THE ROLE OF ACUTE PHOSPHATE SUPPLEMENTATION IN ENDOTHELIUM-DEPENDENT AND -INDEPENDENT VASODILATION

by

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Abstract

Phosphate is a mineral component of the human diet that is essential for life. However, elevated blood phosphate levels are associated with cardiovascular disease and death in the general population. The mechanisms through which phosphate may be detrimental to cardiovascular health remain unclear, however there is emerging evidence that suggests phosphate may impair the function of human arteries. The purpose of this study was to investigate whether a single phosphate supplement affects the ability of the brachial artery to dilate in response to: a) increases in blood flow (flow-mediated dilation, FMD), b) a drug that causes dilation (GTN), or c) both FMD and GTN. 17 healthy male participants (22.9 ±3.07 years old) were exposed to phosphate (PHOS) and placebo (PLAC) conditions over two experimental days (one each visit) in a within-subjects, randomized, double-blinded, placebo-controlled trial. Subjects drank a liquid containing 1200 mg of phosphorus or a placebo. FMD tests were performed pre- and 20 min, 60 min and 120 min post-supplement or placebo ingestion by a standard procedure involving the occlusion of blood flow to the forearm (reactive hyperemia). Blood phosphate concentrations were significantly greater in PHOS vs. PLAC, but did not increase differently between the two groups. Blood phosphate concentrations were significantly greater 60 min vs. 20 min post-supplement in both conditions. Phosphate excretion increased 315% in the PHOS vs. PLAC condition, indicating that the kidney rapidly removed phosphate from the blood. There was no difference in FMD between PHOS and PLAC conditions at any post-supplement time point. There was no effect of PHOS on the GTN tests. These findings indicate that a single phosphate supplement did not impair brachial artery dilation in response to increases in blood flow or the administration of a drug that induces dilation. Also, the phosphate supplement did not substantially increase phosphate concentrations in the blood likely due to a
large excretion of phosphate by the kidneys. Further research is needed to better understand how phosphate may be linked to vascular function.
Co-Authorship

Brendan Levac was responsible for writing all of the chapters in this thesis with comments and revisions provided by Dr. Kyra Pyke.

The manuscript in Chapter 3 entitled ‘The role of acute phosphate supplementation on endothelium-dependent and -independent vasodilation’ is the work of Brendan Levac in collaboration with his co-authors. The co-authors are Dr. Kyra Pyke (School of Kinesiology and Health Studies, Queen’s University) and Dr. Michael Adams (Department of Biomedical and Molecular Sciences, Queen's University).

Brendan Levac was responsible for developing the research question, reviewing the background literature, designing the study, conducting the data collection, interpreting the results and statistical analysis, and composing the primary manuscript. Dr. Kyra Pyke contributed guidance in the design of the study, assisted with the interpretation of the results and provided feedback and revisions on the manuscript for important intellectual content. Dr. Kyra Pyke was also the principal investigator on the research grant funding this study. Dr. Michael Adams provided guidance in the study design and offered his laboratory services for serum and urine analysis.
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List of Abbreviations

absFMD- Absolute Diameter Change in FMD
ANOVA- Analysis of Variance
AUC- Area Under the Curve
BAEC- Bovine Aortic Endothelial Cells
BMI- Body Mass Index
cGMP- Cyclic Guanosine Monophosphate
CKD- Chronic Kidney Disease
CVD- Cardiovascular Disease
EDHF- Endothelial-derived Hyperpolarizing Factor
ELISA- Enzyme Linked Immunosorbent Assay
eNOS- Endothelial Nitric Oxide Synthase
ET-1- Endothelin
FEP- Fractional Excretion of Phosphate
FMD- Flow-mediated Dilation
GTN- Glyceryl Trinitrate
HDL- High-density Lipoprotein
HUVEC- Human Umbilical Vein Endothelial Cells
LDL- Low-density Lipoprotein
MAP- Mean Arterial Pressure
MI- Myocardial Infarction
NADPH- Nicotinamide Adenine Dinucleotide Phosphate
NO- Nitric Oxide
PGI₂- Prostacyclin

PHOS- Phosphate Condition

PKC- Protein Kinase C

PLAC- Placebo Condition

PO₄- Phosphate (the primary form of elemental phosphorus present in the body)

ROS- Reactive Oxygen Species

SS-AUC- Shear Stress Area Under the Curve

VC- Vascular Calcifications

VSMC- Vascular Smooth Muscle Cell
Chapter 1

Introduction

Cardiovascular disease (CVD) is a prominent health concern worldwide. Each year in North America, CVD accounts for approximately 30% of all deaths, with heart disease being the leading cause of death (67). Advances in medical care, medical technologies, and pharmaceuticals contribute to the prevention of approximately 10% of all premature deaths in North America, however modifiable behavioral risk factors, primarily poor dietary and lifestyle habits, account for 40% of all-cause mortality in North America (67, 92). The focus of reducing CVD prevalence has shifted towards a greater emphasis on preventing poor dietary and lifestyle habits compared to the more traditional pharmacological treatment approach (70, 98). Numerous components of the North American diet have been scrutinized as CVD risk factors, including cholesterol, fats, sugar-sweetened beverages and alcohol. More recently however phosphate, a mineral constituent of the diet, has been associated with CVD mortality and labeled a novel CVD risk factor (54, 70, 89).

Phosphate is involved in a variety of cell functions including cellular signaling, nucleic acid synthesis and energy metabolism (88). Phosphate is regulated in a dynamic balance between intestinal absorption and renal excretion. Traditionally, reduced renal function was the purported initiator of a deleterious high phosphate environment linked to adverse cardiovascular events (1, 27), but more recently serum phosphate, even within the clinically normal range (2.5 to 4.5
mg/dl), has been shown to predict cardiovascular morbidity and mortality in individuals with and without renal impairments (25, 77, 104).

In the healthy, general population, the underlying mechanisms by which serum phosphate may increase cardiovascular risk are incompletely understood, however convincing mechanistic in vitro, and in vivo evidence has emerged to suggest that phosphate-induced endothelial dysfunction may be a critical link between elevated serum phosphate levels and increased CVD risk (73). The current understanding is that serum phosphate is transported into the vascular endothelium where it attenuates vasoprotection by causing a decrease in NO production (63, 97).

The endothelium is a single layer of cells situated on the luminal surface of blood vessel walls. This unique location exposes endothelial cells to hemodynamic forces that are significantly larger than fluid forces experienced elsewhere in the body and the endothelium has developed to respond to these hemodynamic forces in both the acute and chronic regulation of vascular tone and structure (23). Specifically, endothelial cells are sensitive to shear stress, which is the frictional force created by blood flow through an artery (17). In response to increases in shear stress, the endothelium releases vasoactive substances, including nitric oxide (NO; 11, 29). NO is critical for regulating vasoprotection and the local control of vascular tone (56, 69, 107). NO and other vasodilators produced by the endothelium in a shear-stress-dependent manner signal the surrounding vascular smooth muscle cells to relax, thereby producing a dilation of the blood vessel (50, 76). This dilatory response is termed flow-mediated dilation (FMD; 14). Increasing blood flow and the associated shear stress can be exploited experimentally, such that measuring the magnitude of the subsequent conduit artery dilation (FMD) provides an assessment of endothelial cell function (16).
Reactive hyperemia is the established technique used to create an increase in shear stress for FMD assessment (16, 23, 50). Typically, reactive hyperemia involves a 5-minute period of forearm vascular occlusion and subsequent re-establishment of forearm blood flow, which produces a large transient increase in shear stress within the brachial artery. A strong positive correlation exists between changes in shear stress and changes in blood vessel lumen diameter (FMD), therefore, it is important to measure the shear stress stimulus that the endothelium receives in order to effectively interpret an FMD response as an index of endothelial function (8, 71, 103). The induced shear stress stimulus elicits an FMD response that can be measured by vascular ultrasound techniques (103). To ensure that FMD is indicative of endothelial function specifically, it is important to assess the dilatory response of the vascular smooth muscle cells (VSMC) to an endogenously administered vasodilator, such as the NO donor glyceryl trinitrate (GTN). Assessing VSMC sensitivity to GTN in conjunction with FMD tests allows an investigator to determine whether a measured impairment in vasodilation is the result of dysfunctional VSMC, endothelial cells, or a combined impairment.

Endothelial dysfunction is characterized by diminished vasoprotection resulting in maladaptive changes within the vascular environment, and is recognized as an early stage in the development of CVD. The loss of vasoprotection precedes the overt manifestation of clinically significant atherosclerosis and can be measured using FMD tests to assess CVD risk (24). In long-term population-based studies monitoring the occurrence of cardiovascular events (ie. mortality, myocardial infarction, stroke, and revascularization procedures), individuals with higher FMD responses experienced fewer cardiovascular events than their counterparts with lower FMD responses (90, 94, 112, 113). FMD data in these studies provided significant
prognostic value for the prediction of future cardiovascular events even after adjustment for known cardiovascular risk factors (90, 94, 113). Endothelial function is impaired in the presence of CVD, but repeated exposure of a healthy endothelium to various environmental (cigarette smoke; 2, 25) and dietary (fat/lipids, suboptimal vitamin C, alcohol; 36, 48, 60) components can precipitate transient episodes of endothelial dysfunction independent of underlying disease. Recurring exposure to negative environmental and dietary factors may contribute to the development of clinically relevant atherosclerosis and cardiovascular risk through cumulative attenuation of endothelium-dependent vasoprotection.

In the sole study, to date, investigating the impact of acute dietary phosphate consumption on endothelial function in human subjects, reactive hyperemia FMD was impaired two hours following a high phosphate meal and was associated with elevated serum phosphate levels in young, healthy men (97). While the seminal work by Shuto et al. (97) highlights a potentially large consequence of the high phosphate Western diet on vascular health (51, 106), it should be noted that these authors did not measure VSMC function or the shear stress stimulus during reactive hyperemia. Therefore, the observed impairments in the calculated FMD response are not necessarily a result of reduced endothelial cell function, but may be attributed to impairments in conduit artery VSMC function or a decrease in the blood flow-associated shear stress stimulus during post-phosphate supplement FMD assessments. Because the VSMC controls vascular tone and the dilatory response to cellular signals (ie. NO), elucidating the impact of phosphate on both the conduit artery VSMC function, and the endothelium is critical for a complete understanding of the interaction between phosphate and vascular function.
The purpose of the investigation described in chapter 3 of this thesis was to determine the impact of acute phosphate supplementation on both endothelium-dependent and -independent dilatory responses in the brachial artery. To achieve this, each subject participated in a series of vascular function tests under two experimental conditions (phosphate supplement and placebo) performed on separate days in counterbalanced order.

1.1 Specific Objectives
1) To determine the acute impact of ingestion of a phosphate supplement on brachial artery FMD (endothelial dependent dilation) and GTN mediated vasodilation (endothelial independent dilation).
2) To identify the impact of phosphate supplementation on reactive hyperemia stimulus magnitude, and to use this when interpreting the impact of the phosphate supplement on the FMD response.
3) To examine the relationship between serum phosphate concentration and changes in vascular function.

1.2 Hypotheses
It was hypothesized that:
1) Endothelium-dependent vasodilation (FMD) but not VSMC function would be impaired following the acute phosphate supplement.
2) The reactive hyperemia stimulus would not be significantly affected by acute phosphate supplementation.
3) The time course of FMD impairment would correspond to the time course of increasing serum phosphate concentration, demonstrating a concentration-dependent impairment of FMD.
Chapter 2

Literature Review

2.1 The endothelium, shear stress and the resultant vasodilatory response

Endothelial cells form the inner surface of the human vascular system and establish the functionally and metabolically active endothelium. Direct contact between the blood and the endothelial cells enables the endothelium to be a major regulator of platelet activation, inflammation, angiogenesis and vascular structure and function (38, 56, 69). Specifically, hemodynamic forces exerted directly on the endothelial cells are a primary contributor to the regulation of vascular tone (8, 66). Shear stress, the frictional force created by blood flow, activates a cellular response mediated by various components of the endothelial cell’s glycocalyx, cell-surface proteins, ion channels and cytoskeleton (101). The transmission of shear stress into a cellular response is termed mechanotransduction and facilitates vasoregulation via the synthesis and release of both vasodilatory and vasoconstrictor substances. A multitude of vasoactive substances are produced and the net balance of vasodilatory and vasoconstriction signals determines the overall vascular tone. The primary vasoactive substances released by endothelial cells in response to shear stress are nitric oxide (NO; 30, 39, 62), prostacyclin (PGI₂; 53), endothelial-derived hyperpolarizing factor (EDHF; 24) and endothelin-1 (ET-1; 44).

2.2 Measuring endothelial function using flow-mediated dilation

In 1992, Celermajer and colleagues published a seminal paper highlighting a newly developed and non-invasive technique for examining endothelial function via flow-mediated
dilation (FMD). Using high resolution ultrasound techniques, the diameter of a peripheral conduit artery can be measured before, during and after ischemia-inducing circulatory occlusion of the downstream vasculature (ie. forearm or calf) via pneumatic cuff inflation (16). Release of the pneumatic cuff re-establishes blood flow to the ischemic tissues and generates a large increase in blood flow-associated shear stress in the feeding conduit artery (reactive hyperemia). This shear stress stimulus causes endothelium-dependent dilation (FMD) (12, 50, 66). The equation used to estimate the shear stress stimulus triggering an FMD response within a conduit artery is: shear stress=blood viscosity x blood velocity/arterial diameter (81).

The relationship between peripheral artery FMD and vascular health was first established by Celermajer and colleagues by demonstrating an impairment in FMD in individuals with risk factors for CVD, such as hypercholesterolemia and smoking (15, 16). Additional studies have identified a correlation between brachial artery FMD and coronary artery endothelial function (3, 100). Anderson and colleagues were the first to show a weak, albeit significant correlation (r=0.36, p=0.01) between brachial artery FMD and coronary response to acetylcholine, an endothelium dependent vasodilator (3). A stronger relationship between brachial and coronary artery endothelial function (r=0.78, p<0.001) was found when FMD was used as the index of endothelial function in both arteries (100). As a result, brachial artery FMD is interpreted as a surrogate measure of endothelial function for the clinically relevant coronary circulation (Figure 1).
Figure 1: Summary figure of the relationship between brachial artery FMD, endothelial function and cardiovascular disease.

2.3 Nitric oxide and flow-mediated dilation

Initially, the brachial artery FMD response to reactive hyperemia stimulated by the release of vascular occlusion distal to the site of ultrasound measurement was found to be NO-dependent (50, 71). This was identified by observations that, in comparison to saline infusion, the infusion of L-NMMA, an antagonist of eNOS, did not affect peak blood flow after occlusion release, but abolished any FMD response to increased shear stress in the radial artery (50, 71). However, there is now emerging evidence to suggest that NO is not obligatory for FMD even when ‘standard’ (5 minute vascular occlusion with cuff below ultrasound probe) protocols are followed (84, 111). The current evidence therefore does not support that FMD in response to any singular shear stress profile is mediated by one endothelial-derived vasodilator and FMD is unlikely to be a reliable index of NO bioavailability. NO bioavailability remains an important component of vascular function and health, however it is likely that the physiologically-relevant ability to dilate in response to an increase in shear stress (via the effect of any vasodilatory molecule) contributes to the capability of FMD to predict future cardiovascular events (32, 47, 52). In support of this, a recent meta-analysis identified that both FMD elicited by a standard reactive hyperemia protocol and FMD elicited with proximal cuff occlusion placement (upstream of ultrasound measurement site, and substantially NO independent) were predictive of cardiovascular events. In summary, while the vasodilatory mechanisms remain an area of active study, there is little doubt that FMD provides valuable information regarding vascular health and is an important non-invasive tool for assessing endothelial function in humans.
2.4 The shear stress stimulus and FMD response magnitude

When measuring FMD using a reactive hyperemia stimulus, the magnitude of the FMD response is a composite of the magnitude of the shear stress stimulus applied to the endothelial cell surface, the transduction of the shear stimulus to synthesize or release vasoactive substances, and the ability of the vasoactive substances to modify VSMC tone (4, 55). However, the shear stress stimulus following the release of forearm occlusion cannot be tightly controlled and variable hyperemic shear stimuli may be a source of between-subject or pre- to post-intervention variability when measuring FMD (103). Potential differences in the imposed shear stress stimulus can make interpretation of changes in FMD (between-subject or pre- to post-intervention) challenging. It is important to consider whether an observed reduction in FMD magnitude from pre- to post-intervention is a product of endothelial dysfunction or a reduction in the shear stress stimulus applied to the endothelium. Consequently, investigators have implemented a method of normalizing the magnitude of the FMD response to the shear stress stimulus (normalized FMD= %FMD/shear stress; Pyke & Tschakovsky 2007; Padilla et al. 2009). Normalization has not been adopted by all investigators because of potential statistical limitations pertaining to the degree of linearity in the relationship between shear stress and FMD (5). Nonetheless, examining the shear stress stimulus, the magnitude of the FMD response and the relationship between the shear stress and vasodilatory response is important for a comprehensive interpretation of endothelial function.
2.5 Endothelium-independent vasodilation and FMD

In the seminal paper by Celermajer and colleagues (1992), the authors recognized the importance of assessing the sensitivity of the VSMCs when measuring FMD because the effector component of the dilatory response is predicated on healthy VSMC function. A measurable reduction in FMD may not represent endothelial dysfunction if the VSMC is dysfunctional, and does not relax appropriately in response to the vasodilators released from the endothelium. Therefore, it is crucial to measure both endothelium-dependent and -independent vasodilation in order to attribute a decrease in FMD magnitude to endothelial dysfunction.

Assessing endothelium-independent vasodilation is performed by continuously measuring arterial diameter using high-resolution ultrasound techniques before, during and after the administration of glyceryl trinitrate (GTN; also nitroglycerin or NTG). GTN is a nitrate compound that must be bioactivated, via mitochondrial aldehyde dehydrogenase, to produce an exogenous source of NO (18). This exogenous source of NO can enter VSMCs to induce vasodilation through the same cGMP-mediated pathway as endothelial-derived NO (65). While exogenously administered NO is a very useful tool to assess the VSMC sensitivity to NO, it does not provide a comprehensive assessment of VSMC function. It is possible that the VSMC could exhibit impaired responses to NO, while retaining sensitivity to other vasodilatory substances or vice versa. With that potential limitation acknowledged, GTN administration remains the standard test of VSMC function performed in conjunction with FMD (20).

The time course of peak dilation following GTN-mediated vasodilation has been a point of contention within the literature. A report from the International Brachial Artery Reactivity Task Force highlights that peak dilation occurs 3 to 4 minutes following GTN administration.
(20), however it has been shown that continuous measurement of arterial diameter should be performed for at least 5 minutes in order to capture the peak brachial artery diameter (11, 59, 102).

Endothelium-independent vasodilation has been shown to be impaired in individuals with CVD risk factors (26, 44, 72). Patients with primary hyperparathyroidism (abnormally high calcium levels) are at risk for myocardial ischemia and were reported to have significantly lower endothelium-independent vasodilation than controls (72). However, studies investigating the acute impact of CVD risk factors on vascular function do not frequently observe impaired VSMC function (2, 39), therefore it was hypothesized that endothelium-independent vasodilation would not be impaired by phosphate supplementation. VSMC dysfunction can confound the interpretation of FMD responses and may present difficulties in assessing endothelial function. One approach to account for possible differences in VSMC function is to assess endothelial function in the context of VSMC sensitivity to NO by using the ratio of FMD to GTN (9). In this manner, investigators may quantify observed changes in FMD with respect to the effector component of the FMD response (VSMC) and may allow for a broader assessment of the vascular environment before interpreting an impaired FMD response as solely endothelial dysfunction.

2.6 Phosphate

Phosphate (PO₄) is a highly regulated anion that is essential for nucleic acid structure, energy systems, mineral metabolism, protein phosphorylation and the skeletal system. Traditional Western diets contain an abundance of phosphate, which is absorbed from the
intestinal tract into the blood via sodium-phosphate type IIb transporters (109). Sequestration of phosphate into intracellular stores, mineralization within the skeletal system and circulation in the blood constitute the major phosphate pools in the human body, and are mediated by sodium-phosphate type III transporters (41, 109). The primary mechanisms maintaining appropriate phosphate concentrations are intestinal absorption, bone formation or resorption, and renal excretion (6). Signaling pathways linking the kidney-intestine-bone axis enable renal excretion to match intestinal uptake in order to manage a wide range of dietary phosphate intake (7). In turn, postprandial increases in serum phosphate may be returned towards resting levels by inhibition of renal phosphate reabsorption in the proximal tubule, which can respond to homeostatic disturbances within one hour (60, 74). Inappropriate phosphate handling, or prolonged exposure to excessive circulating phosphate, termed hyperphosphatemia, can be detrimental to cardiovascular function and health, and is exemplified by CKD (1, 48).

2.7 The link between phosphate, CKD and CVD

CKD is renal disease characterized by a loss of functional nephrons and progressively worsening uremia, including electrolyte imbalances leading to the accumulation of various serum electrolytes, such as phosphate. CVD is frequently associated with CKD and individuals with CKD are more likely to die of CVD than to develop fatal kidney failure (96). A significant graded relationship exists between CKD severity and the rates of all-cause mortality, adverse cardiovascular outcomes and hospitalization (43). Furthermore, the traditional risk factors for CVD in the general population with normal renal function do not explain the increased CVD risk in CKD (43, 91). Thus, alternative non-traditional risk factors, such as phosphate, have been
investigated to elucidate the relationship between CKD and CVD. Changes in hormonal signaling through the kidney-intestine-bone axis via parathyroid hormone, vitamin D and fibroblast growth factor 23 can initially upregulate renal excretion of phosphate, however once kidney function declines below a glomerular filtration rate of ~30 ml/min/1.73m² then the kidney can no longer maintain normophosphatemia (22). Consequently, overt hyperphosphatemia develops and an attempt to re-establish phosphate homeostasis results in the pathological deposition of phosphate into soft tissues (19, 61). The major soft tissue reservoir of concern is the vasculature, which is susceptible to the formation of vascular calcifications (VC) (93). The relationship between hyperphosphatemia, VC and the progression of CVD is frequently reported in the CKD literature (91, 93). VCs are ectopic mineral complexes of calcium and phosphate, called hydroxyapatite, within the medial smooth muscle layer of blood vessel walls (40). VC alter the structural properties of the blood vessel by increasing arterial wall stiffness leading to increased pulse pressure, systolic blood pressure and maladaptive left ventricular hypertrophy (61, 73). The combination of these cardiovascular pathologies may play an important role in the heightened CVD morbidity and mortality in CKD (34, 43).

2.8 Phosphate as a non-traditional risk factor for CVD

CKD generates a dysfunctional serum electrolyte balance that promotes VCs and adverse cardiovascular events, however these findings cannot explain the recently reported epidemiological findings that link elevated serum phosphate levels, within clinically-normal range (2.5-4.5 mg/dl), to CVD risk in individuals with normal renal function (25, 77, 104). In a 5-year follow-up study of 4127 individuals with normal renal function and prior myocardial
infarction, a 1 mg/dl increase in serum phosphate was associated with a 27% increase in all-cause mortality (104). When categorized into quartiles of resting phosphate levels and adjustment for age, sex and race, an independent graded relationship between serum phosphate and mortality was observed (104). Individuals in the highest serum phosphate quartile (> 4mg/dl) had 42% increased all-cause mortality risk compared to the referent second quartile (2.5-3.4 mg/dl). The third quartile, with serum phosphate levels in the normal-to-high clinical range (3.5-3.9 mg/dl), also had a 25% increased risk of mortality. Similar graded relationships were observed between serum phosphate and fatal and non-fatal myocardial infarctions (MI), coronary death or non-fatal MI and development of congestive heart failure (104). Additional investigations into the association between serum phosphate and the development of CVD (CVD morbidity and mortality) in individuals without identifiable CVD or CKD in the general population further confirmed this graded relationship (25, 77). Despite an aggregate of observational studies indicating higher serum phosphate levels, even within clinically acceptable ranges, increase cardiovascular morbidity and mortality independent of renal function, the underlying mechanisms by which serum phosphate may enhance CVD risk are largely unknown. The mechanism of CVD development in CKD compared to the general population may be distinct. While hyperphosphatemia has been associated with VC in CKD (1, 40), the mechanisms of CVD development in the general population are more elusive and are most likely a product of repeated phosphate-induced attenuation of endothelial function, leading to transient reductions in vasoprotection. Repetitive cycles of attenuated endothelial function and a loss of vasoprotection from phosphate exposure likely lead to chronic endothelial dysfunction and atherosclerotic plaque formation (24, 97).
2.9 Phosphate and the endothelium

Studies indicate a mechanistic link between elevated serum phosphate and ectopic calcification of cardiovascular tissues (45, 87), however these hazardous pathologies may not be present, and therefore would not account for enhanced CVD risk independent of a uremic environment. In a population-based study from the Atherosclerosis Risk in Communities study, hyperphosphatemia was associated with increased coronary intima-media thickness, a measure of subclinical atherosclerosis, in healthy men, but not women (77). While no conclusive mechanism can be determined from the prior study, the findings suggest that early modifications to the structure and functionality of the vasculature may be an initiating step in hyperphosphatemia-related CVD risk. Recent research has provided some intriguing in vitro and in vivo evidence to suggest the involvement of endothelial dysfunction connecting phosphate to CVD (63, 64, 97).

In a series of highly technical and well-designed studies, Di Marco and colleagues investigated the impact of exposing human umbilical vein endothelial cells (HUVEC) to hyperphosphatemic conditions on endothelial cell apoptosis. Exposure of HUVECs to 2.5 mM (~7 mg/dl) phosphate-containing media produced a 40% increase in apoptosis compared to controls as measured by phosphatidylserine externalization, and was confirmed by direct observation under transmission electron microscopy (63). The purported mechanism for apoptosis was that it occurred via a reactive oxygen species (ROS) mediated disruption of mitochondrial membrane potential. Inhibition of caspase, a primary component of the apoptotic pathways, before phosphate exposure prevented endothelial cell apoptosis, but did not affect
generation of ROS or mitochondrial membrane potential disruption. Further, all of the apoptotic signaling events could be prevented by inhibiting the phosphate transport into the cell, suggesting that elevated concentrations of intracellular phosphate is a requirement for endothelial cell apoptosis under hyperphosphatemic conditions (63).

Shuto et al. (2009) investigated whether dietary phosphate exposure could be implicated in increased risk for CVD and performed a novel set of experiments to identify potential pathophysiological mechanisms associated with short-term, post-prandial increases in phosphate. Eleven healthy male subjects (age: 24, BMI: 20.9kg/m$^2$) were given meals containing 400 or 1200 mg of phosphate (same 400 mg base meal, but 1200 mg meal was supplemented with 800 mg phosphate taken as an oral liquid solution) in a double-blind crossover design and assessed serum phosphate and FMD of the brachial artery before and two hours following meal ingestion. The meal containing 1200 mg of phosphate increased serum phosphate levels in all subjects at two hours post-ingestion and significantly decreased FMD (pre: 9.26% vs. post: 5.02%). The 400 mg meal did not increase serum phosphate levels two hours post-ingestion and there were no changes in FMD. This was the first in vivo evidence identifying that a large acute phosphate intake can impair FMD, and that post-prandial serum phosphate levels may be implicated as a risk factor for CVD through impairments in endothelial function. This study was a small sample of young, healthy males, and due to some methodological limitations, the interpretation of impaired FMD following phosphate consumption requires further validation. VSMC function was not independently assessed; therefore it is important to determine if impairment of VSMC function contributes to the observed reduction in FMD. Also, the shear stress stimulus responsible for eliciting the FMD response was not quantified and needs to be established by
high-resolution ultrasound capable of simultaneously measuring brachial artery diameter and blood velocity (for determination of the shear stimulus). It is crucial that the shear stress stimulus is consistent between pre- and post-intervention assessments in order to clearly interpret any phosphate-related reductions in FMD as indication of endothelial dysfunction.

Shuto et al. (2009) strengthened the in vivo results of their study with well-controlled in vitro mechanistic experiments in order to elucidate potential mechanisms for the attenuated FMD response (97). By exposing bovine aortic endothelial cells (BAEC) to high phosphate loads, these investigators were able to identify that phosphate uptake by BAECs was dependent on the sodium-phosphate co-transporter and elevated intracellular phosphate increased protein kinase C (PKC) activity. Increased PKC activity contributed to an increase in NADPH oxidase activity and phosphorylation of an inhibitory threonine residue of endothelial nitric oxide synthase (eNOS) resulting in increased ROS and decreased NO production, respectively (Figure 2). Release of NO from the endothelium in response to an increase in blood flow-associated shear stress is an important process for vasoregulation and vascular health (35, 50, 80). A reduction in NO bioavailability, either through increased ROS converting NO to a vaso-inactive radical or direct inhibition of the enzyme responsible for NO production could significantly impair the ability of NO to protect the vascular system from harmful substances or conditions. The proposed mechanism that phosphate uptake into endothelial cells reduces NO bioavailability and generates endothelial dysfunction was further substantiated by an observed reduction in vasodilatory responses to graded doses of acetylcholine applied to rat aortic rings pretreated in hyperphosphatemic conditions (2.5mM phosphate; 77). FMD stimulated by a standard reactive hyperemia test may not be wholly NO-dependent, therefore a decrease in FMD within an acute
hyperphosphatemic environment may be due to a reduction in NO bioavailability, and/or an impact of phosphate on other vasodilatory pathways (eg. PGI₂ or EDHF).

Di Marco and colleagues (2013) have provided additional evidence for the role of hyperphosphatemia in the pathogenesis of CVD. Endothelial cell stiffness (a biomarker of both the structural and functional integrity of endothelial cells) and release of endothelial microparticles are both emerging markers of endothelial dysfunction, atherogenesis and impaired angiogenesis (13, 64). Endothelial stiffness and release of microparticles were significantly increased after exposing human coronary artery endothelial cells to 2.5 mM phosphate for 24 hours (64). Cumulatively, these findings provide substantial support for the role of hyperphosphatemia in creating a dysfunctional endothelium.
Figure 2: The purported mechanism for phosphate-induced endothelial dysfunction
2.10 Summary

Phosphate is an important cellular molecule for protein phosphorylation, energy systems and nucleic acids. It is also an increasing component of the Western diet. While adequate dietary intake of phosphate is required for proper cellular function, elevated serum phosphate can have adverse cardiovascular effects for not only individuals at risk for hyperphosphatemia (ie. CKD), but also for apparently healthy individuals. The mechanisms by which serum phosphate may increase cardiovascular risk in the general population are still being discovered, however phosphate-induced endothelial dysfunction may be a key contributor. Specific focus should be given to quantifying the reactive hyperemia shear stress stimulus for FMD tests before and after phosphate ingestion. Also, endothelium-independent vasodilatory function assessments before and after phosphate ingestion should be used to identify whether phosphate affects VSMC function or sensitivity to NO. These considerations would enable a more confident interpretation regarding the impact of phosphate on endothelial function.

The following chapter describes the study conducted for this thesis. It was designed to address the limitations of previous work investigating the impact of acute ingestion of phosphate on endothelial function by including assessments of the impact of acute phosphate supplementation on VSMC function and the shear stress stimulus for reactive hyperemia FMD.
Chapter 3

The role of acute phosphate supplementation in endothelium-dependent and -independent vasodilation
Abstract

Elevated serum phosphate levels are associated with cardiovascular morbidity and mortality in the general population. The mechanisms through which phosphate may be detrimental to cardiovascular health remain unclear, however there is emerging evidence that suggests endothelial dysfunction may be an early step in the onset of phosphate related cardiovascular disease. The purpose of this study was to investigate the effects of acute phosphate supplementation on endothelial-dependent (FMD) and -independent (GTN) vasodilation in young, healthy males. 17 healthy male participants (22.9 ±3.07 years old) were exposed to phosphate (PHOS) and placebo (PLAC) conditions over two experimental days (one each visit) in a within-subjects, randomized, double-blinded, placebo-controlled trial. A liquid supplement (Phoslax Oral Solution) containing 1200 mg of phosphorus or a placebo was consumed orally. FMD and GTN tests were performed pre- and 20 min, 60 min and 120 min post-supplement or placebo ingestion using a standard brachial artery reactive hyperemia flow-mediated dilation test (FMD). Serum phosphate concentrations were significantly greater in PHOS vs. PLAC (p=0.03), but only increased modestly following supplement ingestion. Serum phosphate concentrations did not change over time in the PHOS or PLAC condition (p=0.037). Phosphate excretion increased significantly in the PHOS vs. PLAC condition, indicating that phosphate was rapidly removed from the serum via the kidney (PHOS: 315% vs. PLAC: 49%, p<0.05). FMD in the PHOS condition was not significantly different from the PLAC condition at any post-supplement time point (PHOS vs. PLAC, pre-supplement: 7.4±4.3% vs. 6.4±3.0%, 20 min post: 3.7±2.8% vs. 4.4±1.9%, 60 min post: 5.3±3.0% vs. 5.4±2.4%, 120 min post: 6.7±3.1%
vs. 6.7 ± 2.8%; main effect of condition p=0.905, condition*time p=0.411). There was no effect of PHOS on GTN-mediated vasodilation (120 min post-supplement PHOS: 20.1 ± 5.0% vs. PLAC: 22.4 ± 5.9%, condition p=0.132, condition*time p=0.284). These findings indicate that acute phosphate supplementation did not impair endothelium-dependent or -independent vasodilation, and only modest increases serum phosphate concentrations were observed in this group of young healthy males. Further research is warranted to better understand the relationship between dietary phosphate and endothelial function.
3.1 Introduction

Large-scale studies have established that elevated serum phosphate levels are associated with increased atherosclerosis and cardiovascular mortality, even in the absence of clinically significant hyperphosphatemia (25, 77, 104). The mechanisms linking serum phosphate to the development of atherosclerosis have focused on endothelial dysfunction, but are still unclear.

Endothelial dysfunction is described as the pathological shift away from the pro-vasodilatory, anti-coagulative, and anti-inflammatory endothelial phenotype that exerts a vasoprotective role in the circulation. Recently investigators have been assessing the endothelium as a key player in the pathological connection between phosphate and CVD (63, 64, 97). It has been demonstrated in vitro that hyperphosphatemic conditions impair microvascular function, angiogenesis, and endothelial nitric oxide production and induce endothelial cell apoptosis via increases in reactive oxygen species (63, 64, 97).

Only one study to date has assessed endothelial function following high phosphate intake in humans. Impaired endothelium-dependent flow-mediated dilation (FMD) was observed following a single high phosphate meal in young, healthy men, and FMD was negatively associated with serum phosphate levels (97). These findings support a role for endothelial dysfunction in dietary phosphate related cardiovascular risk in the general population. However, to be more conclusive, assessments of FMD must be accompanied by measures of endothelium-independent vasodilation and the shear stress stimulus eliciting FMD. Without these measures it is possible that a depressed post-phosphate ingestion FMD could be due to a diminished shear stress stimulus or impaired smooth muscle relaxation rather than a dysfunctional endothelial response.
With this as background, the aim of the present study was to expand on the current understanding of the acute impact of phosphate ingestion on brachial artery endothelial function. This was achieved by assessing 1) brachial artery FMD and the shear stress stimulus, and 2) endothelium independent vasodilation, prior to and following ingestion of a phosphate supplement or placebo.

3.2 Methods

3.2.1 Subjects

Seventeen healthy, recreationally active male volunteers between the ages of 18-30 years old were recruited from the Queen’s University community (Kingston, Ontario). Individual health status was assessed for the presence of risk factors for endothelial dysfunction, cardiovascular and renal disease using a medical screening questionnaire. The Health Science Research Ethics Board at Queen’s University, which operates in accordance with the Declaration of Helsinki, approved the study protocol, medical screening questionnaire and consent form (Appendix 1).

During the initial visit to the laboratory, subjects were introduced to the study protocol and provided written and verbal informed consent prior to participating. Subjects were screened to ensure a clear image of the brachial artery, and strong blood velocity signal could be obtained. 31 individuals were screened initially, and 14 individuals were screened out because an optimal brachial artery image and/or blood velocity signal could not be obtained. Blood pressure was assessed using the average of three measurements in a seated position (BpTRU BPM-100,
BpTRU Medical Devices, Coquitlam, BC). Hypertensive (>140/90 mmHg) and hypotensive (<90/60 mmHg) subjects were excluded.

### 3.2.2 Experimental Design

Subjects completed two experimental visits separated by a period of at least 48 hours and no more than two weeks. The experiment had a within subjects design such that each subject participated in both conditions: phosphate supplement and placebo; one visit each. The condition order was counterbalanced and double-blinded (Figure 3). All experimental visits took place between 1200 and 1800 hours to minimize the potential impact of circadian variation on serum phosphate, renal reabsorption and excretion of phosphate.
Each participant participated in all of the above outlined sessions. The assigned experimental condition (phosphate or control) for each of the two experimental visits was counterbalanced.

Figure 3: Study timeline
Each participant participated in all of the above outlined sessions. The assigned experimental condition (phosphate or control) for each of the two experimental visits was counterbalanced.
Endothelial independent (ingestion of glycercyl trinitrate (GTN 400 μg)) and endothelial dependent (FMD) brachial artery vasodilation was performed pre- and post-ingestion of a phosphate supplement or placebo as shown in Figure 4. Blood and urine samples were collected as shown in Figure 4.
Figure 4: Outline of the protocol for each experimental condition
FMD: flow-mediated dilation test, GTN: glyceryl trinitrate test.
3.2.3 Experimental Procedures

In the 24-hour period prior to the experimental visit, subjects were instructed to avoid exercise, alcohol and caffeine. Subjects were provided with a standardized low-phosphate breakfast to be consumed 6 hours before the experiment, followed by fasting until arrival at the laboratory. The standardized breakfast contained two regular pieces (~60g) of commercial prepared white bread (Dempster’s Canada, Maple Leaf Foods, Montreal, QC; total phosphorus content is ~47 mg) and one ~50g serving of strawberry jam (Smucker’s Pure Strawberry Jam, Smucker Food of Canada, Markham, ON; total phosphorus content is ~10 mg). All experimental visits occurred in a quiet, temperature-controlled room (21±1°C).

3.2.3.1 Subject Monitoring

Heart rate (HR) was measured continuously using three ECG electrodes placed on the upper chest and lower left abdomen. Blood pressure was monitored using an automated blood pressure device (BPM-100, BpTRU Medical Devices, Coquitlam, BC).

3.2.3.2 Brachial Artery Blood Velocity and Diameter Measurements

Brachial blood velocity was determined using Doppler ultrasound operating at 4MHz (Vivid i2 GE Medical Systems). The Doppler shift frequency spectrum was analyzed via 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. The resultant voltage output from the Multigon spectral analyzer was continuously sampled and recorded (PowerLab, AD Instruments) for future analysis.
Brachial artery diameter was obtained using 12MHz B-mode ultrasound (Vivid i2 GE Medical Systems). Ultrasound parameters were set to optimize longitudinal B-mode images of the lumen/arterial wall interface. Data was collected using an insonation angle of 68°, and remained constant between all trials. All ultrasound images were acquired from the ultrasound device using a VGA to USB frame grabber (VGA2USB-LR, Epiphan Systems Inc., Ottawa, ON) and recorded as a video file on an independent computer using commercially available software (Camtasia Studio 7, TechSmith Corporation, Okemos, MI).

3.2.3.3 Phosphate and Placebo Supplements

Depending on the experimental visit, the subject ingested either the phosphate supplement or placebo supplement. According to Figure 4, the phosphate or placebo supplement was ingested after a 10 min rest period (time 0 min) following the baseline assessment of endothelial-dependent (FMD) and -independent (GTN) vasodilation. The phosphate supplement (Phoslax Oral Sodium Phosphates, Odan Laboratories) consisted of a total of 1200 mg of phosphorus (a neutral mixture of 4.48g monobasic sodium phosphate and 1.68g dibasic sodium phosphate, Appendix 2) consumed orally in liquid form. The phosphate and placebo supplements were flavoured with Kraft Food’s Crystal Light to make the placebo supplement indistinguishable from the phosphate supplement based on taste and colour. The placebo supplement was water with Crystal Light dissolved in solution. Subjects were given 125-250 ml of water every hour to maintain hydration throughout the experimental visits.
3.2.3.4 Brachial Artery Reactive Hyperemia FMD

All FMD tests were conducted on the left arm. Following a twenty-minute rest period, brachial artery diameter and blood velocity were recorded for one minute of baseline, the final minute of a 5-minute forearm occlusion (cuff inflation to 250 mmHg) and for three minutes following the release of vascular occlusion. The cuff was placed on the subject’s forearm at the antecubital fossa (distal to the site of brachial artery ultrasound measurement). The subject rested until the next vascular function test (either FMD or GTN, Figure 4).

3.2.3.5 Glyceryl Trinitrate-mediated Vasodilation

After a ten-minute rest period, arterial diameter and blood velocity measurements were recorded for one minute before a single metered 400-μg dose of GTN was administered sublingually. Continuous brachial artery diameter and blood velocity recordings were obtained for ten minutes following dosing to determine the peak change in arterial diameter and provide an index of NO-dependent VSMC function.

3.2.3.6 Blood Sampling

The following technique was utilized for all blood samples, as indicated in Figure 4. A venous blood sample was withdrawn using a closed intravenous single port catheter system (BD Nexiva, 20 GA). A non-latex tourniquet was briefly applied to the upper arm in order to identify a vein for catheterization. Following sample collection, the catheter was flushed with a saline solution (BD Posiflush SP Syringe, 0.9% NaCl, 5 mL). Blood samples were centrifuged at 4°C for 10 minutes at 2500 RPM and the serum was separated, aliquotted and stored at -80°C for
future analyses of phosphate and creatinine. Whole blood viscosity was analyzed at a shear rate of 225 s\(^{-1}\) at 37 ± 2°C (Brookfield Viscometer DV-II+ Pro, Middleboro, MA).

3.2.3.7 Urine Sampling

A mid-stream urine sample was collected from subjects when they arrived at the laboratory, and immediately after the last assessment of GTN-mediated vasodilation. Urine samples were centrifuged at 4°C for 10 minutes at 2500 RPM and the sample was aliquotted and stored at -80°C for future analyses of phosphate and creatinine.

3.2.4 Data Analysis

3.2.4.1 Brachial Artery Blood Velocity

Blood velocity was analyzed offline in 3-second average time bins using data acquisition software (LabChart, AD Instruments, Colorado Springs, CO), as previously described (86).

3.2.4.2 Brachial Artery Diameter

Vessel diameter was measured offline using automated edge-detection and wall-tracking software (Encoder Analysis FMD, Reed Electronics) designed to detect and track the inner walls of the artery within an investigator-defined region of the image, as previously described (86). Brachial artery diameters were inserted into a 3-second average time bin spreadsheet and matched with the corresponding 3-second average blood velocities in order to calculate shear stress.
3.2.4.3 Shear Stress

Brachial artery shear stress was determined using the following formula: \( \tau = 4 \cdot \mu \cdot \frac{v}{d} \);

where \( \tau \) = shear stress, \( \mu \) = blood viscosity, \( v \) = mean blood velocity, \( d \) = brachial artery diameter (42). The shear stress stimulus was quantified as the shear stress area-under-the-curve (AUC) until peak diameter following the release of vascular occlusion.

3.2.4.4 Flow-mediated Dilation

The same investigator analyzed all images, and the image files were blinded to the subject number, experimental condition and temporal order used during data collection. Absolute peak change (absFMD) and peak percent change (FMD) in artery diameter was assessed to characterize the arterial response to increased shear stress. \%FMD was calculated as the percent change in artery diameter from baseline before vascular occlusion to the peak artery diameter following release of vascular occlusion. absFMD was quantified as the difference between peak artery diameter and baseline artery diameter. In six of the 136 total FMD tests, \%FMD was calculated as the percent change in artery diameter from vascular occlusion to the peak artery diameter following release of vascular occlusion. In these six scans, a poor baseline scan was obtained and the occlusion artery diameter was determined to most accurately represent the artery diameter prior to release of vascular occlusion. The following formula is used in calculating \%FMD. The same formula can be used for calculating the change in arterial diameter in response to GTN administration.

\[
%FMD = \frac{Peak \ Diameter - Baseline \ Diameter}{Baseline \ Diameter} \times 100\%
\]
3.2.4.5 Blood and Urine Samples

Frozen serum samples were outsourced to the Adams-Holden Research Group (Queen's University, Kingston Ontario) for analysis of phosphate and creatinine. Serum and urine phosphate concentrations were analyzed using a standard laboratory malachite green phosphate colorimetric assay. This assay has been tested against common laboratory contaminants to ensure accuracy. Serum and urine creatinine concentrations were analyzed using an enzyme-linked immunosorbent assay (ELISA, QuantiChrom Creatinine Assay Kit, Bioassay Systems, Hayward, CA). Cholesterol, triglycerides and glucose were analyzed in-house using a Cholestech LDX system (Cholestech) from whole blood. Blood samples were analyzed for blood viscosity using a viscometer (Brookfield Viscometer DV-II+ Pro) in order to calculate the shear stress. Fractional excretion of phosphate (FEP) was calculated as the ratio of the urinary phosphate excretion rate to the renal filtration rate of phosphate according to the following formula (21):

\[
FEP = \frac{\text{urinary phosphate} \times \text{serum creatinine}}{\text{serum phosphate} \times \text{urinary creatinine}} \times 100\% 
\]

FEP was calculated in 9 subjects because complete blood and urine samples were only obtained successfully in those subjects.

3.2.5 Statistical Analysis

All statistical analyses were performed using Sigmaplot 11 computer software. All values are expressed, as the mean ± SD. Statistical significance was set at p < 0.05. The variables FMD, GTN mediated vasodilation, shear stress AUC, and serum phosphate levels were compared using
a two-way, repeated measures ANOVA. The factors were condition (phosphate (PHOS) vs. placebo (PLAC)) and time (pre-supplement, and 20, 60 and 120 min post-supplement).

Anthropometric data, and percent change in FEP were compared between conditions using a paired t-test.

3.3 Results

3.3.1 Subject Characteristics

Subjects were 23±4 years old with a BMI of 23.4±2.7. Subject characteristics did not differ between the visits to complete the PHOS and PLAC conditions (p>0.05) (Table 1).
Table 1: Anthropometric Data

All data are expressed as the mean ± SD (n=17). PHOS: phosphate condition (liquid supplement containing 1200 mg liquid phosphorus), PLAC: placebo condition (water), BMI: body mass index, HDL: high-density lipoprotein, LDL: low-density lipoprotein.

<table>
<thead>
<tr>
<th></th>
<th>PHOS</th>
<th>PLAC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy expenditure (kcal/kg/week)</td>
<td>235.1±11.8</td>
<td>237.8±14.7</td>
<td>0.301</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>152.8±37.7</td>
<td>146.1±30.7</td>
<td>0.063</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>86.8±62.9</td>
<td>73.0±32.0</td>
<td>0.292</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>46.2±14.4</td>
<td>43.9±10.8</td>
<td>0.205</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>92.6±23.7</td>
<td>90.9±25.8</td>
<td>0.510</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>82.9±10.4</td>
<td>85.1±5.5</td>
<td>0.402</td>
</tr>
</tbody>
</table>
3.3.2 Mean Arterial Pressure (MAP)

MAP (Figure 5) was not significantly different between the PHOS and PLAC conditions (p=0.548), however it increased significantly over time (p<0.001). Post hoc analysis revealed that MAP at 120 min post-supplement was significantly greater than pre-supplement, 20 min post-, and 60 min post-supplement MAP (p<0.001), and MAP at 60 min post-supplement was significantly greater than pre-supplement (p<0.001) and 20 min post-supplement (p=0.004) MAP. There was no interaction between time and condition for MAP (p=0.475).
Figure 5: Mean Arterial Pressure
MAP recorded before each FMD trial in both the PHOS and PLAC conditions (n=17). Times indicate the number of minutes post-supplement ingestion. MAP increased significantly over the duration of the experiment, but was not significantly different between conditions. PHOS: phosphate condition, PLAC: placebo condition. * p<0.001 vs. pre-supplement, # p<0.001 vs. 20 min, & p<0.001 vs. 60 min, $ p<0.05 vs. 20 min. All data are mean ± SD.
3.3.3 Serum Phosphate Concentration

Serum phosphate concentrations (Figure 6) were significantly greater in the PHOS compared to the PLAC condition (p=0.030). There was also a significant main effect of time (p=0.037) on serum phosphate levels, however there was no significant interaction between time and condition (p=0.780). Post hoc analysis regarding the effect of time identified that serum phosphate concentration at 60 min post-supplement was greater than at 20 min post-supplement with borderline significance (p=0.050).
Figure 6: Serum Phosphate Concentration
Serum phosphate concentration measured before each FMD trial in both the PHOS and PLAC conditions (n=10). Times indicate the number of minutes post-supplement ingestion. PHOS: phosphate condition, PLAC: placebo condition. * p<0.05 vs. PLAC condition, # p=0.050 vs. 20 min. All data are mean ± SD.
3.3.4 Fractional Excretion of Phosphate (FEP)

A significant interaction between time and condition was detected for FEP (p=0.016; Figure 7). Post hoc analysis identified that FEP was not significantly different between the PHOS and PLAC conditions at either the pre-supplement (p=0.093) or 120 min post-supplement (p=0.078) time points. However in the PHOS condition, 120 min post-supplement FEP was significantly greater than pre-supplement FEP (p<0.001), while no significant difference was detected pre- to post-supplement in the PLAC condition (p=0.130). In agreement with this, percent change in FEP from pre- to post-supplement was significantly greater in the PHOS compared to the PLAC condition (%ΔFEP in PHOS: 314.6% vs. PLAC: 49.2%, p=0.016).
Fractional excretion of phosphate (FEP) is presented as the ratio of the urinary phosphate excretion rate to the renal filtration rate of phosphate (n=9). FEP increased 315% from pre- to post-supplement in the PHOS condition compared to 49% in the PLAC condition (p<0.05).

PHOS: phosphate condition, PLAC: placebo condition. * p<0.001 vs. PHOS pre-supplement. All data are mean ± SD.

Figure 7: Fractional Excretion of Phosphate

Fractional excretion rate to the renal filtration rate of phosphate (n=9). FEP increased 315% from pre- to post-supplement in the PHOS condition compared to 49% in the PLAC condition (p<0.05).

PHOS: phosphate condition, PLAC: placebo condition. * p<0.001 vs. PHOS pre-supplement. All data are mean ± SD.
3.3.5 *Brachial Artery Baseline Diameter*

There was no main effect of condition, or interaction between condition and time on brachial artery baseline diameter (p=0.390 and p=0.084, respectively; Figure 8). A main effect of time was detected (p<0.001) and post hoc analysis revealed that brachial artery baseline diameter was significantly greater at 20 min post-supplement compared to the pre-supplement, 60 and 120 min post-supplement (p<0.001). Baseline diameter at 60 min post-supplement was also significantly greater than pre-supplement (p=0.016).
Figure 8: Brachial Artery Baseline Diameter
Brachial artery baseline diameter at the beginning of each FMD trial. Times indicate the number of minutes post-supplement ingestion. PHOS: phosphate condition, PLAC: placebo condition. * p<0.05 vs. pre-supplement, # p<0.001 vs. 60 min post-supplement, & p<0.001 vs. 120 min post-supplement. All data are mean ± SD.
3.3.6 Endothelium-independent Vasodilation (GTN)

There was no main effect of time (p=0.879), or condition (p=0.132), and there was no significant interaction between time and condition for endothelium-independent vasodilation (p=0.284; Figure 9).
Figure 9: Endothelium-independent Vasodilation
%GTN measured pre- and post-supplement. Phosphate supplementation did not affect VSMC sensitivity to endogenously administered NO. PHOS: phosphate condition (1200 mg liquid oral phosphorus), PLAC: placebo condition (water). All data are mean ± SD.
3.3.7 Endothelium-dependent Vasodilation (FMD)

No difference between conditions, or interaction between condition and time was found for %FMD (p=0.905 and p=0.411, respectively; Figure 10). A main effect of time was detected (p<0.001), and post hoc analysis revealed that the 20 min post-supplement %FMD was significantly less than the pre-supplement and 120 min post-supplement %FMD (p<0.001). No significant effect of time (p=0.119) or interaction between time and condition (p=0.639) was detected for the time from cuff release to peak diameter (Table 2). A main effect of condition was found (p=0.030), such that the time from cuff release to peak diameter in the PHOS condition was significantly greater than the PLAC condition. absFMD results reflect the same findings as the %FMD (Table 2 presents absFMD and peak diameter results).

%FMD was not significantly correlated to serum phosphate concentrations in either the PHOS or PLAC conditions (r²=0.04, p=0.228 and r²=0.01, p=0.508, respectively). An exploratory analysis comparing subjects with the highest vs. lowest post-supplement change in FEP (responders vs. non-responders) did not reveal any significant differences in FMD responses.
Table 2: Brachial Artery FMD Characteristics
All brachial artery FMD variables were measured on the full data set (n=17). Times indicate the number of minutes post-supplement ingestion. All data are mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>Pre-supplement</th>
<th>20 min</th>
<th>60 min</th>
<th>120 min</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peak diameter (mm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Time: &lt;0.001</td>
</tr>
<tr>
<td>PHOS</td>
<td>3.94 ± 0.29</td>
<td>4.13 ± 0.36</td>
<td>3.98 ± 0.34</td>
<td>3.89 ± 0.30</td>
<td>Condition: 0.723</td>
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<tr>
<td>PLAC</td>
<td>3.90 ± 0.26</td>
<td>4.16 ± 0.29</td>
<td>3.98 ± 0.31</td>
<td>3.85 ± 0.33</td>
<td>Time*Condition: 0.496</td>
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<tr>
<td><strong>absFMD (mm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Time: &lt;0.001</td>
</tr>
<tr>
<td>PHOS</td>
<td>0.266 ± 0.148</td>
<td>0.140 ± 0.096</td>
<td>0.192 ± 0.099</td>
<td>0.240 ± 0.010</td>
<td>Condition: 0.952</td>
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<tr>
<td>PLAC</td>
<td>0.228 ± 0.100</td>
<td>0.171 ± 0.070</td>
<td>0.198 ± 0.084</td>
<td>0.237 ± 0.093</td>
<td>Time*Condition: 0.326</td>
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<tr>
<td><strong>Time to peak dilation (s)</strong></td>
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<td></td>
<td></td>
<td></td>
<td>Time: 0.119</td>
</tr>
<tr>
<td>PHOS</td>
<td>51.9 ± 21.3</td>
<td>42.4 ± 19.3</td>
<td>45.7 ± 19.3</td>
<td>43.4 ± 26.2</td>
<td>Condition: 0.030</td>
</tr>
<tr>
<td>PLAC</td>
<td>40.9 ± 14.5</td>
<td>35.6 ± 10.7</td>
<td>34.8 ± 8.5</td>
<td>38.8 ± 8.9</td>
<td>Time*Condition: 0.639</td>
</tr>
</tbody>
</table>
Figure 10: Flow-mediated Dilation
Percent change in brachial artery diameter (%FMD). Times indicate the number of minutes post-supplement ingestion. PHOS: phosphate condition, PLAC: placebo condition. * p<0.001 vs. pre-supplement, # p<0.001 vs. 120 min post-supplement. All data are mean ± SD.
3.3.8 Reactive Hyperemia Shear Stress Stimulus (SS-AUC)

There was no main effect of condition (p=0.064), or interaction between time and condition (p=0.444) for SS-AUC until peak dilation (Figure 11). There was a main effect of time (p<0.001) and post hoc analysis revealed that pre-supplement SS-AUC was significantly greater than the 20 min (p<0.001), 60 min (p<0.001) and 120 min (p=0.008) post-supplement.
Figure 11: Reactive Hyperemia Shear Stress Stimulus
The shear stress stimulus elicited by reactive hyperemia of the forearm, and measured as SS-AUC until peak dilation. Times indicate the number of minutes post-supplement ingestion.
PHOS: phosphate condition, PLAC: placebo condition. * p<0.001 vs. 20 min post-supplement, # p<0.001 vs. 60 min post-supplement, & p<0.05 vs. 120 min post-supplement. All data are mean ± SD.
**3.4 Discussion**

The purpose of this study was to determine the acute impact of a single phosphate supplement on brachial artery endothelium-independent vasodilation, the shear stress stimulus elicited with a standard reactive hyperemia protocol, and the resulting FMD response. The main findings from the study were: 1) the phosphate supplement did not affect VSMC responsiveness to endogenously administered NO, the reactive hyperemia shear stress stimulus, or the FMD response, and 2) serum phosphate concentrations were modestly elevated at 60 min post-supplement in both conditions. It is possible that the vasculature was not sufficiently exposed to phosphate concentrations that could induce detectable impairments in vascular function because of a marked increase in renal phosphate excretion within the phosphate condition.

**3.4.1 The Impact of Acute Phosphorus Intake on FMD and GTN-mediated Vasodilation**

This study was designed to confirm and extend the findings of the only previous investigation of acute phosphate loading on human endothelial function (97). The primary findings from the present study, that neither FMD nor GTN mediated dilation differed between the PHOS and PLAC conditions (Figure 9 & 10), is in contrast to Shuto et al. (97), who found impaired brachial artery FMD two hours after phosphate loading (Pre vs. postprandial: 9.3% vs. 5.0%).

When bovine aortic endothelial cells were incubated in a high phosphate media *in vitro* Shuto et al. (97) observed a decrease in NO production via inhibitory phosphorylation of eNOS (97). Therefore, it was posited that impairment in the NO pathway was the primary mechanism by which phosphate induces a deleterious impact on FMD. While NO signaling is considered an integral component to an FMD response, there is evidence that young, healthy humans
demonstrate heterogeneity in the vasodilatory mechanisms that contribute to the FMD response (82, 84, 99). Under conditions of impaired NO bioavailability (eg. with pharmaceutical eNOS inhibition), it has been shown that other vasodilatory pathways may compensate and maintain an intact FMD response (82, 84, 99). Therefore, in the present study it is possible that numerous shear stress-dependent vasodilatory pathways contributed to FMD and an upregulation of non-NO pathways in the PHOS condition may have masked any phosphate-induced impairments in NO-mediated FMD.

It is difficult to determine the degree to which phosphate may have entered the endothelium when considering the relatively stable serum phosphate profile. Rapid renal responses could serve a protective role by eliminating phosphate before it can negatively impact endothelial function. Differences in the serum phosphate profile between the study by Shuto et al. (97) and the present study support a role for distinct phosphate handling in the disparate FMD responses.

Serum phosphate increased 20% from 3.79 pre-phosphate loading to 4.56 mg/dl at 2h hour post-loading in the study by Shuto et al. (97), which places the peak serum phosphate concentrations at the upper limit of clinically normal ranges. In contrast, in the present study there was a more modest increase of 12% from 3.40 (pre-supplement) to 3.80 mg/dl, and peak serum phosphate concentrations occurred one hour after phosphate loading and placebo. Importantly however, while serum phosphate concentrations were significantly higher in the PHOS compared to the PLAC condition, the difference was apparent pre-supplement and did not widen appreciably at any time point post-supplement (Figure 6) following the same pattern in both conditions. This suggests that the phosphate was either not absorbed or, if absorbed, was
efficiently taken up by tissue and/or removed via the kidney. Putative explanations for the stability in serum phosphate in the PHOS condition are discussed below.

The between-condition difference in baseline serum phosphate concentration was an unexpected finding given the standardized pre-study meal and the within-subjects study design. There are numerous factors that impact serum phosphate concentrations, including diet, time of day, intrarenal mechanisms, age and genetics (53, 58). No dietary records were collected for pre-study meals in advance of the breakfast provided, therefore it is possible that subjects ate differently in the days preceding each visit. Dietary restriction of phosphate has been demonstrated to decrease the average diurnal serum phosphate concentrations in healthy men (83).

Several notable differences between the present study and that of Shuto et al. (97) may at least partially explain the conflicting serum phosphate observations. First, in the present study, an exclusively inorganic phosphate supplement was administered, while Shuto et al. (97) provided one-third of the phosphate load as a meal and supplemented the remaining two-thirds of the phosphate dose in an inorganic form identical to the present study. Inorganic phosphate salts, such as the liquid oral supplement in this study, have a high bioavailability and are readily absorbed (90-100% vs. 10-60% of organic sources) by sodium-phosphate co-transporters in the small intestine (36, 75, 109). The inorganic phosphate supplement was selected instead of a meal to provide the most bioavailable form of phosphate in an attempt to cause the most rapid and substantial increase in serum phosphate concentrations. Therefore, while the total phosphate dose (1200 mg of phosphorus) was consistent between studies, the compositional differences of the ingested phosphate could have affected the quantity absorbed, the rate of absorption within the
small intestine, and ultimately endothelial exposure to the phosphate in the circulation. However, a 20 min post-supplement spike in serum phosphate levels was not detected in the present study indicating that the administered phosphate supplement may have resulted in a rapid increase in blood levels that peaked outside of the measured time points. However, it is also possible that no significant peak occurred due to a) incomplete absorption or, b) rapid distribution to a different phosphate pool upon absorption.

An increased FEP in the PHOS condition suggests that the phosphate load was largely absorbed because a significant increase in FEP and rising serum phosphate concentration would be unlikely to occur in the absence of a substantial influx of phosphate into the circulatory system. The ability of the kidney to rapidly upregulate renal phosphate excretion with increased dietary phosphate is well-documented (7, 29, 74, 108) and most probably blunted the expected accumulation of phosphate in the serum within an hour of supplementation (74). Consequently, the discrepancies in serum phosphate concentrations between the current study and other studies (74, 97) are likely the product of a more readily absorbed phosphate load (all inorganic) producing a greater stimulus for upregulation of renal phosphate excretion. Thus, the phosphate load delivered to the vasculature and endothelium was arguably modest and minimized the potential for phosphate to induce endothelial or VSMC dysfunction.

Additionally, regional differences in chronic diet may have contributed to the disparate serum phosphate profiles between the Canadian subject sample in the present study and the Japanese subject sample in Shuto et al. (97). The typical western diet that most Canadians consume has a higher dietary phosphorus content (1488 mg/day vs. 1232 mg/day; 27, 111). Chronic exposure to high dietary phosphorus is a stimulus for downregulating the expression of
sodium phosphate co-transporters in the proximal tubule, which acts to decrease phosphate reabsorption (60, 83, 105). Therefore, regional variability in chronic dietary phosphate intake may have contributed to enhanced phosphate elimination in the present study, and ultimately disparities in the phosphate load delivered to the endothelium. Exploratory analysis of between subject variability in serum phosphate concentrations within the current study did not reveal any differences in FMD, however this is not conclusive given the small number of participants.

As mentioned above, phosphate distribution to pools other than the urine could also have contributed to the modest rise in serum phosphate. Subjects in the present study were fasted for 6 hours, which may have established a gradient for phosphate distribution to the extracellular space. Subjects were not however exposed hyperglycemic or hyperinsulinemic conditions, which may promote cellular phosphate uptake (6, 95). Excess phosphate is considered to be detrimental to cardiovascular health, and it is possible that the deleterious effects of phosphate are more apparent when consumed with a meal to provide a hyperglycemic or hyperinsulinemic signal for cellular phosphate uptake. The impaired post-prandial endothelial function reported by Shuto et al. (97) may have been facilitated in part by glucose or insulin mediated cellular phosphate uptake, thereby increasing the intracellular phosphate exposure to the endothelium.

3.4.2 Interpreting FMD with Variations in Brachial Artery Baseline Diameter

In the present study, brachial artery baseline diameter was significantly greater than pre-supplement baseline diameters at both of the 20 and 60 min post-supplement FMD tests (Figure 10). This was an unanticipated outcome of GTN administration, which had a longer lasting vasodilatory effect than expected (39). GTN produces vasodilation by releasing NO, which
enters VSMC to activate guanylyl cyclase and increase cGMP production. However, the GTN-mediated vasodilation may not be produced entirely through activation of guanylyl cyclase. NO and endothelin-1 (ET-1), a potent vasoconstrictor, coexist in a dynamic balance to regulate vascular tone (10). Specifically, NO plays a fundamental role in modulating ET-1 activity through tonic inhibition of ET-1 production, receptor interactions and second messenger signaling (10). In the present study, sustained GTN action, as evidenced by a prolonged increase in brachial artery diameter, may have been induced by persistent NO-mediated inhibition of ET-1 action within the brachial artery even after the metabolism of NO. A changing baseline diameter can influence %FMD in that the same absolute change will yield a smaller %FMD with larger starting diameter. However, a similar pattern in %FMD and absFMD over time suggests that the depressed %FMD at 20 and 60 min post-supplement was not driven wholly by this mathematical issue. Conduit artery diameter influences the shear stress stimulus, such that for the same flow, a larger diameter will yield a smaller shear stress. The shear stress stimulus was lower post-supplement and this was likely due in part to the larger diameter and probably contributed to the lower FMD magnitude observed at 20 min post-supplement.

3.4.3 Limitations

In the present study sample, only young, healthy males were recruited for data collection. Thus, the impact of phosphate on endothelium-dependent and -independent vasodilation remains unclear in females, older adults, and individuals in the early predialysis stage of CKD. Serum phosphate concentrations are have been shown to be higher in females compared to males (104), therefore it is important to determine if gender differences in phosphate regulation exist and if
these differences translate into heightened phosphate-related CVD risk. In predialysis, deteriorated renal function would diminish the upregulation of phosphate excretion, thereby increasing the serum phosphate concentrations, enhancing endothelial exposure to phosphate and increasing the likelihood of phosphate induced endothelial dysfunction. Because we did not observe the hypothesized deleterious impact of phosphate loading on FMD, the present study was not able to isolate phosphate mediated endothelial dysfunction from VSMC dysfunction. A study that reproduces phosphate induced FMD impairment and includes GTN assessments will be required to achieve this.

Serum and urine analyses were performed to grant insight into the pharmacokinetics of the phosphate supplement used in this study. Serum and urine analyses are the most common sampling pools for assessing pharmacokinetics (49), however the phosphate dose could have been distributed to other tissues or remained in the intestine (48). It is not possible to determine intestinal absorption or tissue distribution from this study. 24-hour urine collections and dietary records could have assisted in understanding net phosphate balance by comparing net influx to efflux rather than comparing single time point measures of renal phosphate excretion. The combination of urine output and dietary intake would have enabled a more accurate estimation of how dietary patterns influenced baseline renal phosphate handling and serum phosphate concentrations, and how acute phosphate supplementation modified renal phosphate handling in this setting.

3.4.4 Conclusion
In conclusion, acute phosphate supplementation did not impair FMD or GTN-mediated dilation. This was observed in the presence of a fairly stable phosphate concentration and a marked increase in FEP with phosphate ingestion. These findings are in contrast to a recent report that links phosphate supplementation to endothelial dysfunction, and our data suggest that rapid adaptations in renal phosphate handling may be a protective mechanism against hyperphosphatemia and FMD impairment. Further investigations should assess acute phosphate supplementation in combination with different dietary patterns, meal composition, and in groups that are considered to be at-risk for phosphate-related CVD (individuals with CVD risk factors, mild renal impairment). This will permit a more comprehensive understanding of the impact of dietary phosphate on vascular function and the role of the kidney in protecting endothelium-dependent and -independent vasodilation following a high phosphate load.
Chapter 4

General Discussion

As mentioned previously, phosphate is a tightly regulated mineral that is essential for proper functioning of nearly all of our cellular systems. Dysregulation of phosphate homeostasis has been associated with atherosclerosis, vascular calcifications and the development and progression of CVD (25, 33, 63, 77, 104). Therefore, an understanding of how phosphate may affect endothelial function, specifically FMD, can provide a more comprehensive insight into the dynamic, yet unknown, events preceding the pathological cardiovascular changes associated with hyperphosphatemia.

4.1 Strengths and Weaknesses

It is becoming widely recognized that serum phosphate levels are associated with poor cardiovascular outcomes independent of renal function. To date, the study described in Chapter 3 is the second investigation into how an acute phosphate load affects the ability of the vascular endothelium to respond to increases in blood flow in young, healthy subjects. Therefore, this thesis was designed to specifically address limitations acknowledged by Shuto et al. (97) in order to characterize the role of phosphate loading on endothelium-independent vasodilation and the reactive hyperemia shear stress stimulus.

An important strength of the study presented in Chapter 3 was the randomized, double-blinded, placebo-controlled design that incorporated assessments of both endothelium-dependent and -independent vasodilation at three follow-up time points. Furthermore, simultaneous measurements of brachial artery diameter and blood velocity were recorded for calculation of the
shear stress stimulus elicited by reactive hyperemia, and blinded automated continuous diameter analysis enabled the investigator to accurately determine brachial artery baseline and peak diameter, as well as time-to-peak diameter, without biasing the analysis.

Limitations to the study in Chapter 3 are centered on the difficulty in making conclusions regarding the impact of phosphate on the primary objectives of this thesis, including \%FMD, \%GTN and the shear stress stimulus eliciting FMD, because increases in renal phosphate excretion blunted the expected increase in serum phosphate in the PHOS condition. Preliminary assessments of phosphate supplementation were not conducted prior to this investigation, and may have provided an early indication that renal adaptations to the phosphate load could significantly blunt a rise in serum phosphate concentrations. However, it is possible that endothelial cell phosphate uptake could occur in the absence of elevated serum phosphate concentrations, and may not be obligatory for phosphate-induced impairments in endothelial function. Additionally, there is no ethically acceptable method for sampling phosphate uptake into endothelial cells, therefore determining whether the phosphate supplement affected intracellular phosphate concentrations and NO vasodilatory pathways is unknown. Finally, the unexpected prolonged vasodilation induced by GTN administration resulted in a significant change in baseline diameter over time, which complicated the interpretation of the FMD response.

4.2 Practical Insights for Endothelium-independent Assessments

In addressing some of the limitations from the Shuto et al. (97) study, it was discovered that GTN has a long lasting and persistent dilatory effect on conduit arteries for at least 45
minutes after administration. This finding is very important to vascular ultrasound research because GTN is a commonly used drug for the assessment of endothelium-independent vasodilation. Prolonged vasodilation or disruption of NO/ET-1 balance in the control of arterial diameter could produce unintended changes in baseline artery diameter if used for pre- to post-intervention assessments of endothelium-independent vasodilation. Consequently, GTN administration should be reserved for comparing post-intervention assessments of endothelium-independent vasodilation in a control group assessed on a separate day.

4.3 Regional Dietary Differences Between Study Populations

The human body is sensitive to dietary modifications and responds with robust physiological adaptations in order to maintain proper function and homeostasis. A variety of hormonal feedback cascades exist in order to properly regulate homeostatic disturbances, such as post-prandial nutrient uptake. Specifically, phosphate has a complex regulatory system that enables a stable supply of phosphate for biological processes. Following periods of phosphate restriction, adaptive mechanisms trigger the upregulation of sodium phosphate co-transporter expression in the apical membrane of the renal proximal tubule and intestinal microvilli (110). This enables a greater amount of phosphate absorption from the gut and reabsorption of phosphate from the renal filtrate, thereby conserving total body phosphate. On the contrary, chronic exposure to high levels of dietary phosphate is an important stimulus for decreasing the expression of sodium phosphate co-transporters in the proximal tubule (60, 105), which minimizes phosphate reabsorption because retention of phosphate is detrimental under conditions
of dietary excess (83). Consequently, different dietary phosphate intakes can be accommodated by adaptations in renal phosphate handling.

In the study performed for this thesis, the study sample was a group of young, healthy Canadian males that most likely consume a typical western-type diet, characterized by a high intake of animal protein (meat and dairy), fatty foods and refined grains and sugars (28, 114), however the lack of chronic dietary records prevents confirmation of the actual diet consumed.

In contrast to the diet of the study population used by Shuto et al. (97), typical Japanese diets involve a high intake of fish protein, rice, noodles and vegetables (28). The INTERMAP study conducted in the late 1990s investigated the differences in nutrient intake amongst Japanese, Chinese, UK and US populations, and reported that US vs. Japanese male diets had higher animal protein intake (10.2% kcal vs. 8.9% kcal), lower vegetable protein intake (5.0% kcal vs. 6.9% kcal) and a higher dietary phosphorus consumption (1488 mg/day vs. 1232 mg/day; 27, 111). Further analyses of this dataset found that the two largest dietary groups contributing to the Japanese daily phosphate consumption were fish/shellfish, and pasta, rice and noodles (28).

These primary phosphate sources are, in part, animal protein, which exhibits 40-60% phosphate bioavailability, while vegetarian protein is 10-30% bioavailable. In the US, milk and cheese, and meat and poultry were the highest sources of dietary phosphate (28). Both of these dietary phosphate sources are 40-60% bioavailable (75). Additionally, dairy and meat products in the US often undergo food processing or enhancement that involves the addition of inorganic phosphates to foods that are already rich in phosphate. As mentioned in Chapter 3, the lack of a prominent peak in serum phosphate in this study may be attributable to the regional differences in phosphate consumption. Individuals chronically consuming a western diet may be more adapted
to high phosphate loads, and therefore less likely to experience a perturbation in phosphate homeostasis (in the presence of normal renal function). Individuals frequently exposed to high quantities and bioavailable dietary phosphates are more likely to respond with a more rapid upregulation of renal phosphate excretion. For example, individuals that usually eat smaller meals do not experience large phosphate doses and may be more challenged to mitigate perturbations to phosphate homeostasis. Consequently, to deal with these challenges to phosphate homeostasis, distribution of phosphate to extracellular pools, or the endothelium, could be involved in the compensatory mechanism to restore serum phosphate concentration, and inadvertently produce impairments in FMD.

4.4 Future Directions

With long-term studies demonstrating an association between serum phosphate and CVD, it is important to further investigate how phosphate impacts endothelial function because endothelial dysfunction precedes the development of atherosclerosis and CVD (24). Further studies should conduct an experiment similar to the one performed in Chapter 3, however a few minor protocol amendments should be made to enable a more clear interpretation of the results. First, endothelium-independent vasodilation should be assessed in both the PHOS and PLAC conditions, but only after the final FMD test, thereby eliminating the chance that brachial artery diameter may not return to pre-supplement values. Second, strict pre-study dietary reports should be recorded and, if possible, a dietician should be consulted to devise a standardized set of meals for subjects to consume in the day preceding the experimental visit. This would ensure that phosphate homeostasis has been stable in the pre-study period and minimize variability in
baseline serum phosphate concentrations and FEP. Third, to complement the dietary reports, 24-hour urine collection should be obtained in order to calculate net phosphate balance in the body, and facilitate a more concise understanding of the distribution of the administered phosphate load.

4.5 Conclusions

In summary, it was originally hypothesized that FMD would be impaired following an acute phosphate supplement and that the time course of FMD impairment would correspond to the time course of increasing serum phosphate concentration, demonstrating a concentration-dependent impairment of FMD. The findings in Chapter 3 demonstrate that serum phosphate concentrations did not increase after phosphate supplementation and there were no impairments in FMD in the PHOS vs. PLAC condition. No differences in FMD between the PHOS and PLAC conditions for all post-supplement time points suggests that endothelial dysfunction is not apparent in young, healthy males that are presumably exposed to dietary phosphate levels (~1500 mg/day or greater; 73, 104, 112) above nutritional guidelines (~900 mg/day; 14). While neither endothelial dysfunction, nor a dependence of FMD on serum phosphate concentrations could be detected, it was intriguing that the phosphate supplement induced increases in renal phosphate excretion to the extent that hyperphosphatemia was only mildly evident in the PHOS condition. Moreover, rapid renal adaptations in young healthy individuals likely functions as a protective mechanism responsible for the prevention of perturbations in phosphate homeostasis. This finding lends support to the existing literature that states phosphate-related CVD risk is most apparent in older individuals with CVD risk factors or impaired renal function (27, 77, 104). The
results of the study in Chapter 3 are important in that they demonstrate heterogeneity in the response to phosphate loading when compared to subjects sampled from another region of the world (97), and that acute high phosphate intakes may not be universally detrimental to endothelial function.
References


29. **Farrow EG, White KE.** Recent Advances in Renal Phosphate Handling. 6: 207–217, 2011.


Appendix A

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

Tel: 613-533-6000 x. 79631
Fax: 613-533-2009
E-Mail: pykek@queensu.ca

CONSENT FORM

FOR RESEARCH PROJECT ENTITLED:

The role of acute phosphate supplementation on endothelium-dependent and -independent vasodilation

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 impact of phosphate (an inorganic molecule found in food) intake on the function of the main artery of the upper arm (brachial artery). 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 and Affiliated Teaching Hospitals Research Ethics Board.

The purpose of the study is to identify whether acute exposure to elevated blood phosphate levels impairs vasodilation (vessel widening) via the endothelium (a single layer of cells that lines arteries), vascular smooth muscle, or both.

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 6 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.

**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) arteries 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 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.

ARTERIAL PRESSURE MEASUREMENTS:

A small pencil like pressure transducer will be placed over your pulse on the left side of your neck and on your femoral artery pulse in your upper thigh. A small infrared (light) sensor will be taped to the top of your foot to take continuous measurements of the pulse in the artery on the top of your foot. These recordings will be used to measure how quickly the pressure wave created by your beating heart travels through your arteries. This tells us about the stiffness of your arteries. These measurements will be performed continuously for ~5 min while you rest.
RISKS: This technique is non-invasive and poses no risk.

PHOSPHOROUS AND PLACEBO SUPPLEMENT:
The phosphorous supplement (Phoslax by Odan Laboratories Ltd) will be obtained from the Kingston General Hospital Pharmacy and administered orally in liquid form as intended by the manufacturer (Odan Laboratories), but will be flavored with Kraft Food’s Crystal Light. Phoslax is marketed or labeled for use as a laxative or dietary supplement. The dose to be administered in this study is 9.3 ml and is substantially below the labeled dose for laxative purposes (20ml). This dose contains 1200mg of phosphorous which has been previously used in published peer-reviewed research studies examining similar aspects of vascular function. The placebo supplement will be water, but will be flavored with Kraft Food’s Crystal Light in the same manner as the phosphate supplement, thereby making them indistinguishable by colour and taste. Neither you nor the experimenter will know whether you are consuming the phosphorous or placebo supplement. The selection of the supplement to be given is a random process that has been predetermined to avoid potential bias in the study.

RISKS: Although the dose is low (less than half of recommended laxative dose), it is possible that the supplement may have a laxative effect. Dehydration is the only risk for healthy individuals without cardiovascular or renal (kidney) disease. You have previously indicated in your medical questionnaire that you do not have cardiovascular or renal disease. If your health status has changed, please inform the person obtaining consent (Brendan Levac) immediately. The administered dose of phosphorous is not expected to change blood phosphorous levels beyond clinically-normal ranges, therefore the risks are slight dehydration associated with a small movement of water into the gastrointestinal tract. Water will be provided with your supplement to combat any possible dehydration.

GLYCERYL TRINITRATE (GTN) ADMINISTRATION:
GTN will be given in a metered sublingual spray that delivers a dose of 400 μg. It will be given twice during each visit with a period of 2.5 hours separating each dose. GTN is an antianginal (anti chest pain) medication typically prescribed for its ability to produce dilation of arteries and veins, thereby decreasing the work of the heart and alleviating symptoms of coronary heart disease. GTN should not be administered to participants with anemia, head trauma, hypovolemia, or hypotension. Consumption of alcohol within the previous 12 hours or 12 hours following the study could produce hypotension, thereby increasing the risk of fainting or falling. If your health status has changed since the medical questionnaire or if you have not strictly adhered to the study restrictions (no alcohol 12 hours prior to the study) please notify the person obtaining consent (Brendan Levac) immediately. The administered dose of GTN is not expected to produce more pronounced vasodilation or decreases in blood pressure. These substances should have been strictly avoided within the 24-hour period preceding this session. Individuals taking medication that contains dihydroergotamine or phenothiazines should also not be given GTN. If you did not indicate any medications containing these on your medical screening form and these conditions have changed, please notify the person obtaining consent (Brendan Levac).

BLOOD SAMPLING: To measure phosphorous levels and related factors in your blood, the thickness of your blood (blood viscosity), and your blood lipid profile, we will draw a venous blood sample. This will be done via sampling with a needle from vein at your elbow. This will be done by a registered nurse. 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. This needle will be removed but a flexible tube (catheter) will remain in your arm for the duration of the study. 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 Brendan Levac or Dr. Pyke.

URINE SAMPLES

Phosphorous is cleared from the body via the urine. To assess how much phosphorous you have retained from the supplement we will require two urine samples. You will be provided with a sample container to capture your first urination upon waking on each experimental visit and you will be asked for a second sample each visit when the laboratory protocol is completed.

RISKS - This poses no risk

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

How long will it take?

On an initial visit you will be asked to sit down and have your blood pressure measured. Next, you will 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. 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. The next trial will consist of spraying a drug (glyceryl trinitrate) under your tongue (sublingual) that will dilate your arteries in a different manner than the previous forearm occlusion trial. You will have simultaneous measurements of your upper arm blood flow with ultrasound. You will then consume a fluid supplement with a pleasant flavour (300 ml). This supplement may be either the active ingredient of interest (phosphate) or a placebo. Neither the experimenter nor the participant (you) will know if it was the active ingredient supplement or placebo. You will then perform 2-4 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. Finally, one last trial involving the administration of glyceryl trinitrate beneath the tongue and measurement of upper arm blood flow will take place.

Experimental Visit #2: This visit will take a maximum of 3.5 hours and will be identical to Experimental Visit #1.

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, we ask that you do not consume any food during the 6 hours preceding the experiments. The two experimental visits will take place in the afternoon and we ask that you consume the same low phosphorous breakfast (options provided) a minimum of 6h prior to each of your visit start times. 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 Form:

This questionnaire asks 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 or renal diseases, 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 consent form 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. Also, the Queen’s University Health Sciences Research Ethics Board may review the records as part of their research audit responsibilities. 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 a $10/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

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:

Brendan Levac (Student Investigator)
8bml1@queensu.ca
Room 401 D, SKHS 28 Division St.
Queen’s University, Kingston, ON, K7L 3N6
Tel: (613) 533-6000, ext, 79377

Kyra E. Pyke, Ph.D. (Principal Investigator)
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Room 301C, SKHS 28 Division
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Tel: (613) 533-6000, ext, 79631

Jean Cote, Ph.D. (School of Kinesiology and Health Studies Director)
Room 206, KHS
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 B

Elemental Phosphorus Content in PHOS Supplement

Monobasic sodium phosphate (NaH$_2$PO$_4$.H$_2$O) 2.4 g/5mL → 480 mg/mL

Dibasic sodium phosphate (Na$_2$HPO$_4$.7H$_2$O) 0.9 g/5mL → 180 mg/mL

Molecular Mass Calculation:

Monobasic sodium phosphate (NaH$_2$PO$_4$.H$_2$O): 137.98 mg/mmol,

Dibasic sodium phosphate (Na$_2$HPO$_4$.7H$_2$O): 268.07 mg/mmol,

Na+: 22.99 mg/mmol, PO$_4^{3-}$: 94.97 mg/mmol.

Monobasic sodium phosphate (NaH$_2$PO$_4$.H$_2$O): 480 mg/mL

→ (480 mg/mL)/(137.98 mg/mmol) = 3.4788 mmol/mL

[Na$^+$] = [PO$_4^{3-}$] = 3.4788 mmol/mL

From dibasic sodium phosphate (Na$_2$HPO$_4$.7H$_2$O): 180 mg/mL

→ (180 mg/mL)/(268.07 mg/mmol) = 0.6715 mmol/mL

[Na$^+$] = 2 x 0.6715 mmol/mL = 1.3430 mmol/mL

[PO$_4^{3-}$] = 0.6715 mmol/mL

Therefore, total in Phoslax Solution:

[Na$^+$] Total = 3.4788 mmol/mL + 1.3430 mmol/mL = 4.82 mmol/mL,

or [Na$^+$] Total = 4.82 mmol/mL x 22.99 mg/mmol = 110.81 mg/mL

[PO$_4^{3-}$] Total = 3.4788 mmol/mL + 0.6715 mmol/mL = 4.15 mmol/mL

Or [PO$_4^{3-}$] Total = 4.15/mL x 94.97 mg/mmol = 394.13 mg/mL.
or \[ [P] = 4.15 \text{ mmol/mL} \times 30.974 \text{ mg/mmol} = 128.54 \text{ mg/dl} \]