11/2014)
- A copy of the revised medical screening form

I have reviewed these amendments and hereby give my approval. Receipt of these amendments will be reported to the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board.

Yours sincerely,

Albert L. Clark.

Albert Clark, Ph.D.
Chair
Health Sciences Research Ethics Board

Appendix B

Consent Form

School of Kinesiology and Health Studies
Queen's University

Kyra E. Pyke, Ph.D., Principal Investigator
Study performed in Room 400D School of Kinesiology and Health Studies

CONSENT FORM
FOR RESEARCH PROJECT ENTITLED:

Can Vitamin C protect against mental stress induced impairments in endothelial function?

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign this form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Purpose of the Study:

You are being invited to participate in a research study directed by Dr. Kyra Pyke to evaluate the role of oral vitamin C, an antioxidant, on vascular function after a brief period of mental stress. Dr. Pyke or a student investigator will read through this consent form with you and describe the procedures in detail and answer any questions you may have. This study has been reviewed for ethical compliance by the Queen's University Health Sciences Research Ethics Board.

The purpose of the study is to examine whether oral vitamin C supplementation attenuates the potentially detrimental transient effects of acute mental stress on vasodilation (vessel widening) mediated by the endothelium (a single layer of cells that lines the arteries).

Benefits For You:

None

Description of Experiment and Risks:

What will happen? During this study, you will take part in the specific experimental procedures outlined below.

HEART RATE MEASUREMENTS:

Heart rate is continuously monitored by an electrocardiogram (ECG) through 3 spot electrodes on the skin surface. The electrodes are placed on the chest and abdomen and they can detect the electrical activity that makes your heart beat.

RISKS: This procedure is entirely safe. In a very small group of individuals, a skin rash might occur from the adhesive on the electrodes. There is no way of knowing this ahead of time. The rash, if it develops, will resolve itself within a day or so. Avoid scratching the rash and keep clean.

BLOOD PRESSURE MEASUREMENTS:

A small cuff is fit around your finger. This cuff inflates to pressures that match the blood pressure in your finger, so you feel the cuff pulsing with your heartbeat. It shines infrared light through your finger to measure changes in the size of your finger with each heartbeat. Your blood pressure will also occasionally be taken with a cuff that inflates around your upper arm.

RISKS: This technique is non-invasive and poses no risk.

LIMB BLOOD FLOW AND BLOOD VESSEL DIAMETER MEASUREMENTS:

The blood flowing through your brachial (above the elbow) artery can be detected, and your artery size measured using Doppler and imaging ultrasound. A probe will be placed on the skin over your artery and the investigator will control adjustments in its position by hand. High frequency sound (ultrasound) will penetrate your skin. The returning sound provides information on blood vessel size and blood flow.

RISKS: This technique is non-invasive and poses no risk.

FOREARM OCCLUSION:

A blood pressure cuff will be secured just above or below your elbow on your left arm. This cuff will be inflated to 250mmHg for 5 min to limit blood flow into your forearm. You may feel a strong pressure and some mild tingling with cuff inflation

but it should not be uncomfortable. If there is pain, immediately notify the investigator and the cuff will be deflated and repositioned. Upon cuff release there will be a large rush of blood into your forearm. This may feel warm and you may experience mild tingling but no discomfort.
RISKS: This technique is non-invasive and poses no risk.

VITAMIN C SUPPLEMENT or PLACEBO:

On one of two experimental visits, commercially available vitamin C, obtained over the counter from a local pharmacy, will be administered in two doses of 500mg separated by 1h (total dose 1000mg). This dosing protocol is used to make sure that the circulating levels of vitamin C in your blood will remain high for the 90min period after the mental challenge task. Vitamin C has properties (it is an antioxidant) that allow it to lessen the action of some highly reactive molecules that may increase in number during stress. On the other of the two experimental visits you will receive a placebo. Neither you, nor the experimenters will know whether you have received the vitamin C or the placebo.

RISKS: Vitamin C is generally considered to be nontoxic, and the vitamin C ingestion in this study has minimal risk in healthy individuals. The 1000mg dose in this study is one half of the tolerable upper intake level of 2000mg/day from food and supplements. For a food reference, a 250 ml glass of orange juice contains approximately 75mg of vitamin C. The tolerable upper intake level is set at the highest level that is not expected to result in any adverse health effects in the general population. The most common symptoms that occur with consumption of vitamin C in doses in excess of 2000mg are diarrhea, nausea, and abdominal cramps. These occur when unabsorbed vitamin C draws water into the intestine. A high intake of vitamin C is not recommended for individuals prone to kidney stones, by individuals who have iron absorption regulation problems or by those with sickle cell anemia. If you have any of these conditions and did not disclose them on your medical screening form, please inform the person obtaining consent. You should not participate in this study. High doses of vitamin C can also alter the effectiveness of analgesics/anti-inflammatory drugs (e.g. aspirin), anticoagulants (warfarin), some chemotherapy and radiation medications, and estrogens (hormone replacement therapy, oral contraceptives). Vitamin C intakes higher than 3000mg per day can interfere with drugs prescribed to slow blood clotting and can interfere with urine

tests used to monitor blood glucose levels. Either the vitamin C supplement or the placebo may contain artificial sweetener that may contain the amino acid phenylalanine. This is contraindicated for individuals with phenylketonuria. If you have phenylketonuria please inform the person obtaining consent.

SALIVARY CORTISOL MEASUREMENTS

Cortisol is a hormone released in response to stress. When you arrive in the laboratory and at specific time points during the experiment we would like to take a sample of your saliva. This is collected via chewing a swab which absorbs your saliva.

RISKS: This procedure is non-invasive and poses no risk.

VENOUS BLOOD DRAW:

Blood samples will be taken to measure: antioxidant levels, levels of factors in the blood that indicate an inflammatory response, an index of the action of highly reactive molecules, stress hormones, the thickness of your blood (blood viscosity), the amount of fats in your blood, and your blood sugar levels. Blood samples will be taken from a vein at your elbow or in your hand following standard venipuncture technique. In order to make the vein easy to identify, a non-latex tourniquet will be briefly applied to your upper arm. A needle will be inserted into the full vein the same way that it is done if you donate blood or have blood taken for medical tests. When repeat samples are required this needle will be removed but a flexible tube (catheter) will remain in your arm for the duration of the study. This will be done by a registered nurse. Blood will be drawn from the tube and the tube will be flushed with sterile saline occasionally to keep it open. At the end of the visit the tube will be removed and a bandage applied. The total amount of blood taken will be about 100ml. This is less than one quarter of the amount of blood drawn when you donate blood.

RISKS: There may be some mild soreness and mild bruising at the site of the needle insertion. In rare cases more significant soreness and significant bruising can occur. Puncturing a blood vessel increases the risk of clot formation, but this is very rare with venous blood sampling. If you have symptoms outside of what is described here please contact Meghan Plotnick or Dr. Pyke.

MENTAL CHALLENGE:

During your visit you may be asked to perform a mentally challenging task. This could include mental arithmetic with verbal report of answers, prepping a speech after hearing a recorded scenario or reading the names of colors that are printed in ink that does not match the color of the written word. Your answers will be video recorded for subsequent analysis. You will be asked to rate your degree of stress on a scale of 1 -10.

RISKS: This task may cause your heart rate and blood pressure to increase as they would in moderate intensity exercise. This does not pose any greater cardiovascular risk than exercise, which has very low risk for healthy individuals.

7-DAY PHYSICAL ACTIVITY RECALL: This is a questionnaire that will ask you to report your physical activity levels over the past 7 days.

RISKS- This poses no risk

Familiarization Visit: On an initial visit you will be asked to lie down while we will use ultrasound to get an image of the artery in your upper arm to make sure that we can get clear pictures. We will also measure your height and weight. This visit will take approximately 20-30 min.

Experimental Visit #1: This visit will take a maximum of 3.5 hours. While lying down and resting, you will be instrumented for heart rate, blood pressure and blood flow (ultrasound) measurements. After a 20 min rest period you will undergo one trial of 5-minute cuff inflation and release on the forearm, while we measure the blood flow in your upper arm (brachial artery). There will be at least 10 min of rest between each trial. You will have simultaneous measurements of your upper arm blood flow with ultrasound. You will then be given a supplement containing either vitamin C or a placebo-composed of sugar or artificial sweetener. After you have taken the supplement, there will be 1 hour in which you will rest while waiting for the vitamin C levels to rise in your blood. At the end of the 1 hour, you will take the second dose of the supplement or placebo to ensure vitamin C levels remain elevated in the blood throughout the remainder of the study in the vitamin C condition. You will then perform 3 more trials of 5-minute cuff inflation and release on the forearm, while we measure the blood flow in your upper arm (brachial artery). Venous blood draws will be taken before each forearm occlusion. Between the first and second trials you will complete the mental challenge. You will receive a snack before leaving the laboratory after each experimental visit.

Experimental Visit #2: This visit will take a maximum of 3.5 hours and will be identical to Experimental Visit #1. Either vitamin C or a placebo will be given randomly (one per each visit).

Talking and Movements:

Talking or moving during the times that we are taking measurements will cause variations in the measurements we are making. If you have any discomfort, please let us

know immediately and we can temporarily break from data collection. However, if everything is comfortable, please maintain a very quiet posture. Even very slight movements interfere with our experiments.

Special Instructions:

Participants are asked to not exercise, drink alcohol or caffeine during the 24 hours prior to the study. **Also, please do not consume any vitamin C or multivitamin supplements in the 24h prior to and after each visit**. Also, we ask that you do not consume any food or liquids other than water during the 12 hours preceding the experiments. You should empty your bladder immediately prior to starting the test. When the study is finished, we will have you sit in the laboratory for a short time to allow you to readjust to the upright posture. These precautions should be enough to prevent any sensations of dizziness. Please be aware that sensations of dizziness are not normal and you should let us know if you experience any discomfort before you leave the laboratory. It is also important that you maintain your pre-study physical activity levels and diet throughout this study.

Attached Medical Screening Forms:

These questionnaires ask some simple questions about your health and any medical conditions. This information is used to guide us with your entry into the study. Current health problems indicated on this form, which are related to cardiovascular diseases, or which increase the risk of high vitamin C intake, exclude you from the study.

Safety Precautions:

Safety precautions for the study will include the following:

- ❑ Before entering the study you will be screened using a medical screening form. You will not be able to enter the study if anything is found which indicates that it is dangerous for you to participate.

- ❑ We will continuously monitor your heart rate during testing, and you will be sitting or laying on your back. These precautions allow us to quickly identify if you are experiencing an unusual response and simply stopping the experimental manipulation will allow you to quickly recover.

Confidentiality:

All information obtained during the course of the study is strictly confidential and will not be released in a form traceable to you, except to you and your personal physician upon your request. Your data and any personal health information reported on the health questionnaire will be kept in locked files which are available only to the investigators and research assistants who will perform statistical analysis of the data. There is a possibility that your data file, including identifying information, may be inspected by officials from the Health Protection Branch in Canada in the course of carrying out regular government functions. The study results will be used as anonymous data for scientific publications and presentations, or for the education of students in the School of Kinesiology and Health Studies at Queen's University.

Study Compensation:

You will receive $11 per hour for participation in the study.

Freedom to Withdraw from the Study:

Your participation in this study is voluntary. You may refuse to participate or you may discontinue participation at any time during the duration of the study without penalty and without affecting your future medical care or academic evaluation. Should you

discontinue participation after the first experimental visit, you will be provided with payment for the number of hours you have completed during the first experimental visit.

Participant Statement and Signature Section

I have read and understand the consent form for this study. I have had the purposes, procedures and technical language of this study explained to me. I have been given sufficient time to consider the above information and to seek advice if I choose to do so. I have had the opportunity to ask questions which have been answered to my satisfaction. I am voluntarily signing this form. I will receive a copy of this consent form for my information.

If at any time I have further questions, problems or adverse events, I will contact:

Meghan Plotnick (Graduate student Investigator)
13mdp4@queensu.ca
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If I have any questions concerning research participant's rights, I will contact:

Dr. Albert F. Clark, Chair of the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board
Office of Research Services
Fleming Hall, Jemmett Wing 301
Queen's University, Kingston, ON, K7L 3N6
Tel: (613) 533-6081

By signing this consent form, I am indicating that I agree to participate in this study.

_____ _____

Participant Signature Signature of person obtaining consent

_____	_____
Participant Name (please print)	Name of person obtaining consent (please print)
_____	_____
Date (day/month/year)	Date (day/month/year)

Appendix C

Medical Questionnaire for Research Study

School of Kinesiology and Health Studies

MEDICAL QUESTIONNAIRE FOR RESEARCH STUDY

Can vitamin C protect against mental stress induced impairments in endothelial function?

Faculty Investigators:

Kyra E. Pyke, PhD, School of Kinesiology and Health Studies

Graduate Student Investigator:

Meghan D. Plotnick, MSc. Candidate, School of Kinesiology and Health Studies

To the study participant: Please answer all questions in sections 1 and 2 of this form.

SECTION 1: PERSONAL DATA (please print)

Name: _____

Date of Birth: _____

SECTION 2: MEDICAL HISTORY

1. Y / N Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?

2. Y / N Do you feel pain in your chest when you do physical activity?

3. Y / N In the past month, have you had chest pain when you were not doing physical activity?

4. Y / N Do you lose your balance because of dizziness or do you ever lose consciousness?

5. Y / N Is your doctor currently prescribing drugs (e.g. water pills) for your blood pressure or heart condition?

6. Y / N Do you know of any other reason why you should not do physical activity?

7. Y / N Do you have phenylketonuria (PKU)?

8. Y / N Do you have sickle cell anemia?

9. Do you have, or have you ever had, problems with any of the following?

Yes No

i. Heart or blood vessels ___ ___

(these might include but are not limited to: heart attack, stroke, heart murmur angina, coronary artery disease, high blood pressure, high cholesterol, congenital heart disease, any heart operation, bleeding or clotting disorders)

ii. Nerves or brain ___ ___

iii. Breathing or lungs ___ ___

iv. Hormones, thyroid, or diabetes ___ ___

v. Muscles, joints, or bones ___ ___

vi. Renal or liver impairments ___ ___

vii. regulating iron absorption ___ ___

viii. Other (please list) _____

9. Please list the diagnosis or/or briefly describe any problems identified in #9

10. Are you presently taking any medications? If yes, please list any you are currently taking.

11. Do you have any allergies to medications, adhesive tape, latex, artificial sweeteners, or colors?

12. Do you currently smoke? _____ Y / N Number of cigarettes/day_____

If previous smoker date of last cigarette_____ (year, month)

<u>SECTION 3:</u> TO BE COMPLETED ON THE DAY OF THE EXPERIMENT

13.

Day 1: Have you consumed any alcoholic beverages within the last 12h? yes/no _____ (initials)_____ (date).

Day 2: Have you consumed any alcoholic beverages within the last 12h? yes/no _____ (initials) _____ (date).

14. **Day 1:** Have you had a fever in the last 48 hours? Y / N

Day 2: Have you had a fever in the last 48 hours? Y / N

15. **Day 1:** Have you consumed a supplement containing vitamin C in the past 24 h? Y / N

Day 2: Have you consumed a supplement containing vitamin C in the past 24h? Y / N

I acknowledge that the study investigators completed this form according to my specifications; this information is true to the best of my knowledge.

_____ _____

Participant Name Participant Signature

Date (dd/mm/yyyy) : _____

Appendix D

Physical Activity Recall

Date: ____ / ____ / _____

Human Vascular Control Laboratory

Day of the week form completed:
- ❑ Sunday
- ❑ Monday
- ❑ Tuesday
- ❑ Wednesday
- ❑ Thursday
- ❑ Friday
- ❑ Saturday

Physical Activity Recall

1. Were you employed in the last seven days (paid or volunteer)? ❑ YES ❑ NO → Go to question 4

2. How many days of the last seven did you work? [ ] (round to nearest day)

3. How many total hours did you work in the last seven days? [ ][ ] hours

4. What days of the week do you consider to be your weekend or non-work days? For most people, this would be Saturday and Sunday, but it may be different for you.

❑ Sunday ❑ Monday ❑ Tuesday ❑ Wednesday ❑ Thursday ❑ Friday ❑ Saturday

**********Explain Moderate, Hard, and Very Hard Intensity levels **********

ID# _____ **Interviewer Initials: _____**

Date: _____ / _____ / _____

At the end of the interview:

5. Compared to your physical activity over the past three months, was last week's physical activity more, less or about the same?

- ❑ More
- ❑ Less
- ❑ About the same

ID# _____ **Interviewer Initials: _____**

- Naps [+] / Disruptions [-]

> 30 min

	Yesterday						One Week Ago
Sleep	-	-	-	-	-	-	-
Moderate							
Hard							
Very Hard							
Moderate							
Hard							
Very Hard							
Moderate							
Hard							
Very Hard							

Rounding: 10-22mins = .25hrs 23-37mins = .50hrs 38-52mins= .75hrs 53-1:07mins=1.0hrs 1:08-1:22= 1.25hrs

ID# _____ **Interviewer Initials: _____**

- Naps [+] / Disruptions [-]

> 30 min

7-Day PAR: Interview Evaluation Form

Were there any problems with the 7-Day PAR interview? (circle one)

1. Yes 2. No

Explain:

_____

_____

_____

Do you think this was a valid 7-Day PAR interview?

1. Yes 2. Maybe 3. No

Please list below any activities reported by the participant that you don't know how to classify:

_____

_____

_____

_____

Other comments/concerns:

_____

_____

ID# _____ **Interviewer Initials: _____**