Dairy- and Soy- Derived Bioactive Peptides and the Renin-Angiotensin-Aldosterone System

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Melissa Sue Munn

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Carrie Penland Earthman

May, 2013

Acknowledgements

There are many people who have generously supported the work of this research and who have uniquely contributed to making my research experience a period of personal growth met with challenges and many great opportunities.

First, I would like to thank God for His steady and patient hand in everything in my life. I never imagined I'd be blessed with the opportunity to earn a Master's degree.

I would like to thank Dr. Carrie Earthman, my advisor, for her willingness to accept me as a graduate student in her lab. I've appreciated so much your patience, generosity, guidance, and support. Looking back, I am very grateful for this project and for the challenges it presented me, especially with the systematic review. What once appeared to be an unmovable mountain has left me with an extraordinary sense of accomplishment.

Thank you to my Committee members, Drs. Shalamar Sibley, Baraem Ismail, and Richard Brundage, for your patience, support, and willingness to help anytime. Shalamar, I've appreciated your guidance and feedback throughout these past two years. I feel very fortunate to have had the opportunity to work with you.

I would like to thank my lab mate, Lauren Beckman, who generously and constantly offered help and encouragement. I really appreciate your kindness.

Special thanks to my former lab mates, Sarah Vine and Sarah Kunkel who contributed to the protocol development and data collection for this study, to Wynnie Margatan who prepared the soy proteins, and to Kristina Sandvick who prepared the

cookie treatments. Your commitments are highly appreciated. This study would not have happened without you.

I would also like to recognize and thank the study participants without whom this study could not have been done.

Last, but never least, I would like to thank my parents. Mom and Pa, I would not be where I am today or who I am today if it weren't for you. I am very proud to have you as my parents. Your good humor and listening ears are always appreciated.

Abstract

Hypertension is a chronic, often asymptomatic, and highly prevalent cardiovascular disorder. Medications prescribed to lower blood pressure in hypertensive individuals are successful but are not without side effects or associated costs that render these agents inconvenient to patients. Furthermore, from a public health standpoint, a proactive approach to preventing or delaying progression into a hypertensive state in at-risk individuals is promising. Researchers have discovered bioactivity in peptides derived from food protein sources and the observed potential for blood pressure lowering effects through ACE inhibition has fueled further interest. This thesis focuses on the potential use of dairy- and soy- derived bioactive peptides in lowering blood pressure through ACE inhibition. Chapter 1 provides an overview of hypertension, including its prevalence, clinical definition, associated risk factors, and potential contributors to its complex pathophysiology, as well as current medications and a brief introduction to the use of bioactive peptides in a functional food. Chapter 2 presents a systematic review of the literature, with a strong emphasis on *in vivo* animal and human studies regarding the blood pressure lowering potential of dairy- and soy- derived bioactive peptides. Chapter 3 shares the findings of our study on the acute effects of whey- and soy- derived bioactive peptides administered in the form of a cookie to overweight, pre-hypertensive men and postmenopausal women. Finally, this thesis is concluded with a brief summary provided in Chapter 4.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

MI: Myocardial infarction

BMI: Body mass index

CO: Cardiac output

RAAS: Renin-Angiotensin-Aldosterone-System

ADH: Antidiuretic hormone

NO: Nitric oxide

NOS: Nitric oxide synthase

ROS: Reactive oxygen species

ACE: Angiotensin-Converting Enzyme

ACE-2: Angiotensin-Converting Enzyme-2

DASH: Dietary Approaches to Stop Hypertension

LAB: Lactic acid bacteria

α-La: α-Lactalbumin

β-Lg: β-lactoglobulin

Lfcin B: Lactoferricin B

SHR: Spontaneously hypertensive rats

WKY: Wistar Kyoto rats

SBP: Systolic blood pressure

DBP: Diastolic blood pressure

AUC: Area under the curve

IPP: Isoleucine-Proline-Proline

VPP: Valine-Proline-Proline

eNOS: Endothelial nitric oxide synthase

NOS: nitric oxide synthase

mmHg: Millimeters of mercury

ABPM: Ambulatory blood pressure measurement

WPI: Whey protein isolate

WPH: Whey protein hydrolysate

SPI: Soy protein isolate

SPH: Soy protein hydrolysate

IRB: Institutional Review Board

DCRU: Delaware Clinical Research Unit

cm: centimeters

g: grams

kg: kilograms

ml: milliliters

FAPGG: N-[3-(2-Furyl)acryloyl]- L -phenylalanyl-glycyl-glycine

FAP: furylacryyloylphenylalanine

SST: Serum separator tubes

rpm: revolutions per minute

ANOVA(Repeated measures): Analysis of Variance

SD: Standard deviation

CHAPTER 1: INTRODUCTION

.

Hypertension

Prevalence

Affecting 33% of Americans[1] and approximately 1 billion people worldwide[2], hypertension is considered to be the most prevalent of cardiovascular disorders and a significant public health concern[3]. It has been projected that by the year 2025, approximately 29% (1.56 billion) of the world's population will be hypertensive, particularly in both economically developed and developing countries[2].

Disparities in prevalence exist among races, levels of socioeconomic status, age, and between genders. In the U.S., approximately 43% of African Americans are affected by hypertension compared with both White and Hispanic Americans[1]. Such prevalence within this group is considered to be one of the highest world-wide[4]. Compared with their White and Hispanic American counterparts African Americans are more likely aware of their hypertensive state, yet they are less successful in achieving target blood pressure levels with treatment[1]. Differences within Hispanic American groups have been observed as well, with Puerto Rican and Dominican Americans having the highest rates of hypertension and Cuban Americans having the lowest[5]. In the 2005-2008 National Health and Nutrition Examination Survey, estimates of the prevalence of hypertension among races were reported to be 40% and 43%, 24.6% and 25%, and 30.5% and 28% for African American men and women, Mexican American men and women, and White American men and women, respectively[4]. Within Asian American groups, both Filipino and Japanese Americans were reportedly more likely to be diagnosed with hypertension (27% and 25%, respectively) compared to Chinese (17%) and Koreans (17%)[6]. Low socioeconomic status has been associated with hypertension for reasons that may include

occupational stresses and working conditions and having low birth weight, although the association between maternal malnutrition and hypertension later in life is ambiguous[7]. However, several studies support a fetal programming hypothesis, contending that vitiation of the intrauterine environment (presumably a consequence of maternal malnutrition) results in suboptimal growth that may predestine the fetus to cardiovascular complications later in life[8]. In contrast, those with higher socioeconomic status tend to be better educated in the prevention of hypertension and are more likely to follow medical/pharmacological interventions[7]

Although hypertension is growing alongside the rise in obesity rates among youth populations, it is more common among adults and increases with age with more than 50% of the US adult population identified as hypertensive[1]. The prevalence of hypertension is typically higher in men than women until the age of 45. Between 45 and 64 years of age, men and women have similar rates of hypertension, and beyond 64 years of age, hypertension is more prevalent in women[4].

Definition

Sometimes referred to as 'the silent killer,' hypertension is a chronic, often asymptomatic disorder autonomously correlated with cardiovascular disease risk. Specifically, hypertension imposes greater risk for myocardial infarction (MI), heart failure, and stroke[3], and is a leading cause of kidney failure[1].

Hypertension is clinically defined as having either a systolic pressure of 140 mmHg or higher, a diastolic pressure of 90 mmHg or higher, or both. Many individuals are at an increased risk for hypertension, with either systolic pressures ranging from 120-

130, diastolic pressures of 80–89, or both. These ranges define prehypertension and such individuals are at high risk for developing hypertension[1]. Hypertension can be categorized as Stage I or Stage II, depending on its severity. Stage II is defined as a systolic pressure of 160 mmHg or higher or a diastolic pressure of 100 mm Hg or higher, or both.

Hypertension can be classified as essential (primary) or secondary. Essential hypertension includes all cases in which the origin is unidentified and accounts for 85-90% of all hypertension diagnoses[9,10]. While origination is unclear, it is almost unanimously agreed upon that essential hypertension is multifactorial and ensues from a complexity of interactions, beginning at the genetic level[8,9,11-13]. It has been estimated that, on average, 30-60% of the variation in blood pressure between individuals, after age- and sex- adjustments, results from genetic contributions, including gene polymorphisms. It is currently believed to be under polygenic influence in which the effects of multiple genes ultimately characterize susceptibility[11]. It is imperative to acknowledge the essential contributions of environmental factors that reportedly account for 20% of the observed variations in blood pressure between individuals. While each gene variant may individually offer a small effect on overall blood pressure, together these may result in considerable blood pressure elevation when influenced by certain environmental factors[11]. These factors will be described in subsequent sections. The primary abnormality observed in essential hypertension is increased peripheral resistance resulting from vascular changes that include thickening of the arterial walls and abnormalities in vascular tone[12,14].

It is suggested that approximately 10-15% of individuals have secondary hypertension, so-named because they have another condition that has been identified as the cause. Common causes of secondary hypertension include renal parenchymal disease, renal artery stenosis due to either atherosclerosis or fibromuscular dysplasia (more common in children and young adults), and primary aldosteronism (leading to increased salt and water retention and potassium losses, in response to increased aldosterone). Other identified causes include Phaeochromocytoma and Paraganglioma (tumors in the adrenal medulla and in or outside the sympathetic and parasympathetic paraganglia, respectively) and Cushing's Syndrome (marked by significantly high secretions of glucocorticoids)[9].

Risk factors

Risk factors for the development of essential hypertension include, as previously described, age, race, and ethnicity. Older age is associated with higher risk for hypertension in most populations. African Americans are more likely to develop hypertension earlier in life, with greater severity, and to have higher rates of hypertension-related health complications[1]. Both overweight and obese individuals (as defined by body mass indexes (BMI) of 25.0 to 29.9 and 30 or higher, respectively) have increased risk for hypertension. The obesity epidemic that now spans across all age groups is largely accountable for the increased prevalence of hypertension now observed in youth less than 18 years of age. It has been suggested that risk factors for cardiovascular disease present in youth are strong predictors of early manifestations of atherosclerosis in adulthood and, similarly, blood pressure in childhood is considered a

significant predictor of blood pressure in adulthood[15]. There are a number of lifestyle factors that can increase the risk of hypertension including smoking, alcohol, and television viewing.

In terms of diet, frequent high sodium and low potassium intake might contribute to the development of hypertension[1]. It has also been suggested that magnesium might have a role in the development of hypertension in that small changes in extracellular magnesium concentration or intracellular free magnesium contribute effects on cardiac excitability and vascular tone and contractility. Low levels of magnesium may strengthen arterial response to vasoconstrictors and lower response to vasodilators, leading to increased peripheral resistance and blood pressure[12]. Higher levels of magnesium have opposing effects that ultimately lead to vasodilation and reduced blood pressure[12]. Finally, other risk factors include genetic predisposition and existing pre-hypertension[1].

Hypertension is also a contributing feature of metabolic syndrome, a disorder defined by a cluster of associated risk factors. The prevalence of metabolic syndrome is 22-39% in developing countries and can be a predictor of heart disease and diabetes. The development of hypertension with metabolic syndrome might result from increased weight and impairment in kidney function (with increased sodium reabsorption) and may be both a cause and effect of the associated hypertension[14]. Many individuals with Metabolic Syndrome exhibit some degree of insulin resistance and increased central fat distribution. Some investigators have postulated the contribution of hyperinsulinemia to hypertension in this setting. Possible mechanisms include insulin's ability to stimulate

sodium reabsorption at the kidney (an antinatiuretic effect) to stimulate the sympathetic nervous system[11].

Normal blood pressure regulation vs. pathophysiology

In normal physiology, blood pressure is controlled by many factors and through multiple mechanisms working together to maintain arterial blood pressure within the normal range. Blood flow is dependent on both cardiac output and total peripheral resistance. Heart rate and stroke volume largely determine cardiac output (CO). Heart rate is dependent on β-1 receptors controlled by sympathetic stimulation and cholinergic receptors, controlled by parasympathetic stimulation[11]. Stroke volume is influenced by ventricular force of contraction and the filling pressure determined by intravascular fluid volume and venous capacitance. Total peripheral resistance is influenced by mechanisms that act at local, regional, and systemic levels[11].

Responses to blood pressure elevation can occur in seconds (acute responses), minutes to hours (intermediate-acting responses), or days to weeks (late –acting). Acute responses to increased arterial blood pressure include reduced peripheral resistance and cardiac filling pressure as well as heart rate and myocardial contractility. Chemoreceptors at the vasomotor center also respond to acute changes in blood pressure. Intermediate-acting responses to blood pressure changes involve the Renin-Angiotensin-Aldosterone System (RAAS), antidiuretic hormone (ADH), and the renal juxtaglomerular apparatus. Finally, late-acting mechanisms include pressure natriuresis and diuresis. Specifically, an increase in blood pressure above the homeostatic set point results in an increase in sodium and water excretion[11].

The actual pathogenesis of hypertension remains unclear as multiple inputs may contribute including the sympathetic nervous system, the kidneys, endothelin, nitric oxide, the kallikrein-kinin system, and the RAAS. Each is presented briefly below with the exception of RAAS, which is discussed in greater detail in the following section.

The kidney is ultimately responsible for blood volume and blood pressure control in the long-term. Interestingly, patients with essential hypertension who develop renal failure have been cured of their underlying hypertension upon successful kidney transplantation from a donor with normal blood pressure. Others have suggested a disorder of genetic origin in which there is a reduction in nephron number and hyperfiltration and glomerular pressure damage ultimately impair sodium excretion. Salt sensitivity is less of a concern in youth and appears to increase with age, such that by 70 years of age, almost all hypertensive individuals are salt-sensitive[11].

The sympathetic nervous system influences vascular tone, cardiac output, and peripheral resistance. In the initial increase in blood pressure, both heart rate and stroke volume are increased as well as tone, which might partially explain the initial increase in peripheral vascular resistance[11]. Hyperactivity of the sympathetic nervous system has been reported in some studies on hypertension[11]. Stimulation of this system leads to vasoconstriction, increased heart rate, increased norepinephrine plasma concentrations, and increased blood pressure[10,11]. It has also shown to promote vascular hypertrophy, increasing wall thickness that can contribute to resistance[11].

Endothelin-1 is a peptide with vasoconstriction properties that plays multiple roles in various organs. Endothelin-1 is found in blood vessels and the kidney. It has been suggested to promote inflammation by increasing oxidative stress in the vascular wall,

resulting in vascular remodeling and endothelial dysfunction[16] which can contribute to increased peripheral resistance and organ damage that can occur with chronic hypertension.

Nitric oxide (NO) is produced from nitric oxide synthase (NOS) during the conversion of its substrate L-arginine to L-citrulline. In vascular endothelium, the nitric oxide diffuses to vascular smooth muscle to stimulate vasodilation and to prevent vasoconstriction. Given its contribution to blood pressure regulation, inadequate production of NO might be a factor in the etiology of hypertension[17]. Possible explanations of insufficient NO production include reduced nitric oxide synthase (NOS) expression and strong endogenous inhibitors of the enzyme. These are ordinarily cleared by the kidney, but in renal failure, clearance is impaired. Finally, a deficiency in NO could be due to inactivation by superoxide anions or other reactive oxygen species (ROS)[17].

The kallikrein-kinin system opposes the RAAS. It is not well understood, especially in regards to the pathophysiology of hypertension. Kallidin is a strong vasodilator that is formed from its precursor kininogen via the hydrolytic actions of kallikrein. Kallidin is further hydrolyzed by this enzyme to form other peptides with vasodilatory properties, including bradykinin. It is believed that these then bind to β-receptors resulting in a series of chemical reactions that precede an influx of intracellular calcium ions that stimulate NO production via NO synthase. This pathway can be inhibited by ACE which hydrolyzes bradykinin[18].

The Renin-Angiotensin-Aldosterone System (RAAS)

The RAAS has a significant role in the regulation of blood pressure and involves a cascade of events resulting in increased blood pressure. Prorenin is a precursor to the aspartyl proteolytic enzyme responsible for the initiation of the RAAS pathway, renin. Biosynthesis of renin occurs in the juxtaglomerular cells of the renal glomerulus. Mature renin results with proteolytic removal of a peptide from the N-terminus of its precursor and is secreted into renal and systemic circulation in response to a drop in perfusion pressure or sodium chloride delivery to the kidney and sympathetic stimulation[19]. Renin is present in other tissues including the brain, adrenal glands, visceral adipose tissue, and possibly the heart and vascular tissues[19]. Renin converts angiotensinogen into angiotensin I, a decapeptide with no known bioactivity. Angiotensinogen is primarily made in the liver, but is also thought to be synthesized in the brain, kidney, heart, and adipose tissue. While angiotensinogen plasma levels appear static, increased production results from increased glucocorticoids, estrogens, and inflammatory cytokines[19]. Angiotensin I quickly becomes activated upon removal of its C-terminal dipeptide, forming the octapeptide, angiotensin II. This reaction is facilitated by ACE, a dipeptidyl carboxypeptidase. ACE is thought to be present in vascular endothelial cells, renal proximal tubule cells, and neuroepithelial cells and is membrane-bound[19]. Interestingly, it is suggested that plasma levels of ACE are reflective of clearance of bound ACE[19]. ACE also promotes vasoconstriction through its facilitation of bradykinin degradation. Angiotensin II binds to its type-1 (AT_1) receptor, resulting in increased vasoconstriction, aldosterone secretion, and consequential sodium retention and reabsorption[19-21]. It can

also promote oxidative stress[19]. In contrast, binding to its type-2 (AT_2) receptor has opposing effects[21].

More recently, a new axis of RAAS has been discovered in which eventual conversion of both angiotensin I and angiotensin II result in production of the metabolite angiotensin-(1-7) by angiotensin-converting enzyme-2, ACE2. Endothelium-dependent vasodilation results when the metabolite binds to its receptor, Mas, thereby antagonizing the hypertensive effects resulting from angiotensin II binding to AT_1 receptor. Accordingly, this axis may play a counter-regulatory role against the well-known RAAS pathway[21].

Current interventions

A first recommendation for the management of hypertension is to alter lifestyle habits, especially dietary choices and level of physical activity. A common dietary recommendation for prevention is to reduce sodium intake to less than 1.5 grams (1500 mg) per day. Another approach is the DASH diet, or Dietary Approaches to Stop Hypertension, which promotes the consumption of fresh fruits and vegetables, whole grains, lean meats, and low fat or fat-free dairy products. It recommends low fat, low cholesterol and low sodium foods[1]. Other recommendations include potassium supplementation for lowering blood pressure in those with and without hypertension. It has also been suggested to reduce alcohol consumption (chronic drinkers) and to limit intake to no more than 24 ounces of beer or 10 ounces of wine per day. Less often recommended because of insufficient support includes calcium and fish oil supplements. Dietary advice is usually paired with recommendations to increase physical activity,

specifically to a minimum of 30 minutes each day, or most days of the week. Weight loss for those who are overweight or obese is strongly suggested. Other lifestyle recommendations include smoking cessation[1].

Such recommendations are frequently accompanied by pharmacological interventions that include a wide array of medications used in the employment of various inhibitory or antagonistic strategies or for exploitation of physiological control mechanisms to indirectly regulate blood pressure. The multitude of inputs and consequent dynamic complexity underlying its pathology are invitational to the development of widely varying pharmacological agents for the attenuation of hypertension. These medications include diuretics, beta blockers, calcium channel blockers, alpha blockers, alpha-beta blockers, nervous system inhibitors, vasodilators, angiotensin II receptor blockers, and angiotensin-converting enzyme (ACE) inhibitors[1].

Thiazide diuretics are often first prescribed and may be particularly effective in African Americans, in which salt sensitivity is most prevalent[22]. The mechanistic actions of diuretics include decreased blood volume and cardiac output as a result of increased urinary excretion of sodium and water, as diuretics exert natriuretic effects. Blood volume and cardiac output revert to normal within 6-8 weeks of treatment. Additionally, vasodilation may increase due to reduced sodium and water in vessel walls[23]. Diuretics include carbonic anhydrase inhibitors, loop diuretics, thiazides, and potassium sparing agents, each acting at different sites of the renal tubule[23]. Beta blockers competitively antagonize β-adrenoreceptors to slow heart rate and are often used in combination with other hypotensive agents. Calcium channel blockers antagonize L-type calcium channels of the heart and smooth muscles thereby inhibiting the movement of calcium inward.

They stimulate dilation of coronary arteries (to increase oxygen supply) and peripheral arterioles and decrease cardiac contractility[24]. Alpha blockers are alpha-adrenergic antagonists that relax various muscles and promote vasodilation, countering the effects of norepinephrine[25]. Angiotensin II receptor blockers competitively antagonize angiotensin II receptors to prevent vasoconstriction and promote the release of aldosterone and catecholamines[26]. ACE inhibitors competitively inhibit conversion of angiotensin I into angiotensin II to prevent the potent effects of angiotensin II, as previously described.

While the aforementioned pharmacologic agents may help attenuate hypertension, they are not without side effects that render them inconvenient to patients. Diuretics offer some of the least amount of side effects, but do include the risk for electrolyte imbalances (especially hypokalemia, and hyponatermia), impaired glucose tolerance resulting in hyperglycemia and insulin resistance, metabolic acidosis, and lipid abnormalities among others[23]. Also, there is increased risk for muscle weakness, fatigue, and leg cramps[27] as consequences of increased potassium loss, as well as arrhythmias, a potential side effect of thiazides and loop diuretics[23]. Common shared side effects of alpha and beta blockers include fatigue, dizziness, nausea, headache, upset stomach, and diarrhea or constipation[25]. Side effects of beta blockers also include cold sensations in the extremities (hands and feet) and depression and heart rate may increase when taking alpha blockers[27]. Calcium channel blockers share similar side effects but also increased risks for flushing, drowsiness, and tachycardia. ACE inhibitors can cause similar effects as well as dry cough and sleep disturbances, increased risk for hyperkalemia[25], skin rash and dysgeusia[27].

The need for something more, a functional food

In addition to their potential side effects, pharmacological interventions can be both costly and inconvenient. According to the Center for Disease Control and Prevention, it was estimated that in 2010, $76.6 billion in the U.S. would be spent in hypertension-related health care, medication, and missed work days[28]. In addition, there is insufficient education and instruction provided to hypertensive patients, partly due to a lack of insurance coverage or reimbursements for counselling and referrals to dietitians by primary healthcare providers[1].

Many hypertensive individuals are without healthcare insurance and therefore may be without treatment. Consequently, there is a need for additional efforts in addressing hypertension, ideally in its prevention. A less expensive but more convenient alternative is of interest. Consumers in the US and most other developed countries are becoming acquainted with and accepting of food products offering additional health benefits (so called "functional foods"). Given the high prevalence of hypertension, most consumers would predictably be interested in purchasing something that could help lower blood pressure or, if consumed often, potentially slow or impede progression of hypertension from a pre-hypertensive state. Increased awareness and consumer knowledge of the relationship between diet and health has encouraged greater interest in exploring the possibilities of functional foods[29], and dietary modifications including bioactive food substances are highly appealing. The Academy of Nutrition and Dietetics describes its position as, *"All foods are functional at some physiological level, but it is the position of the American Dietetic Association that functional foods that include whole foods and fortified, enriched, or enhanced foods have a potentially beneficial effect on*

health when consumed as part of a varied diet on a regular basis, at effective levels,"[30]. Immediate benefits of a functional food designed to lower blood pressure would include its affordability, its availability, and its ability to preclude the side effects observed when taking antihypertensive medications.

Bioactive peptides

Researchers have found antihypertensive activity in dairy-derived peptides in *in vitro*, animal, and human studies. The discovered bioactivity has stoked the interest and curiosity of researchers with the idea of developing functional foods that both attenuate high blood pressure and preclude the side effects associated with antihypertensive medications. Bioactive peptides are specific fragments of proteins that have a positive influence on body functions and may enhance health. Upon consumption, bioactive peptides can affect various systems in the body that include the digestive, cardiovascular, immune, and nervous systems and their target systems are contingent upon their individual amino acid compositions and sequences[31]. Regarding hypertensive pathways, the majority of bioactive peptides have shown ACE inhibitory activity.

Interestingly, ACE-inhibitory peptides were first identified in snake venom. Since then, many ACE-inhibitory peptides derived from animal and plants have been discovered. Some sources include eggs through hydrolysis of ovalbumin, fish (salmon, sardine, anchovy, and Alaskan Pollack), as well as fermented fish sauce. Plant sources include soybean protein and sesame powder protein[29]. Milk or dairy-derived proteins are good sources of antihypertensive peptides.

In vitro production and characterization studies provide vital information for optimizing the development of peptides with ACE inhibitory properties. However, the findings from studies examining *in vivo* effects on blood pressure post administration of these peptides reveal their true value and the probability for success of a functional food. **The aim of this thesis project** was to investigate the acute effects of dairy and soy protein-derived bioactive peptides on blood pressure in adult humans. A systematic review of the current literature on dairy- and soy- derived ACE inhibitory peptides in animals and humans was conducted and is presented in Chapter 2. Chapter 3 presents the pilot human study that was conducted for this thesis project, and Chapter 4 presents conclusions and directions for future research.

CHAPTER 2: A SYSTEMATIC REVIEW OF THE LITERATURE*

* Portions of Chapter 2 are in preparation as a manuscript for possible publication as a systematic review.

Introduction

Referred to as 'the silent killer,' hypertension is the most prevalent cardiovascular disorder that is both chronic and often asymptomatic. It is estimated to affect 33% of Americans[1] and 1 billion people worldwide[2]. A first recommendation for management is to address lifestyle habits, especially diet and physical inactivity. Sodium restrictions to less than 1500 mg per day and the Dietary Approaches to Stop Hypertension (DASH) diet, which promotes the consumption of fresh fruits and vegetables, whole grains, lean meats, and low fat or fat-free dairy products have been prescribed[1]. Pharmacological interventions are effective treatments, but are often accompanied by undesirable side effects. According to the Center for Disease Control and Prevention, it was estimated that in 2010, $76.6 billion in the U.S. would be spent on hypertension-related health care, medication, and missed work days[28]. There is a need for alternative approaches to address hypertension, ideally focusing on its prevention. A less expensive and more convenient alternative to antihypertensive medications is of interest. Increased consumer awareness of the relationship between diet and health supports investigation into the possibilities of functional foods[29] and dietary modifications including foods that contain bioactive substances. Immediate benefits of a functional food designed to lower blood pressure or impede progression of a hypertensive state would include its affordability, convenience and availability, and its lack of undesirable side effects compared to pharmacologic approaches.

Researchers have found antihypertensive abilities in dairy-derived peptides in *in vitro* and *in vivo* animal and human studies. This potential bioactivity has stoked the interest and curiosity of researchers with the idea of developing functional foods that both

attenuate blood pressure and preclude medication-related side effects. Bioactive peptides are protein fragments encrypted within an inactive protein sequence with biological functions that may enhance health. These peptides can affect various systems in the body that include the digestive, cardiovascular, immune, and nervous systems, and have been shown to exert antioxidant effects[31,32]; their target systems are contingent upon their individual amino acid compositions and sequences[31]. The majority of antihypertensive bioactive peptides are believed to exert effects though ACE-inhibition with dairy-derived peptides studied most extensively. Though much less studied, soy-derived peptides are of interest, considering the many potential benefits already associated with soy-based diets.

In vitro production and characterization studies provide vital information for optimal development of peptides with ACE inhibition. Consequently, much attention has been paid to this area of research. However, the true worth of these peptides, and by extension, the success of a functional food containing these peptides, requires the evaluation of their blood pressure lowering capabilities *in vivo*, particularly in human subjects. In order to assess current understanding of their *in vivo* potential, we reviewed the literature on dairy- and soy- derived ACE inhibitory peptides in animals and in humans with the purpose of presenting the findings of animal and human-based studies on blood pressure reduction and/or ACE-inhibition following acute or chronic administration of dairy- or soy-derived bioactive peptides.

Methodological approach to systematic review

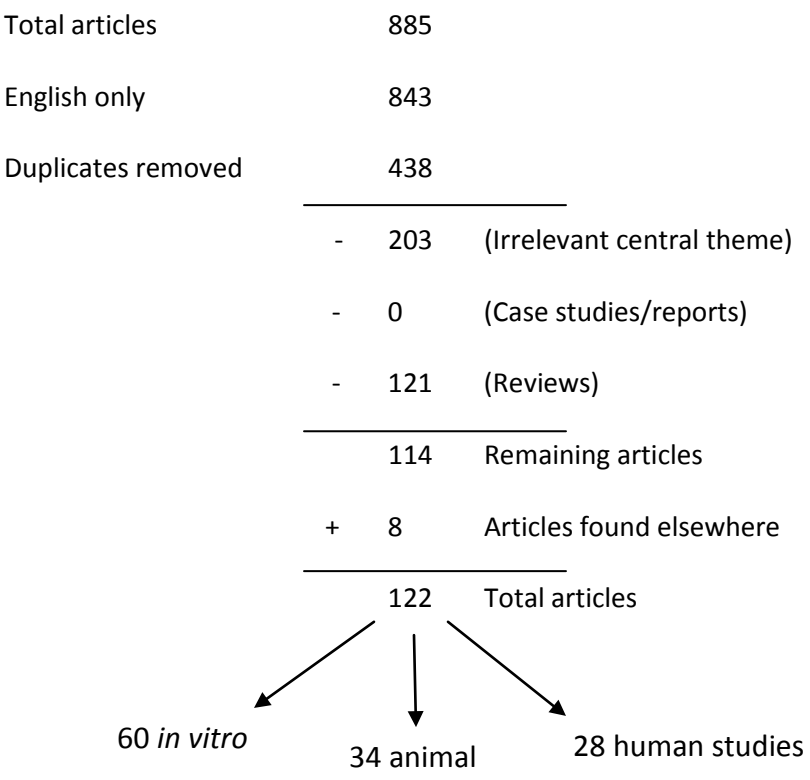
A systematic review was conducted on the *in vivo* animal and human-based studies on the antihypertensive effects of dairy- and soy-derived bioactive peptides and the renin-angiotensin system, specifically ACE-inhibiting peptides using a multi-database search with the following databases with no limitations by year: In Process, Ovid Medline (R), Agricola, Agris, Biological Abstracts, Biosis Previews, CAB Abstracts, and Food Science and Technology Abstracts. Additionally, Cochrane, Web of Science, and Pubmed were also searched. The following sets of search terms were entered in the formats shown below and were searched separately:

> hypertension OR blood pressure* OR antihypertensive agent* OR antihypertens* OR antihypertensive activity* OR angiotensin-converting enzyme inhibition OR angiotensin-converting enzyme inhibitor* OR ACE inhibitor* OR peptidyl-dipeptidase A OR kininase II OR angiotensin-converting enzyme* OR angiotensin* OR renin-angiotensin system OR angiotensinogen OR renin
>
> bioactive peptide* OR bioactive amino acid* OR physiologically active peptide*
>
> soybean protein* OR soy protein* OR soybean derived peptide* OR soya protein* OR glycinin OR conglycinin* OR beta conglycinin*
>
> milk protein* OR milk peptide* OR dairy protein* OR whey isolate* OR whey protein isolate* OR whey protein hydrolysate* OR whey hydrolysate* OR casein* OR lactotripeptide* OR lactoglobulin* OR alpha lactalbumin* OR alpha-La OR lactalbumin*

The search strategy involved combining the above term sets as described: the set of terms that include “bioactive peptide*” and the set beginning with “milk protein*” were searched together joined by AND. The set beginning with “bioactive peptide*” was also searched along with the set that begins with “soybean protein*” using AND in the databases. The results from the aforementioned two searches were combined by OR and these results were finally joined with the set of terms beginning with “hypertension,” joined by AND. Terms that could be exploded were so, in an effort to optimize database searches.

Following this strategy, a total of 843 from 885 articles were found after limiting to articles in the English language. After removing duplicates, a total of 438 articles remained. Articles whose central theme did not pertain to dairy- or soy- derived peptides and ACE inhibition or blood pressure lowering were excluded, as were case studies and reviews. Figure 1 below presents the exclusion process:

Figure 1. Multi-database search results and the exclusion process



Production of bioactive peptides

Bioactive peptides are usually inactive within the parent (protein) sequence, necessitating hydrolytic release for bioactivity. These peptides are produced primarily through hydrolysis during bacterial fermentative processes or when treated with isolated or combined proteases derived from various sources. An important benefit to the identification and use of various enzymes or enzyme systems is the formation of a variety of peptides, each possessing different bioactive potential. Two frequently used methods for production, as previously mentioned, rely on fermentation with bacterial sources and treatment with isolated or combined enzymes from various sources and an overview of sources of each are presented.

Dairy-derived ACE inhibitory peptides: sources for fermentation

Hydrolysis can occur with bacterial fermentation, most often using lactic acid bacteria (LAB), in which several proteolytic enzymes released from starter cultures assist in protein degradation[33]. The type of starter culture used in dairy products is an influential factor in successful development of these peptides[34]. Consequently, investigations on bacteria strains and peptide yields are of great interest to researchers in this area. Bacteria used in fermentation processes in the production of bioactive peptides have included (often mixed) cultures containing strains of *Streptococcus thermophilus*[34], *Lactobacillus delbrueckii subsp. bulgaricus*[34,35], *Streptococcus thermophilus* TH4, *Lactobacillus acidophilus* LA5, and *Bifidobacterium bifidum* Bb12[34]. Others include various species of Lactobacillus, such as *Lb. helveticus*[36-38], *Lb. brevis, Lb. paracasei, Lb. acidophilus,* and *Lb. bifermentan, L. lactis* subsp. *cremoris* FT4[37-39]. The microflora of various cheeses have also been used for fermentation[33] as well as isolated strains of *Enterococcus faecalis* from raw cow milk[40]. Other studies have used probiotics during cheese ripening processes to determine ACE-inhibitory peptides and their activities[41] or have examined the food-grade production and stability of casein-derived ACE-inhibitory peptides[42]. Finally, efforts toward optimization of peptide yields have led to the advent of combined method strategies, including the use of LAB fermentation with added protease[43].

Dairy-derived ACE inhibitory peptides: sources for enzymatic hydrolysis

Enzymatic hydrolysis is used for bioactive peptide production. Some investigators have conducted hydrolysis with single digestive proteases or varying

combinations of pepsin, trypsin, and chymotrypsin[33,34,44-51]. Others have used combined enzyme treatments such as pepsin and pancreatin to digest human milk and infant formula[52]. The incubation of proteins with isolated bacteria-derived enzymes is another common method of enzymatic hydrolysis. Such proteases are derived from the frequently used *Bacillus thermoproteolyticus*-derived thermolysin,[53], Flavourzyme[54], or other fungal proteases derived from *Aspergillus oryzae*[43,55], proteinase K, from *Tritirachium album*[45,46], *Bacillus licheniformis* protease[45], and Actinase-E , derived from *Actinomyces ssp*[47]. Some investigators have used a commercial proteolytic mixture from *Bacillus subtilis*, Protease N Amano[56] for the development of ACE-inhibitory peptides. Finally, interesting alternative origins of enzymes include plant sources, such as *C. cardunculus*[57] and papain from *Carica papaya*[47]. Additionally, the enzyme Corolase PP, a proteolytic mixture from the pancreas glands of pigs that includes trypsin, chymotrypsin, and several amino and carboxypeptidases[58] and its ACE-inhibitory peptide yield have been included in studies[44,49].

Soy-derived ACE inhibitory peptides: sources for enzymatic hydrolysis

The production methods for yielding soy peptides mirror those employed for the dairy-derived counterparts in that processes include the use of mammalian digestive, bacteria- and mold-derived enzymes and fermentation, or a combination of these. Specifically, some investigators subjected soy protein to pepsin and pancreatin treatments for peptide production[59,60]. Other enzymes used in the production of soy-derived peptides have come from bacterial sources including *Bacillus subtilis*[61] and *Bacillus licheniformis*, responsible for producing the commonly used enzyme, alcalase[62,63]. Other

researchers have produced peptides using treatments with proteases derived from molds, specifically *Monascus purpureus*, a commonly used fungus for the production of certain fermented foods[64]and strains of *Aspergillus oryza*[61,63,65]. Furthermore, peptides produced from probiotics including *Lactobacillus acidophilus* in soy whey medium have been investigated[66]. Finally, others have compared bioactive peptide productions from various enzymes using digestive enzymes, pepsin, trypsin and chymotrypsin, with various commercial enzymes[63,67] and ginger protease from ginger, *Zingiber officinale*[67].

The development of dairy- and soy- derived ACE inhibitory peptides has largely relied on proteolytic systems of fermentative processes as well as hydrolysis with isolated or combined enzymes. Success and diversity in their production can be at least partly ascribed to the use of a number of bacterial, fungal, and plant sources. An important benefit to the wide array of enzymes and enzyme systems used for production is the ability to derive bioactive peptides with varying potential, thereby increasing the likelihood of discovering peptides with potent bioactivity.

ACE inhibition *in vitro*

How is ACE inhibition expressed?

The determination of ACE inhibition by an antagonist, such as a bioactive peptide, in an *in vitro* study is most often conveyed as the half maximal inhibitory concentration (IC_{50}) of the inhibitor. Essentially, this value describes the concentration of the substance needed to inhibit the enzyme by half of its original activity. Accordingly, a lower IC_{50} value signifies greater potency, as less of the inhibitor is required to inhibit to half the enzymatic activity[68]. Conversely, a higher value would imply less potency. As

expected, these values are highly variable among bioactive peptides. Although much more potent than any bioactive peptide studied, Captopril, an ACE-inhibitor drug, is often used for comparison, with an IC_{50} reported to be around 0.006µmol/L[51]. It is also often expressed as percentage (%) of ACE inhibition at a given protein concentration. Though there are other methods to arrive at this value, several studies have employed spectrophotometric absorption measures and have used the following formula (or some version of this):

% Inhibition activity = (A-C) / (A-B) X 100; where:

A = the optical density without the peptide fraction

B = the optical density without ACE

C = the optical density with both the peptide fraction and ACE

What effects do time and degree of hydrolysis have on *in vitro* ACE-inhibition?

As the generation of bioactive peptides is dependent on hydrolysis of the parent protein, speculation about the effects of hydrolysis time on their production has led to further investigation. Equally as important as the enzymes selected for production of ACE inhibitory peptides is the determination of optimal time of exposure to the enzyme and the degree of hydrolysis.

The plausibility that increased hydrolysis time yields more potent bioactive peptides has been supported by researchers observing lower IC_{50} values or higher percent (%) inhibitory activities with increased hydrolysis time, or degree of hydrolysis[34,45,51,54,56,61,69]. However, some researchers infer that a maximum ACE

inhibition exists, minimizing or eliminating the need for extended hydrolysis time[45,70] though ACE inhibition has been observed to correlate with degree of hydrolysis [71]. Observations of plateaus in ACE-inhibitory activities with time[56] and early release of such peptides without further production with increased hydrolysis[44] are congruent with the previous suggestions. Furthermore, the maintenance of the ACE inhibition with increased hydrolysis time could result from resistance to further degradation or, conversely, the formation of new peptides upon degradation, thereby lending balance[45]. Associations may partly depend on the enzyme or enzyme mixture and its proteolytic activities; given that it has been observed that some hydrolysates associated with the highest degrees of hydrolysis resulted in lowest ACE -inhibitory activities, possibly owing to a combination of endopeptidase and exopeptidase activities[63]. The presence of both could result in cleavages at various sites of the peptide chain, precluding bioactivity[63]. In contrast, others found that increased growth of a probiotic in a soy-based medium correlated with increased ACE -inhibitory activity, potentially due to a proteolytic system comprised of several peptidases and proteases essential for cell growth and survival that releases such peptides[72]. Importantly, the degree of hydrolysis does not necessarily correlate with enzyme exposure time as nearly immediate increases in degree of hydrolysis followed by plateaus with further time advancement has been reported[73]. Similar trends have been observed with ACE- inhibitory activity[73]. Finally, researchers have observed changes in ACE inhibitory activity upon subsequent treatments with other enzymes[73,74] and time of exposure and degree of hydrolysis with one treatment may affect susceptibility to cleavage of peptides during exposure to a second enzyme treatment[74]. Interestingly, it has been observed that heat pre-treatments of peptides might

enhance digestibility/proteolysis[74]. Nonetheless, greater complexity is introduced when differences in protein and enzyme types and their associated hydrolysis times for maximum ACE -inhibitory peptide release are considered[46,51]. Such complexity presents challenges for the production of bioactive peptides with optimal potencies.

Do the bioactive peptides survive digestion?

While *in vitro* ACE inhibition observed in food-derived substances inspires thoughts of functional food opportunities in industry, researchers recognize that appreciable *in vivo* effects require that the ingested bioactive peptides surmount an important obstacle: digestion. Though results of many *in vitro* studies have readily revealed peptides with ACE-inhibitory activity, only some have subjected these to the digestive proteases pepsin, trypsin, and chymotrypsin. As one might expect, some peptides lost potency upon hydrolysis[57,64], while other studies reported peptide survival from enzymatic cleavage, likely due to short peptide chains (4-7 amino acids)[35,38,75-77]. In accordance with previous speculations, other investigations revealed little or no changes in inhibitory ability[33,38,57,58,62-64,67,77,78], or *increased* ACE -inhibitory potential[43,57,64,77] after exposure to digestive proteases. An explanation, as previously mentioned, for the latter observation is that either the original peptide resisted further hydrolysis or its hydrolysis yielded smaller bioactive peptides with greater chance for survival from further proteolytic cleavage[33,43,49,57,64,77]. Importantly, it has been suggested that the presence of other peptides may impose a change of fate upon a peptide previously tested in isolation. Specifically, Roufik et al[79] observed that when long- and short- chain peptides are combined (as seen *in vivo*) there is increased hydrolysis, even among those

that, when alone, resist hydrolysis. Furthermore, these researchers also considered the influence of pH changes (i.e. stomach to small intestine), noting altered susceptibility to hydrolysis as a consequence of modified charges within the peptide[79]. Other researchers have suggested the potential influence on potency of the stage of digestive processes at which peptides are absorbed on potency and the possibility of absorption of long peptide chains that then may act in synergy or cumulatively with shorter peptides to enhance overall blood pressure lowering effects[74]. In effect, researchers assert that the applications of theoretical specificity regarding digestive proteases and *in vitro* digestion warrant caution in their prediction of outcomes of bioactive peptides after digestion, and that important variables, including chain length and the presence of other peptides, support the need for *in vivo* studies[79]. Interestingly, researchers have suggested that the ability of small peptides to be absorbed easily in intact form may be due to unique uptake systems[62] or through transcytosis from the gut into circulation [80].

What is known about peptide sequence and structure, in relation to *in vitro* ACE inhibition?

It is well-accepted that hydrophobic amino acids, especially with aromatic or branched side chains occupying the three C-terminal positions of a peptide instill potent inhibitory potential[38,48-51,65,74,76,77] and are likely accommodated by the three subsites of the carboxyl domain of the catalytic site, S1, S1', and S2', like the three C-terminal residues of angiotensin I (phenylalanine, histidine, and leucine)[34,53,57,81]. It has also been suggested that the most potent include aromatic residues (tryptophan, tyrosine, or phenylalanine), the (imino) amino acid proline, arginine, with the positive charge on its

guanidine group side chain at the ultimate position[34,37,51,53,64,74], as well as leucine, owing to the positive charge on the epsilon-amino group[56]. It has also been suggested that a proline residue at the antepenultimate position contributes significantly to potency[34,38,48-50,77]. It is recognized that dicarboxylic amino acids are least potent[34,82]. Most ACE-inhibitory peptides are comprised of 2-12 residues, but they can be as many as 27 residues in length[71]. Regardless, ACE appears to have wide substrate specificity, a possible consequence to the presence of two active sites[62,67]. While it seems that much has been learned regarding substrate preference for ACE, the actual structure-function relationships of these peptides is unclear[38,67,71,82] and individual and occasionally conflicting observations contribute to the complexity of the picture. For example, positive correlations between side chain hydrophobicity and potency and between side chains without positive charges occupying the C-terminal end and potency have been suggested by some[74]. Others observed a reduction in potency associated with the size of the side chains of the neighboring residue of the C-terminus and a positive contribution from residues with positive charges[74]. Furthermore, some researchers suggest a possible influence on peptide potency of N-terminal residues, potentially enhancing potency or greater ACE affinity when occupied by branched chain amino acids, though this appears to be neither essential[62,65,67], nor well-supported[77] and most studies focus on the C-terminal occupants. Potency could also be influenced by the conformers resulting from the preparation of bioactive peptides, particularly of those containing proline, most often when present at the C-terminus. Specifically, it was found that the *trans* form was more potent than the three *cis* conformers studied, offering a reason for differences found in ACE inhibition between peptides with the same primary sequences[83]. Finally, although

some studies report mixed inhibition[43] most support the competitive inhibition of bioactive peptides[34,40,84,85]. In contrast to the identification and characterization of ACE-inhibitory peptides, other investigations identified casein-derived peptides with bradykinin-potentiating abilities and that may act as opiates[86]. While *in vitro* findings are important for optimal production of peptides that survive digestion and absorption processes with predictive ACE- inhibitory effects, inarguably, the *in vivo* effects ultimately define their worth.

Assessing the significance of blood pressure reduction in humans

Investigators have evaluated the *in vitro* and *in vivo* potential of bioactive peptides to inhibit ACE and/or lower blood pressure. Before discussing the relevant human studies of bioactive peptides, in particular, it is useful to consider the potential clinical relevance or public health implications when evaluating blood pressure reductions, even when these may not be found to be statistically significant in studies with small sample sizes. A reduction in SBP of 2 mm Hg may reduce risks for stroke and MI by about 4%[87,88], and reductions of 3-5 mmHg in SBP is associated with reduced risks for stroke and MI by 15% and 10%, respectively[89]. It has also been found in clinical trials on blood pressure medications that a mean reduction in DBP by 6 mmHg is associated with a 40% reduction in stroke risk[90].

Whey-derived bioactive peptides

Whey protein

A by-product of cheese making[68,91], whey is a heterogeneous mixture of proteins with bioactive and functional food potential that accounts for approximately 20% of total milk protein[68,92]. Once considered a waste product or something to be made into low value products[68,92], advancements in technology are unveiling whey protein's true worth through further discovery of its physiological potential[92]. Interestingly, its use in products only began in the early 1990's, primarily as an additive to sports nutrition products. Its use was favorable because of its digestibility, efficient absorption, and ample supply of branched chain amino acids[68]. Devoid of flavor, whey proteins are also used to enhance the protein content of food products without sacrificing or altering product taste[68]. With employment of various technologies, whey proteins can be processed into powders, concentrates, isolates, and hydrolysates[68] for various purposes.

Whey protein is a composite of various protein fractions including α-lactoglobulin, immunoglobulin, and bovine serum[68,93]. Its main fractions, comprising 70-80% of whey protein, are α-lactalbumin (α-La) and β-lactoglobulin (β-Lg)[92-94]. More specifically, β-Lg is a small globular protein of 162 amino acids in length and is considered the major bovine whey protein, accounting for about 50% of the total whey[91,92,94], though an estimate of 60% contribution has been previously suggested[82]. α-La is the second major protein (accounting for 20-25% of bovine whey), comprised of 123 amino acids[91]. Furthermore, minor whey proteins include lactoferrin, immunoglobulins, and proteose-peptones that are believed to have antimicrobial

activities[93]. Whey-derived bioactive peptides with ACE -inhibitory or blood pressure lowering abilities have been termed lactokinins[34]. Though not as well-studied as casein, current research supports whey as an excellent source of bioactive peptides, thereby keeping researchers resolute in their quest for further discovery.

Antihypertensive effects of whey-derived peptides *in vivo*: animal studies

At present, the vast majority of work on whey-derived peptides with ACE inhibition/blood pressure lowering potential has been *in vitro*-based studies. This section discusses the limited number of *in vivo* and *ex vivo* investigations that tested for blood pressure lowering effects using animal models.

Ex vivo ACE inhibition

Although *ex vivo* studies do not take into account the susceptibility of bioactive peptides to further hydrolysis during digestion and absorption processes, they allow for the ability to directly examine and confirm the potential for peptides to inhibit ACE in animal tissue. A study by Centeno et al[81] used *in vitro* studies on ACE inhibition from lactoferricin B- related peptides compared to synthetic hexapeptides to determine the most potent ACE inhibitors that were then further examined in *ex vivo* functional assays. Lactoferricin B (Lfcin B) is a peptide consisting of 25 amino acid residues derived from the whey fraction, lactoferrin[81]. The *ex vivo* studies required dissection of the common carotid arteries of 30 male white rabbits. Following washout from exposure to angiotensin I, each segment was exposed to one of the following: a second exposure to angiotensin I, two exposures to angiotensin II, separated by exposure to Captopril as a

negative control, pre-incubation with Captopril (from 1nM to 1 μM) followed by a second exposure to angiotensin I, or pre-incubation with one of the ACE inhibitory peptides (20 μM) followed by a second exposure to angiotensin I. A second measurement of contractile capacity response followed the treatments, expressed as the percent of the respective first contraction. As expected, angiotensin I provoked transient contractions in arterial segments. As one might expect from a potent ACE inhibitor, in segments pre-incubated with Captopril at 1 μM, this contraction was unobserved, though returned following wash-out. In contrast, Captopril did not affect the angiotensin II-induced contractions, supporting that angiotensin I is responsible for ACE-dependent contractions and that formation of angiotensin II is required for the vasoconstriction. Interestingly, the 5 peptides exerted different inhibitory effects on contractions, only one of the two Lfcin B-derived peptides of which significantly reduced angiotensin-I induced contractions along with two of the three synthetic peptides.

Results from this study support many *in vitro* studies which suggest that whey-derived blood pressure lowering peptides may exert their effects through ACE inhibition, in part by inhibiting angiotensin-I induced contractions. However, other mechanisms have been explored and are presented next.

The potential for other mechanisms for blood pressure reduction

Investigations into the antihypertensive abilities of bioactive peptides largely focus on ACE inhibition. However, some researchers have investigated the potential for other mechanisms behind the observed blood pressure lowering effects of these peptides. These other mechanisms include the ability of certain peptides to antagonize the AT_1 and

AT_2 subtype receptors of angiotensin II[95]. Another suggestion is the potential ability of certain peptides to bind to opioid receptors; some have been found to lower blood pressure through vaso-relaxation, as described next.

Derived from hydrolyzed α-La, α-lactorphin is a peptide comprised of four residues (Tyr-Gly-Leu-Phe) that is believed to possess both opioid-like and ACE-inhibitory potential[96]. In a multifaceted study, relationships between subcutaneous administrations of α-lactorphin and blood pressure in spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto rats (WKY) were examined and revealed dose-dependent reductions in the blood pressure of SHR. The lowest dose capable of attenuating blood pressure was 10μg/kg. Interestingly, maximum reductions in blood pressure did not result from the highest dose administered (1mg/kg), but after injection of 100 μg/kg. This maximal effect was reached 50-100 minutes post-administration and reverted to baseline values by 200 minutes. No significant blood pressure lowering effect was observed after administration of any of the individual amino acids that comprise α-lactorphin. The dose-response relationship for blood pressure in WKY rats was quite similar to SHR, resulting in reductions of 16 and 12 mmHg for systolic and diastolic blood pressures, respectively, at a dose of 100 μg/kg. A unique feature to this study is the use of antagonists to help establish a mechanism, or to rule out potential contributors to the observed blood pressure lowering effect. Researchers employed pre-treatment experiments in which SHR received either a bradykinin B2 receptor antagonist, an opioid receptor antagonist, or saline 30 minutes prior to administration of 100 μg/kg α-lactorphin. The bradykinin B2 receptor antagonist did not affect blood pressure or the effect of α-lactorphin administration on blood pressure. Similarly, blood pressure was

not affected by the lower doses of the opioid antagonist; however, a significant decrease in both SBP and DBP at higher doses was observed. Interestingly, the hypotensive effects exerted by α-lactorphin at its most influential dose of 100 µg/kg was antagonized and reversed, resulting in a hypertensive response. These findings suggest that the mechanism behind the ability of α-lactorphin to reduce blood pressure involves opioid receptors.

The effects of acute and chronic whey treatments in SHR

Some studies have investigated the *in vivo* blood pressure lowering effects of a single dose of whey protein treatment. In a study by Muguerza et al[40], after fermentation with wild strains of bacteria and assessment of *in vitro* ACE-I activity, samples of whey fractions with highest ACE inhibition were administered to male SHR and WKY through gastric intubation as a single dose of 5 ml/kg (or about 1.5 ml) of either water or unfermented milk or as 50 mg/kg Captopril in a deionized water solution. Treatments with the fermented milk samples resulted in a similar degree of reduction in DBP to that observed with Captopril, but a lower reduction in SBP. In addition, the maximum lowering effects were observed in the fermented milk groups sooner, around 4 hours post-administration, compared to Captopril (6 hours). Blood pressure returned to near baseline values 24 hours after treatments. In contrast to findings from other studies[96], these researchers observed that none of the treatments lowered blood pressure in normotensive rats, suggesting safety for use as a functional food without posing risk to normotensive individuals[40].

A study conducted by Tsai et al[43] employed a combined method of lactic acid fermentation and proteolysis of milk to determine the blood pressure lowering effects of a chronic administration of fermented whey. Using the most potent fraction, oral administration of 150ml of diluted fermented milk whey was given to 8 SHR. The increase in SBP by 12 weeks in the control and treatment groups was significantly different; the SBP in the fermented whey group was lower, signifying a slowed progression in the development of hypertension. After continued oral administration for 8 weeks, the SBP of the treatment group was significantly lower than the control, with an average reduction of 15.9 mmHg. A similar trend was observed in DBP, with values increasing with age and significant differences not apparent until later in the experiment. The use of analytical techniques allowing for determination of the fraction sequence, further use of *in vitro* ACE inhibition assays, and confirmation of survival from digestion allowed researchers to confidently infer that this fraction of the whey hydrolysate was the main contributor to the observed blood pressure lowering effects.

The findings of these studies support the ability of acute and chronic doses of hydrolyzed whey to reduce blood pressure in SHR. Furthermore, the potential for safety following ingestion of antihypertensive peptides in normotensive rats is suggested.

Does in vitro ACE inhibition equate to in vivo blood pressure reduction?

The worth of *in vitro* investigations is evident. These studies provide information on optimal production of peptides, their structure-activity relationships, and they have demonstrated that potency can be lost, maintained, or increased following further hydrolysis with digestive proteases. However, as previously mentioned, researchers

suggest that such findings need to be taken with caution and that the potential for other influences including the presence of other peptides, chain length, and pH changes during passage from the stomach to the small intestine may alter susceptibility to further cleavage, rendering peptides more or less potent[79].

Murakami et al[95] examined the *in vivo* effects on SBP of ACE-inhibitory peptides derived from whey, casein, ovalbumin (from egg white), wheat gluten, and soybean administered to SHR through gastric intubation (in a solution of 2 mg peptides/2 ml distilled water). Blood pressure measures were taken before and 6 hours after administration. The greatest change was observed in a whey-derived protein treatment group (- 21.2 ± 16.9 mmHg). It is interesting to note that while a sample from ovalbumin scored the highest percent inhibition of ACE *in vitro* (shared by a whey sample, 78.2%), it exerted the *weakest* antihypertensive effects *in vivo*. In contrast, samples of whey and casein scored medium degrees of ACE inhibition (defined as >51% inhibition), but exerted strong antihypertensive effects, with reductions greater than 18.0 mmHg. Furthermore, when the whey fraction showing the strongest *in vivo* antihypertensive effects was subjected to further fractionation, with its sub-fractions tested for ACE inhibition, only one showed inhibition with an IC_{50} of 928µM (considered very weak inhibition). Upon administration of this peptide fraction to another group of 5 SHR, subsequent blood pressure measurements revealed decreases of about 19.0 mmHg at 6 hours and 21.4 mmHg at 8 hours, both significantly different from the control group. Blood pressure values nearly reached baseline after 10 hours post-treatment. The findings of this study support the need for human-based evaluation as the quantified potencies of certain peptides *in vitro* were not supported by *in vivo* observations.

Accordingly, research on humans is essential before functional foods with blood pressure lowering ability become widely available.

Antihypertensive effects of whey-derived peptides *in vivo*: human studies

Whey-derived bioactive peptides on blood pressure attenuation in humans have not been well-studied, although conflicting results from *in vitro* and *in vivo* studies and the development of functional foods containing these peptides underscore the need for studies in humans. In an extensive search with eleven databases, only two studies investigating whey-derived peptides in humans were found, both assessing the effects of chronic intake of whey hydrolysate.

Pins and Keenan[97] conducted the first human pilot study to determine the antihypertensive and other cardiovascular effects of a whey protein hydrolysate. Thirty subjects were randomized to either the active protein group (containing 20g of hydrolyzed whey protein) or the control protein group (unmodified whey protein) to consume without food each day for 6 weeks. By the end of the first week, there was a mean SBP reduction of about 8.0 mmHg and a DBP reduction of 5.5 mmHg in the treatment group. Analyzing both size and duration of change with an AUC approach, an AUC decrease in SBP during the experimental period (from weeks 1-6) revealed a significant 216 ±24 mmHg reduction for the treatment group, compared to a 27 ±19 mmHg reduction for controls (a difference of 189 mmHg). Significant differences existed for DBP as well, with reduction of about 151 mmHg for treatment and 33 mmHg for control, a net difference of 118 mmHg. Furthermore, it was discovered that the whey protein hydrolysate reduced total cholesterol and LDL-cholesterol (both by 13%).

Findings of this study support general findings from *in vitro* and *in vivo* animal work, that bioactive peptides can lower blood pressure *in vivo* and may offer additional benefits. However, it should be noted that a retraction was made to this study due to reporting of falsified data[98].

Regardless, the results from the Pins and Keenan study were not supported by findings from a study by Lee et al[99]. In this study, 54 hypertensive subjects were asked to ingest either 125 ml of a milk drink treatment that contained whey peptides or placebo in the morning for 12 weeks. It was found that no significant changes occurred in blood pressure from the beginning to the end of the experiment. Although IC_{50} data were not provided, it is worth noting that these peptides were tested for ACE inhibition by other *in vitro* tests. Results of this study do not support the findings of Pins and Keenan. The conflicting results of the two human studies presented exemplify the need for more human-based studies when considering the blood pressure lowering potential of whey-derived bioactive peptides.

Casein-derived bioactive peptides

Casein and the lactotripeptides

Accounting for around 80% of milk protein, casein encompasses a family of phosphorylated proteins that include α_{S1}, α_{S2}, β, and κ-caseins that aggregate together in milk, forming casein micelles[93]. Caseins provide a wealth of amino acids upon digestion as well as calcium and phosphorus; the fragments bound to these minerals are called casein phosphopeptides and have been studied for various interests[93]. Industrial use of casein includes sodium caseinate, a precipitate of casein micelles, as an emulsion

stabilizer[93]. Interestingly, certain casein-derived peptides are found to possess opioid-like activity similar to morphine and have been termed *casomorphins*, with most originating from β-caseins and to increase gastrointestinal transit time and prevent diarrhea[93]. Furthermore, κ-casein derived peptides have been termed *casoxins*, and act like opioid antagonists. Casein-derived peptides, IPP and VPP, are the most extensively studied ACE-inhibitory peptides and are referred to as the *lactotripeptides*. These have been incorporated into commercial fermented beverages, including Calpis (Calpis Co., Ltd, Shibuya, Tokyo) and Evolus (Valio Ltd., Finland).

Antihypertensive effects of casein-derived peptides in vivo: animal studies

Casein-derived peptides may improve renal and overall cardiovascular health

There have been several studies in animal models that have examined the effects of casein-derived peptides on ACE inhibition and blood pressure reduction, several of which report additional improvements to cardiovascular-related and renal health that may contribute to blood pressure reduction.

Some investigators[100] have found that supplementation with β-casein rich hydrolysate from goat milk in young SHR for 12 weeks partly prevented increases in SBP, with significantly lower measurements than the control group. Furthermore, the hydrolysate-supplemented diet strongly prevented renal hypertrophy, significantly increased vasodilation in response to acetylcholine in aortic rings, and significantly reduced ACE activity in the heart, aorta, and kidney of SHR compared to other groups. In agreement, others noted reductions or delayed increases in SBP in SHR after

administrations of casein-derived peptides as well as improved relaxation in precontracted arterial[101,102] and mesenteric[103] rings, and reduced left ventricular hypertrophy[103]. One study observed time-dependent relationships between tripeptide exposure and protective endothelial function effects with improved relaxation and less arterial contractions than control[104].

In contrast to findings of renal and vascular health improvements, other researchers have found significant hypotensive effects on SBP but no changes in relaxation responses or ACE activity[105]. One study found significant reductions in SBP after 8 weeks of ingestion of either of two different milk products: (1) containing tripeptides, though there was no effect on aortic relaxation or arterial relaxation responses, or (2) containing tripeptides and plant sterols, resulting in enhanced aortic relaxation and reduced serum ACE activity in SHR[102].

In summary, there may be additional cardiovascular benefits beyond blood pressure lowering that follow treatment with casein-derived peptides. However, more investigation is needed to establish these benefits more firmly.

The potential for other mechanisms for blood pressure reduction

In support of the widely accepted ACE-inhibiting mechanism for blood pressure lowering abilities of dairy-derived peptides, some investigators reported reductions in both aldosterone and serum ACE concentrations and reduced angiotensin I to angiotensin II contraction ratio in salt-induced hypertensive diabetic mice[104]. Additionally, dose-dependent reductions in blood pressure have been reported with associated findings of non-competitive inhibition of ACE[71]. In contrast, a study by Fuglsang et al[106] examined

the ACE-inhibitory effects, as opposed to hypotensive effects, of various peptides using different models to indirectly measure the conversion of angiotensin I to angiotensin II, or reversion of blood pressure after continuous infusion of angiotensin I, or hydrolysis (deactivation) of bradykinin. No significant effects were observed. It was suggested that the peptides were inactivated upon hydrolysis by serum enzymes. However, these results may support the potential for other blood pressure lowering mechanisms. Interestingly, Ehlers et al[21] found that IPP and proline treatments significantly enhanced Ang-(1-7)-induced relaxation in arterial rings and bradykinin responses. This finding suggests an alternative mechanism for achieving hypotensive effects, that is, through the ACE2-Ang-(1-7)-Mas axis. Through this axis, angiotensin I and II are metabolized to Ang(1-7) by ACE2, thereby inducing vasodilation in vessels through the Mas receptor, an antagonist to the AT_1 receptor that promotes pressor responses[21].

Finally, a unique study[107] used gene profiling of the aorta of SHR after multiple oral doses of lactotripeptides and discovered changes in gene expressions that align with *in vivo* responses to ACE inhibitors, though such changes were much smaller than those induced by pharmacological ACE-inhibitory agents. Although the genes associated with the RAAS were not significantly changed, certain genes associated with vascular function and blood coagulation were. For example, *connexin* and *eNOS* expressions in the aorta of SHR administered the tripeptides significantly increased[107]. Increased *eNOS* expression is reported in other studies as well[103]. Furthermore, these various gene expression changes align with changes observed in previous studies with administration of different ACE inhibitors[107].

Does in vitro ACE inhibition equate to in vivo blood pressure reduction?

Some studies have found that significant reductions in blood pressure correlate with *in vitro* ACE-inhibitory activity[108-110] while others have shown different hypotensive effects *in vivo* of different peptides sharing similar *in vitro* IC_{50} values[111]. Differences in chain length might contribute to such findings, as longer peptides may be more susceptible to gastrointestinal proteolysis, but they can also result in peptides with considerable antihypertensive effects when absorbed[112]. Furthermore, an unobserved significant hypotensive effect *in vivo* may be partly explained by the lack of hydrolysate peptides detected in blood, a potential consequence to rapid degradation[113]. As supported by findings of whey-based studies, it should not be immediately assumed that *in vitro* inhibition will result in *in vivo* blood pressure reduction, underscoring the importance of animal and human based studies.

Studies on safety and toxicity of casein-derived peptides

As with any bioactive functional food candidate, safety and toxicity studies are essential. Anadón et al[114] performed oral toxicity studies on rats that involved a single, acute dose of 2000 $mg \cdot kg^{-1}$ of a powdered casein hydrolysate dissolved in distilled water or repeated daily doses of 1000 $mg \cdot kg^{-1}$ for 4 weeks. After observing for extended periods of time, rats in either study presented with no clinical or behavioral abnormalities, no changes in body weight, food or drink intakes, and no statistical differences in hematological or clinical parameters were noted. Furthermore, there was no mortality, even with the chronic high dose treatment. Such findings support the safety

of incorporating casein hydrolysate derived from food-grade pepsin[114] into a functional food.

Antihypertensive effects of casein-derived peptides *in vivo*: human studies

Administration of casein-derived peptides

Casein-derived peptides are the most extensively studied of the food-derived ACE-inhibitory peptides *in vivo*, with strong focus on the two lactotripeptides, IPP and VPP. Often, casein-derived peptides are incorporated into fermented milk products[88,90,115,115], yogurt-based beverages[116-118], fruit juices[119], commercial drinks *Evolus*[120] or *Calpis*[121], and have even been delivered in a spread[89]. Interestingly, a number of studies have administered casein hydrolyates containing the lactotripeptides IPP and VPP in tablet form[122-125].

Chronic dosing of casein peptides

Although current investigation is insufficient, interest should be paid to potential differences in hypotensive effects between oral administrations of isolated lactotripeptides and hydrolysates containing these (and other) peptides. Nonetheless, concentrations of IPP and VPP in test products have ranged from 0.72 mg to 10 mg/100g[90,125], with treatment trials ranging from 4 weeks to 24 weeks. While most studies have not examined the potential for dose-response effects, Mizuno et al found that both increased doses and prolonged exposure to treatment result in greater reductions in systolic and diastolic blood pressures in 131 Japanese subjects in a randomized single-blind placebo-controlled study[125]. Another study noted that long-term intake (24 weeks)

of fermented milk that contained these tripeptides was required to see reductions in blood pressure that may have clinical relevance in 89 Finish subjects[88]. It is worth noting that IPP and VPP concentrations increased midpoint during the 24-week intervention nearly five-fold, therefore; it is important to consider that the increased concentrations may have also contributed to the observed reductions.

In a double-blind, placebo-controlled randomized clinical trial by Ishida et al[123], 48 Japanese subjects with normal or high-normal blood pressures or mild hypertension ingested placebo tablets or 20 tablets of 5.0 g/d casein hydrolysate, containing 7.5mg VPP and 9.6 mg IPP, estimated to be about 5 times more than the doses determined previously by these investigators to be effective for 4 weeks. It was found that SBP, in general, was significantly reduced compared to placebo and after 4 weeks, a reduction of 8.0 mmHg was observed. Though not statistically significant (but possibly clinically relevant), DBP reduced by almost 4.0 mmHg after 4 weeks compared to the placebo group. Furthermore, SBP of mildly hypertensive subjects reduced significantly, and after 4 weeks, SBP and DBP reduced by almost 15 mmHg and 7.2 mmHg, respectively. No differences were observed in subjects with high-normal blood pressure. Importantly, no adverse effects associated with ingestion of the tablets and no significant changes in blood pressure in normotensive subjects or exaggerated lowering effects were observed. This supports the use of such peptides in food products without imposition upon safety measures.

Seppo et al[120] found significant reductions in systolic blood pressure among hypertensive subjects, both treated and untreated with antihypertensive medications, following treatment of 150 mL of the Finnish product, Evolus, for 21 weeks. There was

also a reduction in diastolic blood pressure compared to control groups. Mean changes in blood pressure were about 14 mmHg (SBP) and 8 mmHg (DBP) in subjects not taking antihypertensive medications and nearly 13 mmHg (SBP) and 7 mmHg (DBP) among those treated with medications. Importantly, the interaction effects of antihypertensive medications and ACE-inhibitory peptide treatments were considered insignificant and no adverse effects were reported. Furthermore, researchers did not discount the possible contribution to blood pressure reduction from the higher calcium content in the treatment drink compared to the control.

In summary, chronic doses of casein-derived peptides may reduce blood pressure in hypertensive individuals. Little is known about the effects of treatment resulting from a single acute dose; therefore, more research on acute dosing may be warranted.

The use of placebo and ambulatory blood pressure monitoring in casein-derived peptide blood pressure studies

There are many challenges when studying the effects of various treatments on blood pressure, because it is easily influenced by a variety of external factors. When studying the effects of casein-derived peptides on blood pressure, several researchers took certain factors into account. Many studies assessing the hypotensive effects of lactotripeptides have included the use of placebo groups[88-90,116-119,121,123,125] to minimize the potential for the placebo effect on blood pressure that has reportedly interfered with observations of significant effects in some studies[115]. Additionally, many studies have assessed blood pressure changes primarily through ambulatory blood pressure monitoring (ABPM), in effort to minimize confounding variables associated with increased

office/clinical blood pressure measurements, often referred to as the *white coat effect*[88,90,115,117-119]. ABPM is considered a better, more reliable method for blood pressure assessments[90]. One study found more than 15 and 10 mmHg increases in SBP and DBP, respectively, in office compared to ambulatory baseline measurements[90]. Another found statistically significant reductions in 24-hour ABPM in subjects after ingestion of IPP and VPP every day for 10 weeks, with reductions by 7.6 mmHg in SBP and 4.1 mm Hg in DBP[90]. Some studies examined the hypotensive effects of IPP and VPP ingestion on different groups of people, categorized by degree of hypertension[90,121,123], some of which resulted in significant reductions in SBP in individuals with mild hypertension compared to high-normotensive subjects[121,123], suggesting that treatment may be more effective among individuals with greater degrees of hypertension. More work is needed to investigate blood pressure responses in highly hypertensive individuals.

Do lactotripeptides reduce RAAS proteins or ACE activity?

A small number of studies of lactotripeptides examined ACE activity and noted no changes[118], or measured angiotensin I and II concentrations and found no changes[121], or slight decreases[118]. While it has been suggested that the typical doses of tripeptides utilized in these studies are too small to have sufficient impact on the RAAS proteins[121], investigators have considered the possibility of blood pressure lowering effects of bioactive peptides exerted by unidentified mechanisms[118,119,121].

Other vascular improvements as a result of casein peptide treatments

Similar to animal studies, some researchers have examined other parameters for measurement of cardiovascular health benefits of casein-derived peptides in addition to arterial blood pressure in humans. One study investigated the effects on plasma lipid profiles following peptide treatment but found no significant changes[90,119]. Another study considered the effects of tripeptide consumption on arterial stiffness and endothelial function in 89 subjects and found significant decreases in the aortic augmentation index after 24 weeks of treatment and that this reduction was most pronounced in individuals exhibiting metabolic syndrome. No changes were found in endothelial function but serum total cholesterol was also lowered in the treatment group compared to placebo[88].

Interestingly, others examined effects on central arterial systolic blood pressure as it might provide a better picture for cardiovascular risk and treatment response and found significant reductions of almost 18 mmHg and 22 mmHg at 6 and 9 weeks, respectively. These reductions in central SBP resulted sooner and were more profound than those observed in brachial SBP. Finally, reduced arterial stiffness was also observed[122].

Other casein-derived hydrolysates

While the lactotripeptides remain the most extensively studied of the dairy-derived peptides in human subjects, some researchers investigated the effects of other peptides derived from casein. In a study by Townsend et al[124], significant reductions in average SBP (by 9.2 mmHg) and DBP (by 6 mmHg) in 10 subjects of various ethnicities (African American, White, and Asian) as recorded by ABPM after ingestion of 6 tablets containing a peptide derived from bovine casein, known as C12 (100mg) and alginic acid

(1754 mg), a polysaccharide of certain algae. Reductions of 4.5 and 6.5 for SBP and DBP corresponded to intakes of 6 tablets of C12 alone (200 mg), though these reductions were not statistically significant. These findings provide insight to an alternative casein-derived peptide with potential hypotensive effects.

Fermented milks and yogurts

Dairy-derived peptides have been produced by fermentative processes in milks and yogurts and tested for blood pressure lowering ability. In a study by Pihlanto et al[126], two new peptides produced by milk fermentation with *Lactobacillus jensenii* were identified as being primarily responsible for ACE -inhibitory activity *in vitro*. However, the highest reduction of 10 mmHg in blood pressure observed 90 minutes after administration was considered insignificant. Ramchandran et al[127] examined the influence of probiotics added to yogurts inoculated with starter cultures. ACE -inhibitory activity (*in vitro*) decreased in samples that were freeze-dried but increased in those added to SHR pellet diets. The authors suggested the possibility that post-fermentative processes may influence ACE inhibition. In general, ACE- inhibitory activity was highest in samples containing probiotics. In support of others' findings, significant blood pressure lowering effects were observed in animals who consumed feeds with less potent ACE -inhibitory activity *in vitro* and it was noted that high potency *in vitro* did not positively correlate with high antihypertensive effects. Moreover, in support of others, LDL cholesterol-lowering effects were also observed. Seppo et al[128] found significant reductions in both systolic and diastolic blood pressures, though no changes in heart rate, in subjects after consumption of 150ml sour milk fermented by Lb. helveticus LBK- 16

H, a strain that remains viable in fermented milk and is well-known to produce the hypotensive lactotripeptides IPP and VPP compared to a group consuming an equal quantity of a sour milk control. During a four-week follow-up, blood pressure measurements regressed to near baseline values, and were similar to the control group. Interestingly, 85-95% ACE inhibition activity was determined *in vitro* for the fermented sour milk and 100% for the synthesized VPP and IPP, both considered high inhibitory activities. Given that the significant reduction in blood pressure was only observed after treatment with the sour milk and lactotripeptides, it can be inferred that the attenuation was due to the lactotripeptides. Furthermore, the *in vitro* inhibitory and *in vivo* hypotensive effects were in agreement, reinforcing the suggestion that casein-derived lactotripeptides have the most bioavailability.

Current limitations in studies of casein-derived peptides

Although the effects of casein-derived peptides, IPP and VPP in particular, are the most extensively studied dairy-derived peptides on ACE inhibition, ethnic differences and a lack of awareness of the bioavailability of ACE inhibitory peptides remain an important limitation to this research.

Significant effects exerted by these peptides are mostly reported in Asian subjects and have not been successfully reproduced in Caucasians[117,119]. Suggested reasons include the influences of diet, genetics, and baseline blood pressures among different ethnic groups, as well as inter-study differences in doses of treatment per kg body weight[117]. A study by Engberink et al[118] found no changes in SBP and DBP in 135 Dutch subjects after an 8-week daily consumption of a 200 ml low-fat yogurt drink

containing 4.2-5.4 mg IPP and 5.0-5.8 mg VPP produced by fermented milk, enzymatic hydrolysis, or chemical synthesis. Another study on 162 Scottish subjects found no significant effects[117], but diurnal DBP was significantly reduced in normotensive and high-normotensive Mediterranean subjects[119]. It appears much more research is needed on the effects in various ethnic groups of European, African, and Latin American origins.

These results suggest that there may well be differences in ACE inhibitory potential of these peptides and much more work is needed in order to understand the bioavailability of these proteins.

Soy-Derived Bioactive Peptides

Soy and soy proteins

For centuries, soy has been a mainstay of the diet of many Asian populations[129,130]. Interestingly, although soy foods have been consumed by vegetarians and Seventh-day Adventists in the West for about a century, large scale production of tofu in the United States only began in the 1970s[130]. The introduction of this legume to the general population at this time was largely influenced by its adoption by consumers with health interests and an ecological mindset, believing that soy was a high quality protein source low in saturated fat that was an alternative to animal products, that could be produced more efficiently[130]. Production and consumption of soy foods increased substantially during the 1990s[129,130] with the general recognition of soy's potential health-promoting benefits resulting from various biological activities of phytochemicals, including isoflavones[130]. Furthermore, its popularity reached a high in 1999 after approval by the Food and Drug Administration of a health claim on labels of foods

containing soy protein as protective against coronary heart disease[129-131]. The potential effects of soy proteins and associated phytoestrogens, specifically the isoflavones, on various health conditions beyond hypertension, have been studied and are reviewed elsewhere[129,130,132]. Increased use in industry is matched by increased waste production, particularly following the production of soymilk and tofu. Waste consists of okara and soy whey[72]. Interestingly, because of its high protein and sugar content, investigation into the use of soy whey as growth medium for probiotics to yield bioactive peptides has already been undertaken[72].

Soybean contains about 40% protein consisting primarily of storage proteins glycinin and β-conglycinin[129,132] that contain all essential amino acids required by humans[129]. Contributions of these storage proteins to total protein content in soybean have been estimated to be between 65 and 80%[132] to as much as 90%[129]. Results of various studies have stimulated investigations into the potential bioactivity of soy hydrolysates as shared below.

Antihypertensive effects of soy: *In vivo* animal studies

Does in vitro ACE inhibition equate to in vivo blood pressure reduction?

A meaningful attribute of relatively few studies on soy-derived peptides is the inclusion of *in vivo* experiments preceded by *in vitro* characterization of ACE-inhibitory peptides as they allow for the establishment of relationships between theory and reality, that is, between observed potent ACE inhibiting peptides *in vitro* and consequent physiological effects (blood pressure lowering) *in vivo*.

A study by Kodera et al[80] observed dose-dependent responses in the SBP of SHR after single oral administrations of 50-1000 $mg \cdot kg^{-1}$ body weight of soy protein hydrolysate produced prior to administration, with significant reductions noted after intakes of 100 $mg \cdot kg^{-1}$ or greater occurring within two hours post-ingestion. Additionally, blood pressure attenuation was compared among treatments of hydrolysates derived *in vitro* from casein, soy, milk whey, and wheat gluten protein sources in SHR at a dose of 500 $mg \cdot kg^{-1}$ body weight and found that potency (related as IC_{50} values) mirrored the intensity of the antihypertensive effects for each of the hydrolysates one hour post administration, with intensities greatest for casein and soy hydrolysates (in that order). Furthermore, it was observed that while casein and soy effects were immediately intense, the SBP-lowering effects of whey and gluten outlasted these, though SBP reduction was not significant with whey hydrolysate. These researchers suggested the possibility of a pro-drug-like effect of peptides from the latter sources, in which further intestinal hydrolysis might yield more potent, active fragments from inactive, or less active, forms.

Underscoring the value of *in vivo* studies, Chen et al[133] randomized 24 SHR into one of three diets containing 5% casein hydrolysate, soy acid-precipitated protein hydrolysate, or soy protein isolate for 8 weeks and reported that, although casein and soy protein hydrolysates possessed similar IC_{50} values, their effects were quite different. Specifically, the SBP of casein hydrolysate-fed SHR increased during the study while significant reductions were observed in the other two soy-fed groups. It is worth noting that IC_{50} values were lowest in the soy protein forms which exhibited the greatest

antihypertensive effects but these values are not necessarily strong predictors when used alone.

Taken together, results from these studies demonstrate the discrepancy between *in vitro* ACE inhibitory potential and *in vivo* blood pressure lowering abilities, thereby confirming the importance of *in vivo* investigations.

Alternative mechanisms for blood pressure lowering

Many studies support the mechanism of peptide blood pressure lowering effects through ACE inhibition. A study by Martin et al[134] considered the effect on pressor responses to angiotensin I with ACE inhibition, postulating that if a soy diet (containing soybean oil and unhydrolyzed soy protein) reduced blood pressure through ACE inhibition, pressor responses to angiotensin I should be reduced. To examine this, groups included female SHR who underwent bilateral oophorectomy, sham-operated female SHR, and male SHR with members of each allocated to a casein- control or soy diet that replaced casein protein. It was found that mean arterial pressures were significantly reduced in SHR rats fed the soy diet that were subjected to oophorectomy in comparison to casein-fed counterparts. Dose-response curves comparing angiotensin I with change in mean arterial pressure did not reflect theoretical shifts indicative of ACE -inhibitory activity. Furthermore, dose-response curves regarding angiotensin II revealed significant but minimal reduction in pressor responsiveness in female SHR fed a soy diet with modestly reduced blood pressure. These researchers concluded that the observed blood pressure lowering was not a result of direct ACE inhibition. However, in this study, SHR were fed a soy diet that included unmodified protein and soybean oil, not hydrolyzed soy.

Therefore, the effects of soy peptides alone on blood pressure and ACE inhibition were not assessed. While results of this study suggest the potential for an alternative mechanism for the observed blood pressure reductions, others further support ACE inhibition as the primary mechanism. While results of this study suggest the potential for an alternative mechanism for the observed blood pressure reductions, others further support ACE inhibition as the primary mechanism.

In a study by Wu et al[135], 20 female SHR were divided into 5 groups that included a control without drug or peptide treatments, Captopril-administered at 50 $mg \cdot kg^{-1}$ body weight, and groups receiving soy ACE- inhibitory peptides at 100, 500, and 1000 $mg \cdot kg^{-1}$ body weight per day that replaced protein in the standard chow diet for 30 days. Significant reductions in SBP were found at 6 days into the experiment for SHR consuming 500 and 1000 $mg \cdot kg^{-1}$ soy peptides and 12 days for those consuming the lowest dose with SBP reductions from about 193.25 to 155 mmHg at day 30 for this dose, suggesting a progressive lowering effect. Dose-dependent reductions in blood pressure were also reported. Similar to findings in some whey studies, Wu et al noted no change in SBP for a group of normotensive rats consuming the highest amount of soy peptide, thereby reaffirming the safety in use of bioactive peptides in normotensive individuals. Finally, these researchers found no significant changes in aorta and lung ACE activity in soy-fed SHR but found increased activity in serum ACE and reduced activity in aorta ACE in SHR receiving Captopril for 30 days. Interestingly, this effect of Captopril has been previously reported and, though uncertain, it is suspected that ACE inhibitors may induce ACE expression. Given that serum sodium levels were significantly reduced in soy peptide-fed and Captopril-treated groups, a sodium excretion effect is possible.

Specifically, angiotensin II promotes sodium retention, and inhibition of ACE results in its reduced production and an increase in sodium excretion. Such findings bring forth the additional effects of ACE inhibition beyond reduced vasoconstriction that contribute to the antihypertensive effects.

Yang et al[136] divided 40 rats into groups fed diets with varying contributions of soy protein hydrolysate (control 0, 1%, 3%, and 5%) for 6 weeks. Hypertension was induced through the use of a nitric oxide synthase inhibitor (NOS-I), L-NAME. Previous studies recognize a relationship between angiotensin II and NO, and that inhibition of NOS may induce endothelial dysfunction and hypertension while ACE inhibition may result in up-regulation of eNOS, promoting endothelial health, in addition to the reduction of angiontensin II[136]. As predicted, the SBP and DBP of rats treated with L-NAME inhibitor increased significantly throughout the experiment. However, in comparison, the blood pressure of rats fed 3% and 5% soy protein hydrolysate diets were significantly lower. Comparatively, significant differences in rats fed 5% soy protein hydrolysate included lower ACE activity in the heart, a reduction in heart TNF-α, lower MDA levels (malonaldehyde, a marker for oxidative stress) in the heart and aorta, lower SOD activity (superoxide dismutase, an enzyme that converts highly reactive superoxide radicals into peroxide), and improved vascular remodeling and higher plasma NO. Taking these observations into account, it appears that soy protein hydrolysate may limit the effects of angiotensin II through its AT1 receptor by inhibiting ACE and increasing NO levels and thereby attenuating hypertension and reducing oxidative damage[136].

Limitations to soy-based studies on blood pressure

Most soy-based studies on blood pressure do not exclude the potential contribution of soy isoflavones to observed health effects. Consequently, it is challenging to establish an exclusive relationship between soy protein and blood pressure, even when results support blood pressure reductions. Furthermore, other studies use whole or unmodified soy protein, preventing the determination of a relationship between soy-derived peptides and blood pressure.

A study by Palanissamy et al[137] observed elevations in SBP, DBP, mean arterial pressure, heart rate, and ACE activity in rats fed a high fructose diet containing 20% casein for 60 days but replacement with soy protein attenuated these elevations to near normal levels as well as improved insulin sensitivity and renal function. It was determined that the protein source had a profound effect on blood pressure, though other factors, including the presence of isoflavones may account for some of these beneficial changes[137]. However, Yang et al[138] found that the SBP of 16 8-week old SHR fed diets containing either 0.5% or 1.0% soy protein hydrolysate for 12 weeks was significantly lower than SHR fed a standard diet, with differences apparent as early as the second (SPH 1.0%) and fourth (SPH 0.5%) weeks of the experiment. No further changes in blood pressure occurred 6 weeks into the experiment, implying that the lowered SBP was maintained. These investigators also examined the ACE activities of aorta, heart, lung, and kidney and found that, although aorta ACE activity was highest among all SHR, only heart ACE was lower in soy hydrolysate-fed SHR, signifying the possibility of differing affinities of peptides for tissues[138]. Importantly, plasma electrolyte concentrations were unchanged and may suggest that the hypotensive effects of ACE-inhibitory peptides are

independent of isoflavones as previous reports suggest a role of isoflavones in the reduction of hypertension as a promoter of natriuresis through inhibition of Na-K-Cl co-transporters[138]. This is noteworthy as researchers often attribute effects to isoflavones alone. Finally, Nevala et al[139] observed significant reduction in SBP in 8 male SHR fed a diet consisting of 20g soy protein with 80 g standard chow for five weeks compared with chow-fed control and a 20 g casein-with 80 g chow-fed group. However, these proteins were unhydrolyzed and therefore a relationship between casein-hydrolyzed peptides and blood pressure attenuation cannot be made.

Antihypertensive effects of soy: *In vivo* human studies

The health benefits of soy are of interest to health professionals and organizations as well as the food industry. However, it appears that the phytoestrogens, especially isoflavones, are a central focus. Incidentally, in a search for human studies on soy proteins and blood pressure, nearly all that have been retrieved regard soy diets in general, the use of whole (unhydrolyzed) protein, or isoflavones specifically. Biological effects of isoflavones remain controversial and it is conceivable that soy proteins and their individual peptides might contribute to observed physiological effects and thus warrant further investigation.

A large longitudinal cohort study of middle-aged and elderly Chinese women was conducted by Yang et al[140] to determine the association between soy food intake and blood pressure. Inverse relationships between diastolic or systolic blood pressures and soy intakes were discovered when measuring blood pressure 2-3 years after reporting usual soy food intakes that did not change overtime. After adjusting for age and BMI,

mean systolic and diastolic blood pressures of women with intakes of ≥ 25g soy protein/day were 1.9 mmHg and 0.9 mmHg lower, respectively, than mean pressures of women consuming < 2.5 grams/day. Similar relationships were observed between blood pressure and isoflavone intake as well. Furthermore, the blood pressure lowering effect of soy intake was most pronounced in elderly women in which for women > 60 years of age consumption of ≥ 25 grams soy protein each day was associated with a reduction by 4.9 mmHg and 2.2 mmHg in systolic and diastolic blood pressures, respectively, when compared to those consuming < 2.5 grams/day.

A randomized controlled crossover study by Welty et al[141] aimed to determine the cardiovascular health benefits of supplementation with 0.5 cup of soy nuts containing 25g soy protein and 101 mg aglycone isoflavones throughout the day with adherence to a TLC diet for 8 weeks. The subjects consisted of 12 pre-hypertensive and 48 normotensive women. Supplementation with soy nuts resulted in a significant reduction in both systolic and diastolic blood pressures in all pre-hypertensive women and 40 /48 normotensive women with no changes in BMI or physical activity. Furthermore, LDL cholesterol and apoprotein B were reduced significantly in hypertensive women. Although it was expressed that the likely contributor was the isoflavones, one cannot discount the potential contribution of peptides derived from digestion and absorption of 25 grams of soy protein each day, especially when considering hydrolysis of different proteins produce different peptides.

In a double blind crossover clinical trial by Washburn et al[142], 51 women were randomly assigned to receive one of three treatments: (1) 20 g complex carbohydrate supplement without phytoestrogens, 20 g soy protein supplement with 34 mg

phytoestrogens (2) to be consumed once per day, or (3) the 20 g soy supplement split in half and consumed twice per day, for 6 weeks in duration. A significant reduction in diastolic blood pressure (by an average of 4.9 mmHg) was observed in women consuming the soy protein supplement twice per day.

In a randomized double blind comparative study of consumption of 500 ml of soy milk high in isoflavones (63 mg daidzein and 80 mg genistein) and skimmed cow milk (no isoflavones) twice daily for 3 months in 40 mild to moderate- hypertensive individuals (25 men, 15 women), Rivas et al[143] found significant (but moderate) reductions in systolic, diastolic, and mean blood pressures in those following the soy milk diet compared to cow milk diet controls. More specifically, reductions by about 18 mmHg in SBP, 16 mmHg in DBP, and 17 mmHg in mean blood pressure were determined after adherence to the soy milk diet compared with reductions by 1.4 mmHg in SBP, 4.0 mmHg in DBP, and 3.0 mmHg mean blood pressure found in the control group. Moreover, a strong correlation between urinary genistein excretion and blood pressure lowering was observed, though a contributing role from the soy protein is possible. In contrast to previous reports of blood pressure lowering effects of soy protein supplementations, a double blind randomized placebo-controlled crossover study by Teede et al[144], reported that when 41 hypertensive individuals (26 men, 15 postmenopausal women) were randomized to either a gluten placebo cereal or a soy cereal supplement (containing 40 g soy protein and 118 mg isoflavones) for breakfast consumption for three months each treatment, supplementation with soy did not result in significant changes in 24-hour ambulatory or central blood pressures, in vascular compliance, or endothelial function as assessed by brachial artery flow-mediated dilation.

Thus, the blood pressure lowering potential of soy-derived peptides remains uncertain. More research in humans is warranted.

Conclusion

While the inhibitory activities of various peptides have been widely studied *in vitro*, information on physiological relevance is lacking. New technologies and sophisticated methods in bioactive peptide production continue to hold promise for the discovery of new potent ACE-inhibitory peptides, though they remain unmatched by animal, and especially human, studies. It is evident that *in vitro* inhibition is an insufficient predictor of *in vivo* antihypertensive effects. Prevalent inter-individual differences and the minimal human data in the literature underscore the need for more and larger studies. Furthermore, there is need for more research into the mechanisms of blood pressure lowering effects. While dairy- and soy-derived bioactive peptides have shown blood pressure reductions in animal and human studies, the lack of consistent results and the small number of these studies confirm that this area of research is still in early stages. Though more work is needed, the identification and characterization of bioactive peptides and their *in vitro* effects do provide the necessary guidance for allowing further advancements to be made.

CHAPTER 3: ACE-INHIBITORY EFFECTS OF DAIRY- AND SOY-DERIVED PEPTIDES IN PRE-HYPERTENSIVE OVERWEIGHT MEN AND WOMEN*

* Portions of Chapter 3 have been published as a short report: Melissa S. Munn, Shalamar Sibley, Richard Brundage, Baraem Ismail, Carrie P. Earthman. "Angiotensin-converting enzyme inhibitory effects of dairy- and soy-derived peptides in prehypertensive overweight men and women." Functional Foods in Health and Disease, 2013, Volume 3, Issue 1 (January 27, 2013).

Chapter Summary

Hypertension is considered the most prevalent cardiovascular disorder and a significant public health problem. A functional food that could potentially impede progression into a hypertensive state in pre-hypertensive individuals is of significant interest to clinicians and consumers. *In vitro* and animal studies suggest potential ACE inhibitory activity or blood pressure lowering effects of dairy- and soy- derived bioactive peptides. Very few research studies on the effects of whey and soy hydrolyzed proteins have been conducted. The present pilot study tested the acute effects of 20 g doses of whey and soy hydrolysates in pre-hypertensive, overweight men and postmenopausal women on serum ACE activity and blood pressure. No differences were observed between hydrolyzed proteins and their respective unhydrolyzed counterparts on either ACE activity or blood pressure. These results support a discrepancy between *in vitro* and human based *in vivo* ACE-inhibitory effects of whey and soy protein hydrolysates, underscoring the need for further research to better understand potential explanations for these findings.

Introduction

Considered the most prevalent of cardiovascular disorders, hypertension affects nearly 33% of Americans[1] and approximately 1 billion people worldwide[2], thus, it is a significant public health concern[3]. The renin angiotensin (aldosterone) system (RAAS) plays a significant role in the regulation of blood pressure and involves a cascade of events leading to increased blood pressure as a consequence of the conversion of

angiotensin I into the potent vasoconstrictor, angiotensin II, facilitated by the angiotensin converting enzyme (ACE).

A first recommendation for the management of hypertension is to alter lifestyle habits, especially dietary choices and level of physical activity. Common dietary recommendations include sodium restrictions of less than 1.5 grams (1500 mg) per day and the Dietary Approaches to Stop Hypertension (DASH) diet to promote the consumption of fresh fruits, vegetables, whole grains, lean meats, and low fat or fat-free dairy products. Such recommendations are frequently accompanied by pharmacological medications, including ACE inhibitors. While these agents help attenuate hypertension, they are not without side effects that may render them inconvenient to patients. Furthermore, pharmacological interventions can be costly. A less expensive and more convenient alternative is of interest.

Increased consumer awareness of the relationship between diet and health has spurred significant interest in the health-promoting potential of functional foods[145]. Of these, there is a growing interest in 'bioactive' peptides from various protein sources. Bioactive peptides are fragments of proteins freed from the parent chain upon hydrolysis possessing potential for influence on body functions that may enhance health. Researchers have found antihypertensive potential in dairy-derived peptides. This observation has led to efforts to further investigate the potential of these peptides with an eye towards developing functional foods that may both attenuate pre-existing hypertension and slow or prevent progression into a hypertensive state in pre-hypertensive individuals. Immediate benefits of a functional food designed to lower blood pressure would include its affordability, availability, and preclusion of the side

effects associated with antihypertensive medications. The majority of antihypertensive bioactive peptides have shown ACE-inhibition abilities, largely *in vitro*. To the best of our knowledge, the most extensively studied food-derived peptides with ACE inhibitory potential using human subjects are dairy-derived, particularly the casein-derived lactotripeptides, VPP and IPP, with very few studies testing whey hydrolysates. The only human study of whey hydrolysate to date evaluated the effects of chronic consumption on blood pressure, but not ACE activity. There are no published human studies on soy hydrolysates on blood pressure or ACE activity.

Overweight and obesity are associated with hypertension; the therapeutic potential of a soy- or milk-derived functional food component to reduce blood pressure in this ever-growing population is of substantial interest. Therefore, we aimed to investigate the effects of an acute dose of whey- and soy- derived bioactive peptides on ACE activity and blood pressure in pre-hypertensive, overweight men and postmenopausal women.

Methods

Study Overview

This was a randomized, single-blind cross-over study in which five treatments were compared: 20 grams (g) whey protein hydrolysate (WPH), 20 g whey protein isolate (WPI), 20 g soy protein hydrolysate (SPH), 20 g soy protein isolate (SPI), and 20 g casein. A 20 g dose was expected to elicit a biological effect, based on a study reporting a significant reduction in blood pressure in humans consuming that dose of WPH for 6 weeks[97]. Each treatment consisted of four (4) equicaloric cookies containing one of the treatment proteins (5 g each, 20 g total). Subjects consumed a controlled diet for the 7-

day period preceding the first treatment visit, and during a 3-day washout period between cross-over treatments. Figure 2 illustrates the study design.

Subject selection

Subjects were recruited through the University of Minnesota through Institutional Review Board (IRB)-approved campus fliers. Subjects were eligible to participate if they met the following criteria: had a body mass index (BMI) 25 – 34.9 kg/m^2; were at least 40 years old, had systolic blood pressure values ≥ 90 but ≤ 139 mm Hg and diastolic blood pressure values ≤ 90 (taken as the mean of 2 readings), and, if a woman, had had no menses for at least the past twelve months, was not currently taking any hormone replacement therapy medications, or had had a complete hysterectomy at least 12 months prior to study participation.

Individuals were excluded from the study if they were < 40 years of age; were pre-menopausal; were of normal weight (BMI < 25 kg/m^2) or Class II and III obese (BMI > 35 kg/m^2); had pituitary or hypothalamic disease; had congestive heart failure, abnormal thyroid function indices, liver failure, renal failure, or diabetes; had an eating disorder, clinically significant mental health disease or dementia; were undergoing chemo- or radiotherapy treatment for neoplastic disease; were vegetarian or habitually consume soy or whey protein products; had soy, nut, or milk allergies; had poor dentition; were on diuretics, beta-blockers, angiotensin converting enzyme inhibitors, or angiotensin receptor blockers; had menstruated in the last 12 months; had had a hysterectomy in the last 12 months; or were on hormone replacement therapy.

Individuals who agreed to participate came to the Delaware Clinical Research Unit (DCRU) at the University of Minnesota for a screening visit. During this visit, consent was first obtained and then medical history, blood pressure, and anthropometry were assessed to determine eligibility. Once officially enrolled into the study, each participant met with a registered dietitian from DCRU Nutrition Services to determine energy requirements at an orientation visit. This information was used to develop a controlled diet. Approximately one week after the orientation visit, subjects were asked to adhere to the controlled diet for one week before treatment testing. At the end of the week, study participants underwent testing at five visits to the DCRU, separated by a three-day washout period to evaluate responses to the different treatment arms. These participants adhered to the controlled diet for the duration of the study. The study protocol was reviewed and approved by the IRB and the Clinical and Translational Science Institute at the University of Minnesota.

Testing Visits

Subjects arrived to the DCRU by 0700 the day of their testing visit (for each of the five visits) following a fast from 1900 the day before and were asked to void their bladders and to dress in light weight clothes for weight measurements. Subjects were then instructed to lie supine in a bed with one pillow supporting the head for 30 minutes before the baseline blood draw and throughout the entire testing period. The head of bed could be elevated at most up to 30 degrees plus one pillow. The nursing staff placed a peripheral IV line for blood draws in the non-dominant arm and initiated Dinamap blood pressure readings that were monitored at the baseline blood draw and at each blood

sampling time point. After an initial thirty minute rest, blood was drawn for baseline measures of ACE activity and blood pressure was assessed. Participants then consumed the protein cookies based upon randomized assignment to one of five treatment arms: WPH; WPI; SPH; SPI; or casein. During consumption of the treatment, participants were encouraged to consume approximately 120 ml of water without ice. Consumption of the treatment was completed over a 15 minute period (± 5 minutes), at the end of which began the follow-up period (denoted as Time 0). Blood sampling time points occurred in thirty minute intervals from Time 0: +30, +60, +90, +120, +150, and +180 minutes. These sampling points were determined from a preliminary pilot study (unpublished) in which nine subjects received one of the five treatments (20 g of casein, SPH, SPI, WPH, or WPI) and blood was collected at fifteen and thirty minute intervals over a five hour period. Data from this study suggested that maximal ACE activity suppression could be captured between 60-180 minutes and thus a 180 minute follow-up period was utilized in the cross-over study.

Anthropometric Assessment

Subjects were measured for height (cm) and weight (kg) following standardized procedures. Height was measured by a wall-mounted stadiometer to the nearest 0.1 cm. Body weight was measured on a stand-on digital scale to the nearest 0.1 kg. Body mass index (BMI) was calculated using the formula: BMI = kg/m^2.

Controlled Diet

The controlled diet was designed by the DCRU Nutrition Services to meet each individual's determined energy needs, to be whey-, soy-, and dairy-free, and to provide 1 gram of sodium per 1,000 calories and 1.5 grams of potassium per 1,000 calories daily. A Registered Dietitian determined caloric content for each individual's diet estimated by calculating resting metabolic rate using the Harris Benedict Equation[146] and then multiplying that by the subject's estimated physical activity factor.

During the study period, subjects were provided all their food by the DCRU metabolic kitchen; they picked up their food on a daily basis throughout the study period. They were asked to adhere to the controlled diet and to report off-plan intake to Study Personnel and on food records. At the orientation visit, subjects were instructed by Study Personnel on how to keep accurate dietary records and were provided with simple forms for daily record-keeping. These diet records were reviewed by Study Personnel at testing visits in an effort to assess subject compliance. Additionally, subjects were required to return uneaten food to the DCRU when picking up the following day's meals as an additional effort to track subject compliance.

Protein Treatments

Production of hydrolysates and in vitro ACE inhibition

Davisco Foods International, Inc. (Eden Prairie, MN) provided the whey protein isolate (BiPro) and the whey protein hydrolysate (Biozate 1) to be incorporated into the nutritional cookies for this study. Defatted and minimally heated soy flour samples were

provided by Archer Daniels Midland Company (Decatur, IL) for the preparation of soy protein isolate (SPI). Soy protein hydrolysate (SPH) was produced by selective hydrolysis with papain to β-conglycinin –hydrolyzed SPI as described[147]. *In vitro* ACE inhibitory activity measurements of SPH and WPH were performed in triplicate using N-[3-(2-Furyl)acryloyl]- L -phenylalanyl-glycyl-glycine (FAPGG) as substrate[147].

Preparation of the cookies

Cookies were prepared with the goal of achieving a palatable dose of 20 g of protein incorporated into four cookies. Trials for cookie preparation were repeated several times by the same student who prepared them at each testing visit for each subject to ensure consistency in preparation methods and procedures. Weight was measured using a digital scale and small measures of liquid ingredients were controlled using transfer pipettes. The measured amount of each ingredient was scaled up to account for loss during preparation. The four cookies were baked together in a conventional oven at 350 degrees Fahrenheit for 7 minutes. Furthermore, the treatment cookies were equicaloric, varying only by the type of protein incorporated. (See Table 1 for a detailed description of the formulation of the cookies.)

To guarantee freshness, cookie preparation began by 0530 the morning of each testing visit; they were delivered by the student to the DCRU by 0700.

Plasma ACE Activity Assay

At each time point, 5 ml of blood was collected into serum separator tubes (SST). Each of these samples was allowed to clot and held at room temperature. Samples were

batched cold spun at 3000 rpm for 10 minutes after the final sample clotted. Then, 1ml of serum was aliquotted into cryogenic vials in duplicate, flash frozen with dry ice and alcohol, and stored in an ultracold freezer (-80 degrees F) before being shipped on dry ice to ARUP Laboratories (Salt Lake City, Utah) to be assayed for ACE activity.

ACE activity was measured using the synthetic substrate FAPGG (N-[3-(2-furylacryoyl)]-L-phenylalanine-glycyl-glycine). The ACE enzyme hydrolyzes FAPGG to FAP (furylacryyloylphenylalanine and glycylglycine). Its hydrolysis results in a decrease in absorbance at 340 nm as measured by a spectrophotometer. ACE activity is expressed in units per liter (U/L).

Statistical Analysis

Area under the curve (AUC) was calculated for each subject and for each treatment for both ACE activity and systolic blood pressure from baseline to 180 minutes post consumption of cookie treatments. From these, mean AUCs were calculated for each treatment. Our primary interest was to compare whey and soy hydrolysates (SPH and WPH) with respective isolate counterparts (SPI and WPI) for both ACE activity and systolic blood pressure. However, because it was also possible (although not likely) that *in* vivo digestion of the whey and soy protein isolates as well as the casein could potentially have yielded ACE-inhibitory bioactive peptides, we evaluated the differences across all treatments in ACE activity and blood pressure using repeated measures ANOVA with post-hoc comparisons. AUC data from 0-180 minutes and from 60-180 minutes were evaluated, as we observed in the pilot study that ACE suppression appeared to be maximally captured within this narrowed window of time. A significance level of

0.05 was considered significant. Repeated Measures ANOVA was performed using GraphPad InStat version 3.10 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Results

The *in vitro* ACE inhibitory activity of the SPH and WPH samples were evaluated in the laboratory of Dr. Baraem Ismail. The ACE inhibitory activity was expressed as IC_{50} values (the amount of the inhibitor required to reduce enzyme activity by 50% of its original activity). SPH showed comparable, though slightly greater ACE inhibitory activity compared to WPH, with IC_{50} of 0.177 mg SPH protein/mL compared to 0.200 mg WPH protein/mL[147].

Importantly, throughout the study period, no adverse events were reported. Subjects tolerated the treatment protein cookies fairly well; however, there were several logistical challenges, including the long period of time during which subjects were asked to lie in the supine position, even while consuming the treatment cookies. In addition, staffing changes at the metabolic kitchen, and the early morning schedule for making the treatment cookies with limited staffing in the laboratory of Dr. Ismail also presented some difficulties. Due to these and other challenges, the decision was made to conduct an interim analysis of ACE activity and blood pressure data from the first four subjects who completed the study protocol before proceeding with additional subject recruitment. Based on the lack of any significant differences between the treatment proteins on ACE activity and blood pressure, subject recruitment was halted. Data from these first 4 subjects who completed the entire study protocol are presented here.

The subjects were Caucasian men and postmenopausal women (2 F, 2 M) with a mean ± SD age of 58±1.3 years and a BMI of 28±.7 kg/m^2. Mean systolic and diastolic blood pressures were 118±7 mmHg and 81±6 mmHg, respectively. Subject compliance to the controlled diet was quite good, as reflected by daily food records and weigh back information for each meal, and urinary sodium and potassium intake. Table 2 presents dietary intake, serum, and urinary sodium and potassium data for each study visit. The controlled diet was well tolerated and subjects conveyed a strong liking for the entrees and snacks provided. Tables 3 and 4 present the ACE activity and systolic blood pressure AUC data, respectively, for each subject and each treatment. No differences in ACE activity were found among the mean AUCs of the five treatments between 60-180 minutes ($p = 0.97$) or from 0-180 minutes ($p = 0.76$) post treatment. The power of the test for the comparison of treatments by repeated measures ANOVA was <10%, suggesting there is insufficient data to show that a statistically significant difference exists between the treatments. Mean ACE activity over time by treatment is graphically represented in Figure 3.

When considering the effects on systolic blood pressure, no statistically significant difference was found among the five treatment groups from 60-180 minutes ($p = 0.52$) or from 0-180 minutes ($P = 0.72$) post-treatment. A graphical representation of mean systolic blood pressure for each treatment is presented in Figure 4.

Discussion

An acute dose of 20 grams of hydrolyzed whey or soy protein did not result in a reduction in serum ACE activity or arterial systolic blood pressure in overweight mildly

pre-hypertensive men and postmenopausal women within 180 minutes post treatment consumption despite apparent *in vitro* ACE inhibitory activity. It is probable that the amount of ACE inhibitory peptides found in a 20 g dose of either SPH or WPH was insufficient to achieve notable reductions in either ACE activity or blood pressure as only a fraction of the peptides may possess ACE inhibitory activity. It is also possible that chronic intake of bioactive peptides is required to achieve pronounced reductions. However, we found only two human-based studies on hydrolyzed whey protein and blood pressure. One of these, Lee et al[148], found no differences in blood pressure following daily ingestion of 125 ml of a milk drink supplemented with whey peptides for 12 weeks[148]. The other reported significant reductions in systolic and diastolic blood pressures following daily consumption of a 20 g dose of hydrolyzed whey protein for 6 weeks; however, the paper was later retracted due to the discovery that results were based on falsified data[98]. Thus, in essence there have been no valid reports of whey protein demonstrating blood pressure or ACE inhibitory effects in humans. In addition, the blood pressure lowering potential of hydrolyzed soy proteins in humans is completely unknown.

Interestingly, Lee et al[148] also confirmed ACE inhibitory potential *in vitro* of a whey peptide powder prior to its administration to subjects. The clear discrepancy between the apparent *in vitro* ACE inhibition and the lack of *in vivo* lowering of ACE activity and blood pressure underscores the need for more human-based studies. Whey and soy hydrolysates have been studied for survivability to digestion using pepsin, trypsin, and chymotrypsin treatments as well as elaborate *in vitro* models that simulate *in vivo* digestive processes and have largely been found to remain intact or undergo further

hydrolysis, resulting in the release in smaller more potent inhibitors with lower IC_{50} values[33,35,38,43,57,62-64,67,76-79]. Although di- and tripeptides and some other small oligopeptides are more easily absorbed intact, most are further hydrolyzed by the various luminal, brush border, and cytosolic peptidases of enterocytes into free amino acids prior to entry into systemic circulation. A short half-life (minutes) presents an additional barrier to optimal bioavailability that results from hydrolysis by peptidases in plasma and vascular endothelium[149]. A bioavailability of < 0.1% in pigs and peak concentrations in picomolar amounts in humans administered two well-known tripeptides, isoleucine-proline-proline (IPP) and valine-proline-proline (VPP), both reported to be absorbed intact, illustrates these inherent challenges[149]. Data on human plasma concentrations of whey and soy-derived peptides is severely lacking, though it provides important information to ascertain *in vivo* effects.

Undoubtedly, the most studied mechanism behind hypotensive properties of peptides has been ACE inhibition. However, both *ex vivo* and *in vivo* studies in spontaneously hypertensive rat models have suggested the potential for other mechanisms in reducing blood pressure including interactions with opioid receptors, possibly increasing vasorelaxation[95,96].

It should also be considered that other studies have found discrepancies between *in vitro* ACE inhibition and consequent *in vivo* blood pressure effects upon peptide administration in spontaneously hypertensive rats. Specifically, Murakami et al[95] noted that ovalbumin-derived peptides achieved the highest % ACE inhibition (signifying potent ACE inhibitory potential) but exerted the weakest antihypertensive effects. In contrast, high IC_{50} values corresponded to certain peptide fractions of whey protein

(suggesting weak inhibition) though these peptides reduced blood pressure by 19 mmHg at 6 hours and nearly 22 mmHg at 8 hours post administration. Results of various *in vitro* studies subjecting peptides to digestive proteases suggest that certain peptides may not survive digestive processes as they are susceptible to further hydrolysis (especially if chain lengths are >7-9 amino acids) potentially resulting in loss of activity[57,64]. However, other studies have found peptides that either maintained ACE inhibitory potential post treatment with digestive enzymes[33,38,57,62-64,67,77,78] or peptides with enhanced ACE inhibitory potential as a result of further hydrolysis, likely producing smaller, more potent peptides than may be absorbed intact[43,57,64,77].

The delivery of protein treatments in whey studies included liquid forms such as water or milk beverages[97,148]. Studies on soy protein have included whole protein: soy nuts[141], soy milk[143], soy protein supplement[142], or soy cereal supplementation[144] and the potential contribution of soy isoflavones were recognized. Thus, studies on hydrolyzed soy protein and ACE inhibition and blood pressure lowering effects are needed. The insolubility of soy protein hydrolysate in liquid and the desire to deliver 20 g protein treatments in a palatable form influenced the decision for the unique delivery form of a cookie in our study. Nonetheless, the possibility of thermal or pH instability during the baking process may influence the bioactivity or digestive survivability of the soy and whey peptides as little is known about this[147]. It is possible that heat and alkaline conditions may alter the quaternary and tertiary structures of peptides ('protein unfolding' or denaturation), ultimately exposing other active sites for additional enzymatic cleavage or rendering peptides inactive. We tested ACE inhibition of SPH post subjection to heat treatments of either 120 °C or 175 °C for 10 or 15 minutes, similar

to what would be expected during cookie baking. Experimental methods and procedures are discussed elsewhere[147]. Results confirmed thermal stability as ACE inhibition (IC_{50} values) did not change between heat treatments and did not differ from an unheated [147]. However, we did not test for stability to increased pH (as might be expected with the addition of sodium bicarbonate (baking soda) and baking powder). Furthermore, it is possible that pH and heat may exert a combined effect that jeopardizes peptide bioactivity, though little is known about this. Finally, though little is known, inherent reactions that occur in heat conditions between proteins and reducing sugars, collectively termed non-enzymatic browning reactions, such as the Maillard Reaction, may influence bioactivity[147].

There were several strengths to this study. We employed a randomized cross-over design, allowing that each subject served as his or her own control to ascertain the effects of each treatment. Unlike other studies, we examined the hypotensive potential of two protein sources, hydrolyzed whey and soy, and compared them to unhydrolyzed counterparts and a reference, unhydrolyzed, casein protein. Examination of bioactivity was not limited to blood pressure data, as we expanded our endpoints to include ACE activity. If a hypotensive response to either or both hydrolyzed proteins was observed, changes in ACE activity would have provided important information. A mild activation of the RAAS is important when studying ACE inhibition. Reduced sodium intake may stimulate plasma renin activity and increase aldosterone levels[150,151] and consistent sodium and potassium intake prevented day-to-day fluctuations that could impose unwanted variability within subject data; therefore, subjects adhered to a sodium and potassium controlled diet that was dairy- and soy- free. Additionally, compliance was

strongly reinforced with the use of diet records, the required return of uneaten food, daily pickup of meals, and urine analysis. Finally, subjects were asked to be consistent in choice of activity to pass the time at each visit. For example, if a subject rested with her eyes closed at the first visit, she was asked to do this at each visit and for the duration of that visit. Consistency in activity also minimized unwanted variability.

Our study was not without limitations. This was not a dose-response study. We only tested the effects of a 20 g dose of protein and recognize that this amount could be insufficient. More information could have been obtained examining the effects of various doses to determine a minimum amount of protein treatment required to achieve an observable response. We also did not measure other RAAS proteins, such as serum angiotenin II, which would have provided vital information to support or negate the proposition of ACE inhibition as the primary mechanism for blood pressure lowering effects. Finally, we developed a cookie for treatment delivery as a solution to the insolubility of the soy protein isolate and hydrolysate, which made a liquid form unfeasible. Given the potential susceptibility of peptides to denaturation and further enzymatic hydrolysis as consequences to pH change and the Maillard Reaction, a baked cookie may not have been the most ideal delivery system. Further work should be done to devise and evaluate optimal ways to deliver these bioactive peptides to humans.

Conclusion

In our study, reduced blood pressure and ACE activity did not occur following an acute dose of 20 g of either soy or whey protein hydrolysate. The discrepancy between the positive observations of ACE-inhibitory activity by soy and whey protein

hydrolysates *in vitro*, and the lack of *in vivo* evidence of ACE inhibition or blood pressure lowering in human studies underscores the need for more research, particularly to determine peptide bioavailability for optimal dosing to elicit *in vivo* effects. There is much interest in the production of a functional food for the attenuation of existing hypertension and, perhaps more importantly, for the delayed or prevented progression into a hypertensive state in those with prehypertension. Such a preventative approach could have important public health implications, especially when considering the high rates of obesity. However, the potential efficacy of these whey and soy protein-derived bioactive peptides must be established through rigorous human trials before functional foods containing these peptides become available to consumers.

Figure 2. A sample schedule

← Controlled Diet →									
Pre-Testing Week 1	Testing Visit 1	3-Day Washout	Testing Visit 2	3-Day Washout	Testing Visit 3	3-Day Washout	Testing Visit 4	3-Day Washout	Testing Visit 5
X	Treatment: SPH	X	Treatment: WPI	X	Treatment: SPH	X	Treatment: Casein	X	Treatment: SPI

Each subject received the five treatments in a randomized order, each at a different visit: soy protein isolate (SPI), soy protein hydrolysate (SPH), whey protein isolate (WPI), whey protein hydrolysate (WPH), casein. A controlled diet was followed throughout the study, beginning one week before testing; treatments were separated by 3-day washout periods.

Table 1. Protein Treatment Cookie Ingredients

Ingredient	Amount (g)*
Protein**	24.83
Butter	21.59
Starch	21.59
Brown sugar	16.198
Splenda	1.94
Baking soda	0.539
Baking powder	0.269
Almond flavor	0.2159
Vanilla flavor	2.159
Walnut oil	2.159
Water	18.358
Chocolate chips	15.118
Total[+] per cookie[++]	125[+] 28.94[++]

*Amounts have been scaled up to account for losses during preparation

** Proteins: casein, soy protein isolate (SPI), soy protein hydrolysate (SPH), whey protein isolate (WPI), or whey protein hydrolysate (WPH)

[+]Total weight of dough before dividing into 4 cookies; [++]Weight of each of the 4 cookies

Table 2. Dietary, Serum and Urinary Sodium and Potassium for Each Testing Visit

ID	Testing Visit	Na^+ Intake (mg)*	Serum Na^+ (mmol/L)	Urinary Na^+ (mmol/L)	K^+ Intake (mg)*	Serum K^+ (mmol/L)	Urinary K^+ (mmol/L)
85001	Visit 1	1992	140	80	3099	4.2	64
	Visit 2	1992	139	71	3099	4.2	66
	Visit 3	1992	139	72	3099	4.0	65
	Visit 4	1992	141	93	3099	4.7	71
	Visit 5	1992	140	82	3099	3.9	65
85004	Visit 1	2600	140	48	3125	3.8	19
	Visit 2	2572	140	N/A**	2733	4.0	N/A**
	Visit 3	2736	139	28	3320	3.9	15
	Visit 4	2129	140	36	2444	4.0	24
	Visit 5	2301	140	56	2907	3.7	33
85005	Visit 1	2963	140	41	3895	3.9	34
	Visit 2	2963	140	28	3895	3.8	15
	Visit 3	2963	141	54	3895	3.7	26
	Visit 4	2963	139	22	3895	4.0	31
	Visit 5	2963	140	18	3895	3.6	31
85007	Visit 1	2125	141	N/A**	3134	3.8	N/A**
	Visit 2	2125	139	22	3134	3.7	32
	Visit 3	2125	140	32	3134	3.7	62
	Visit 4	2125	142	31	3134	4.0	21
	Visit 5	2125	141	31	3134	3.7	29

*Dietary intakes of potassium and sodium were determined from weigh-back information taken from one day prior to the testing visit.

** Urine specimen not collected/analyzed.

Subjects 85001, 85005, and 85007 consumed everything provided at each meal. Therefore, sodium and potassium intake values did not vary for these individuals.

Table 3. Angiotensin Converting Enzyme (ACE) Area-Under-the-Curve (AUC) by Treatment

Treatment	Subject ID	AUC 60-180 min	AUC 0-180 min
Casein	85001	3825	6150
	85004	5445	7980
	85005	7110	10785
	85007	4170	6270
	Mean	**5138**	**7796**
	SD	**1488**	**2161**
SPI	85001	3405	4845
	85004	4845	7290
	85005	6892.5	10485
	85007	4575	6900
	Mean	**4958**	**7429**
	SD	**1620**	**2272**
SPH	85001	3405	4845
	85004	4845	7290
	85005	6892.5	10485
	85007	4680	7035
	Mean	**4956**	**7414**
	SD	**1443**	**2323**
WPI	85001	3885.00	5475.00
	85004	5490.00	8310.00
	85005	6630.00	10335.00
	85007	4080.00	6180.00
	Mean	**5021.250**	**7575**
	SD	**1289**	**2200**
WPH	85001	4410.00	6675.00
	85004	4545.00	7155.00
	85005	6930.00	10965.00
	85007	4050.00	6495.00
	Mean	**4984**	**7823**
	SD	**1314**	**2113**

Abbreviations: Soy protein isolate (SPI), soy protein hydrolysate (SPH), whey protein isolate (WPI), whey protein hydrolysate (WPH), casein

Table 4. Summary of Systolic Blood Pressure Area-Under-the-Curve (AUC) by Treatment

Treatment	Subject ID	AUC 60-180 min	AUC 0-180 min
Casein	85001	11280	16800
	85004	15360	22830
	85005	12045	18135
	85007	14580	21195
	Mean	**13316**	**19740**
	SD	**1961**	**2762**
SPI			
	85001	11190	16860
	85004	14790	22245
	85005	13125	20250
	85007	13320	20085
	Mean	**13106**	**19860**
	SD	**1478**	**2228**
SPH			
	85001	11625	17205
	85004	16140	24480
	85005	12810	19020
	85007	13275	19875
	Mean	**13463**	**20145**
	SD	**1915**	**3097**
WPI			
	85001	11055	16770
	85004	14715	22005
	85005	12885	19230
	85007	13320	19740
	Mean	**12994**	**19436**
	SD	**1510**	**2148**
WPH			
	85001	10890	16470
	85004	15495	23850
	85005	11730	17430
	85007	13260	19680
	Mean	**12844**	**19358**
	SD	**2022**	**3283**

Abbreviations: Soy protein isolate (SPI), soy protein hydrolysate (SPH), whey protein isolate (WPI), whey protein hydrolysate (WPH), casein

Figure 3. Mean Angiotensin Converting Enzyme (ACE) Activity vs Time by Treatment

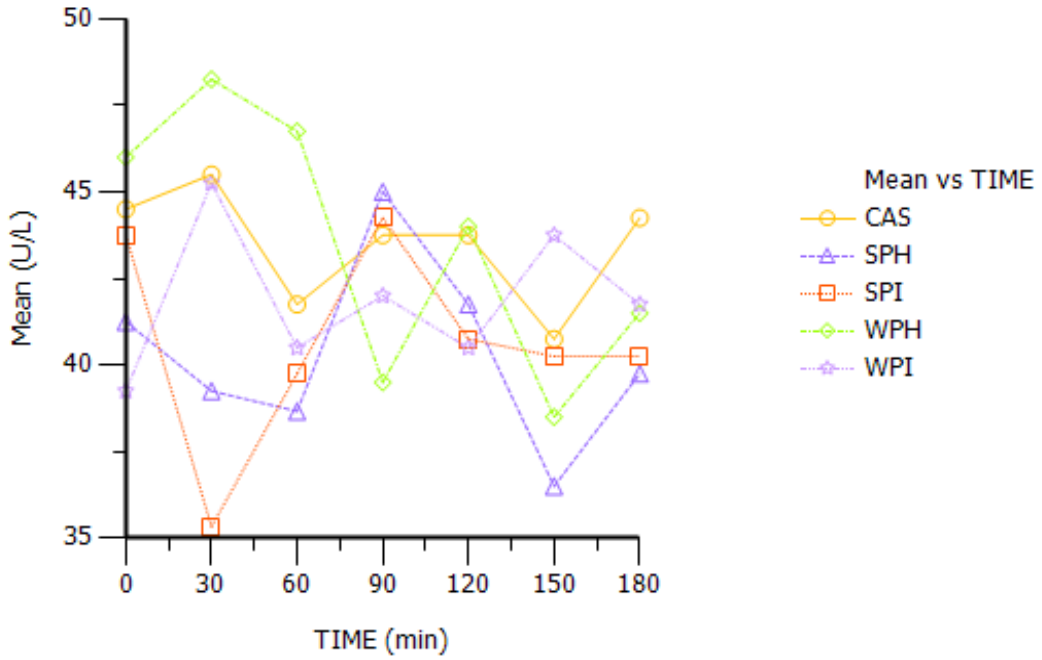
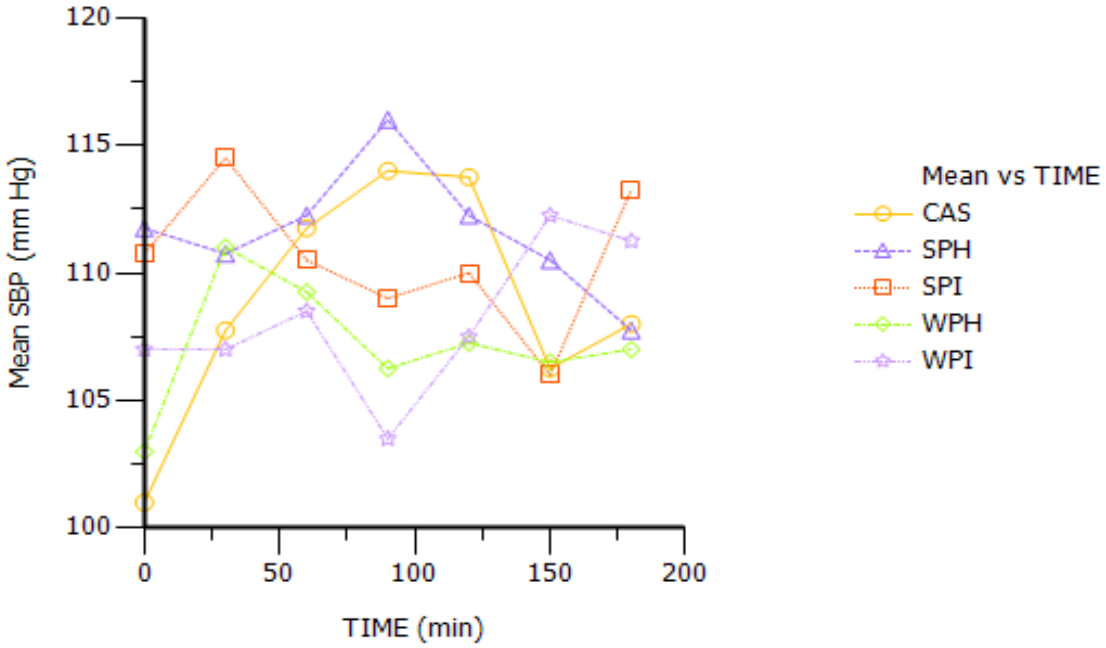


Figure 4. Mean Systolic Blood Pressure (SBP) vs Time by Treatment



CHAPTER 4: CONCLUSIONS

Hypertension, the silent killer, is considered the most prevalent of cardiovascular disorders and is projected to affect almost a third of the world population by 2025. It is recognized as a leading cause of kidney failure and poses significant risks for myocardial infarction, heart failure, and stroke. Essential hypertension was once accepted as a disorder primarily in older adults; however, it now spans across all age groups, affecting children and adolescents parallel to obesity. This is a significant concern for children as childhood blood pressure is a predictor of blood pressure in adulthood. Though certain risk factors cannot be modified, a heart healthy diet and various lifestyle factors can greatly reduce one's risk. Prehypertension is an important risk factor for hypertension and is a focus for preventative approaches.

The RAAS is primarily responsible for blood pressure regulation and thus is a target system for pharmacological interventions. Although blood pressure lowering medications work well, they can be costly and impose unwanted side effects. A significant amount of money is spent in the US on healthcare related to hypertension. Interest in a functional food that can impede progression from prehypertension to a hypertensive state is strong. Many in vitro studies have found ACE inhibitory potential in peptides derived from dairy and soy proteins. Much emphasis has been placed on production and sequencing of the peptides. While important information is obtained in this work, results from in vivo studies determine the ultimate worth of the peptides. Though the existing body of research on bioactive peptides with ACE inhibitory potential is compelling to many scientists in academic institutions and the food industry alike, current research is highly deficient in the most relevant area of all, human-based work. It is this deficiency which ultimately keeps this field in its infancy, regardless of *in vitro*

advancements. Even so, two well-known products already exist in Japan (Calpis) and Finland (Evolus) containing the best studied casein-derived tripeptides, IPP and VPP. Interestingly, results of Japanese and Finnish studies have largely not been reproducible in other ethnic groups.

We conducted a randomized cross-over controlled single blind study to test the effects of 20 g doses of whey and soy hydrolysates on blood pressure and ACE activity over the course of 180 minutes post treatment consumption, using unhydrolyzed soy and whey counterparts as respective controls. An interim analysis on four subjects revealed no differences in ACE activity among the five treatment groups and no differences between hydrolyzed and unhydrolyzed pairs. To the best of our knowledge, after searching eleven databases, only two studies tested the effects of whey hydrolysates on humans and very little has been studied on hydrolyzed soy protein. Given this, much more work is needed.

The idea of incorporating peptides derived from dairy or soy into a functional food that can be consumed daily by individuals at risk for developing hypertension is intriguing. Though very few human studies exist, animal based studies do support the potential for blood pressure lowering effects exerted by peptides. More information is vitally needed regarding bioavailability and efficacy of food-derived ACE inhibitory peptides to positively impact blood pressure in humans. With additional data on these important questions, it is quite possible that functional foods based on dairy and soy-derived peptides may be welcomed by the food industry, academic scientists and consumers as a non-pharmacologic approach to reducing hypertension. The 1970 discovery of peptides in viper snake venom possessing the ability to inhibit ACE signifies

an important milestone for public health and the therapeutic preventative potential of the more recently identified food-derived ACE inhibitory peptides may one day result in another.

REFERENCES

1. NHLBI. National Heart, Lung, and Blood Institute Web site. http://www.nhlbi.nih.gov/index.htm. Accessed May 4, 2012.

2. Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: Analysis of worldwide data. *The Lancet*. 2005; 365(9455): 217-223.

3. Carey RM. Pathophysiology of primary hypertension. In: *Comprehensive physiology*. John Wiley & Sons, Inc.; 2011. 10.1002/cphy.cp020418.

4. Roger VL, Go AS, Lloyd-Jones DM, et al. Heart disease and stroke statistics--2011 update: A report from the american heart association. *Circulation*. 2011; 123(4): e18-e209.

5. Pabon-Nau LP, Cohen A, Meigs JB, Grant RW. Hypertension and diabetes prevalence among U.S. hispanics by country of origin: The national health interview survey 2000-2005. *J Gen Intern Med*. 2010; 25(8): 847-852.

6. Barnes PM, Adams PF, Powell-Griner E. Health characteristics of the asian adult population: United states, 2004-2006. *Adv data*. 2008; 394: 1-22.

7. Grotto I, Huerta M, Sharabi Y. Hypertension and socioeconomic status. *Curr Opin Cardiol*. 2008; 23: 335-339.

8. Kunes J, Zicha J. The interaction of genetic and environmental factors in the etiology of hypertension. *Physiol Res*. 2009; 58: S33-S41.

9. Sukor N. Secondary hypertension: A condition not to be missed. *Postgrad Med J*. 2011; 87: 706-713.

10. Carey R. Overview of endocrine systems in primary hypertension. *Endocrinol Metab Clin N Am*. 2011; 40: 265-277.

11. Singh M, Mensah G, Bakris G. Pathogenesis and clinical physiology of hypertension. *Cardiol Clin*. 2010; 28: 545-559.

12. Touyz R. Role of magnesium in the pathogenesis of hypertension. *Molec Aspects Med*. 2003; 24: 107-136.

13. Montecucco F, Pende A, Quercioli A, Mach F. Inflammation in the pathophysiology of essential hypertension. *J Nephrol*. 2011; 24(1): 23-34.

14. Vykoukal D, Davies MG. Vascular biology of metabolic syndrome. *Journal of Vascular Surgery*. 2011; 54(3): 819-831.

15. Pardee PE, Norman GJ, Lustig RH, Preud'homme D, Schwimmer JB. Television viewing and hypertension in obese children. *Am J Prev Med*. 2007; 33(6): 439-443.

16. Schiffrin EL. Vascular endothelin in hypertension. *Vascul Pharmacol*. 2005; 43(1): 19-29.

17. Thomas GD, Zhang W, Victor RG. Nitric oxide deficiency as a cause of clinical hypertension: Promising new drug targets for refractory hypertension. *JAMA*. 2001; 285(16): 2055-2057.

18. Schmaier AH. The kallikrein-kinin and the renin-angiotensin systems have a multilayered interaction. *Am J Physiol Regul Integr Comp Physiol*. 2003; 285(1): R1.

19. Atlas SA. The renin-angiotensin aldosterone system: Pathophysiological role and pharmacologic inhibition. *J Manag Care Pharm*. 2007; 13:9-20.

20. Jauhiainen T, Korpela R. Milk peptides and blood pressure. *J Nutr*. 2007; 137: 825S-829S.

21. Ehlers PI, Nurmi L, Turpeinen AM, Korpela R, Vapaatalo H. Casein-derived tripeptide ile-pro-pro improves angiotensin-(1-7)- and bradykinin-induced rat mesenteric artery relaxation. *Life Sci*. 2011; 88(5-6): 206-211.

22. Gibbs CR, Beevers DG, Lip GYH. The management of hypertensive disease in black patients. *Q J Med*. 1999; 92: 187-192.

23. Shah SU, Anjum S, Littler WA. Use of diuretics in cardiovascular disease: (2) hypertension. *Postgrad Med J*. 2004; 80: 271-276.

24. Elliott WJ, Ram CVS. Calcium channel blockers. *The Journal of Clinical Hypertension*. 2011; 13(9) :687-689.

25. Mayo Clinic. http://www.mayoclinic.org/diseases-treatments/. Accessed May 4, 2012.

26. Barrerras A, Gurk-Turner C. Angiotensin II receptor blockers. *Proc (Bayl Univ Med Cent)*. 2003; 16(1): 123-126.

27. American Heart Association. http://www.heart.org/HEARTORG/Conditions/Conditions_UCM_001087_SubHomePage.jsp. Accessed May 4, 2012.

28. CDC. http://www.cdc.gov/bloodpressure/faqs.htm#3. Accessed May 4, 2012.

29. Hong F, Ming L, Yi S, Zhanxia L, Yongquan W, Chi L. The antihypertensive effect of peptides: A novel alternative to drugs? *Peptides*. 2008; 29(6): 1062-1071.

30. Hasler CM, Brown AC, American Dietetic Association. Position of the american dietetic association: Functional foods. *J Am Diet Assoc*. 2009; 109(4): 735-746.

31. Pihlanto A. Bioactive peptides: Functionality and production. *Agro Food Industry Hi-Tech*. 2006; 17(6): 24-26.

32. Haque, E., Chand, R.,Kapila, S. Biofunctional properties of bioactive peptides of milk origin. *Food Rev Int*. 2009; 25(1): 28-43.

33. Didelot, S., BordenaveJuchereau, S., Rosenfeld, E., FruitierArnaudin, I., Piot, J. M.,Sannier, F. Preparation of angiotensin-I-converting enzyme inhibitory hydrolysates from unsupplemented caprine whey fermentation by various cheese microflora. *Int Dairy J*. 2006; 16(9, 4th NIZO Dairy Conference -- Prospects for Health, Well-being and Safety): 976-983.

34. Chobert J, El-Zahar K, Sitohy M, et al. Angiotensin I-converting-enzyme (ACE)-inhibitory activity of tryptic peptides of ovine beta-lactoglobulin and of milk yoghurts obtained by using different starters. *Lait*. 2005; 85(3): 141-152.

35. Ashar, M. N.,Chand, R. Antihypertensive peptides purified from milks fermented with *lactobacillusdelbrueckii* ssp. *bulgaricus*. *Milchwissenschaft*. 2004; 59(1--2): 14-17.

36. Kapila S, Jabadolia LN, Dang AK, Kapila R, Arora S. Augmentation of biofunctional properties of whey protein on fermentation with lactobacillus helveticus. *Milchwissenschaft-Milk Science International*. 2009; 64(3): 245-249.

37. Ahn JE, Park SY, Atwal A, Gibbs BF, Lee BH. Angiotensin I-converting enzyme (ace) inhibitory peptides from whey fermented by lactobacillus species. *J Food Biochem*. 2009; 33(4): 587-602.

38. Gobbetti M, Ferranti P, Smacchi E, Goffredi F, Addeo F. Production of angiotensin-I-converting-enzyme-inhibitory peptides in fermented milks started by lactobacillus delbrueckii subsp bulgaricus SS1 and lactococcus lactis subsp cremoris FT4. *Appl Environ Microbiol*. 2000; 66(9): 3898-3904.

39. Donkor, O. N., Henriksson, A., Singh, T. K., Vasiljevic, T., Shah,N.P. ACE-inhibitory activity of probiotic yoghurt. *Int Dairy J*. 2007; 17(11, Rheology and Structure of Fermented Milk): 1321-1331.

40. Muguerza B, Ramos M, Sanchez E, et al. Antihypertensive activity of milk fermented by enterococcus faecalis strains isolated from raw milk. *Int Dairy J*. 2006; 16(1): 61-69.

41. Ong L, Shah NP. Release and identification of angiotensin-converting enzyme-inhibitory peptides as influenced by ripening temperatures and probiotic adjuncts in cheddar cheeses. *Lwt-Food Science and Technology*. 2008; 41(9): 1555-1566.

42. Contreras, M. del M., Sevilla, M. A., MonroyRuiz, J., Amigo, L., GomezSala, B., Molina, E., Ramos, M.,Recio, I. Food-grade production of an antihypertensive casein hydrolysate and resistance of active peptides to drying and storage. *Int Dairy J*. 2011; 21(7): 470-476.

43. Tsai J, Chen T, Pan BS, Gong S, Chung M. Antihypertensive effect of bioactive peptides produced by protease-facilitated lactic acid fermentation of milk. *Food Chem*. 2008; 106(2): 552-558.

44. Mullally M, Meisel H, FitzGerald R. Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins. *Int Dairy J*. 1997; 7(5): 299-303.

45. Otte J, Shalaby SM, Zakora M, Pripp AH, El-Shabrawy SA. Angiotensin-converting enzyme inhibitory activity of milk protein hydrolysates: Effect of substrate, enzyme and time of hydrolysis. *Int Dairy J*. 2007; 17(5): 488-503.

46. HernandezLedesma, B., Recio, I., Ramos, M.,Amigo, L. Preparation of ovine and caprine beta-lactoglobulin hydrolysates with ACE-inhibitory activity. identification of active peptides from caprine beta-lactoglobulin hydrolysed with thermolysin. *Int Dairy J*. 2002; 12(10): 805-812.

47. Abubakar, A., Saito, T., Aimar, M. V.,Itoh, T. New derivation of the inhibitory activity against angiotensin converting enzyme (ACE) from sweet cheese whey. *Tohoku J Agric Res*. 1996; 47(1/2): 1-8.

48. Lopez-Exposito I, Quiros A, Amigo L, Recio I. Casein hydrolysates as a source of antimicrobial, antioxidant and antihypertensive peptides. *Lait*. 2007; 87(4-5): 241-249.

49. GomezRuiz, J. A., Ramos, M.,Recio, I. Identification of novel angiotensin-converting enzyme-inhibitory peptides from ovine milk proteins by CE-MS and chromatographic techniques. *Electrophoresis*. 2007; 28(22): 4202-4211.

50. Tauzin J, Miclo L, Gaillard J. Angiotensin-I-converting enzyme inhibitory peptides from tryptic hydrolysate of bovine alpha(S2)-casein. *FEBS Lett*. 2002; 531(2): 369-374.

51. Ferreira IMPLVO, Pinho O, Mota MV, et al. Preparation of ingredients containing an ACE-inhibitory peptide by tryptic hydrolysis of whey protein concentrates. *Int Dairy J*. 2007; 17(5): 481-487.

52. HernandezLedesma, B., Quiros, A., Amigo, L.,Recio, I. Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. *Int Dairy J*. 2007; 17(1): 42-49.

53. Otte J, Shalaby SMA, Zakora M, Nielsen MS. Fractionation and identification of ACE-inhibitory peptides from alpha-lactalbumin and beta-casein produced by thermolysin-catalysed hydrolysis. *Int Dairy J*. 2007; 17(12): 1460-1472.

54. Janitha P, Wanasundara P, Ross A, et al. Peptides with angiotensin I-converting enzyme (ACE) inhibitory activity from defibrinated hydrolyzed bovine plasma. *J Agric Food Chem*. 2002; 50(24): 6981-6988.

55. Madadlou, A., Sheehan, D., EmamDjomeh, Z., Mousavi,M.E. Ultrasound-assisted generation of ACE-inhibitory peptides from casein hydrolyzed with nanoencapsulated protease. *J Sci Food Agric*. 2011; 91(11): 2112-2116.

56. Ortiz-Chao P, Gomez-Ruiz JA, Rastall RA, et al. Production of novel ACE inhibitory peptides from beta-lactoglobulin using protease N amano. *Int Dairy J*. 2009; 19(2):69-76.

57. Tavares T, del Mar Contreras M, Amorim M, Pintado M, Recio I, Malcata FX. Novel whey-derived peptides with inhibitory effect against angiotensin-converting enzyme: In vitro effect and stability to gastrointestinal enzymes. *Peptides*. 2011; 32(5): 1013-1019.

58. Hernandez-Ledesma B, Amigo L, Ramos M, Recio I. Release of angiotensin converting enzyme-inhibitory peptides by simulated gastrointestinal digestion of infant formulas. *Int Dairy J*. 2004; 14(10): 889-898.

59. Farzamirad V, Aluko RE. Angiotensin-converting enzyme inhibition and free-radical scavenging properties of cationic peptides derived from soybean protein hydrolysates. *Int J Food Sci Nutr*. 2008; 59(5): 428-437.

60. Lo W, Li-Chan E. Angiotensin I converting enzyme inhibitory peptides from in vitro pepsin-pancreatin digestion of soy protein. *J Agric Food Chem*. 2005; 53(9): 3369-3376.

61. Fan J. Preparation of angiotensin I-converting enzyme inhibiting peptides from soybean protein by enzymatic hydrolysis. *Food Sci Tech Res*. 2003; 9(3): 254.

62. Wu J. Characterization of inhibition and stability of soy-protein-derived angiotensin I-converting enzyme inhibitory peptides. *Food Res Int*. 2002; 35(4): 367.

63. Chiang WD. Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor. *Food Chem*. 2006; 98(4): 725.

64. Kuba, M., Tana, C., Tawata, S.,Yasuda, M. Production of angiotensin I-converting enzyme inhibitory peptides from soybean protein with *monascus purpureus* acid proteinase. *Process Biochem*. 2005; 40(6): 2191-2196.

65. Rho SJ, Lee J, Il Chung Y, Kim Y, Lee HG. Purification and identification of an angiotensin I-converting enzyme inhibitory peptide from fermented soybean extract. *Process Biochem*. 2009; 44(4): 490-493.

66. Fung W, Liong M. Evaluation of proteolytic and ACE-inhibitory activity of lactobacillus acidophilus in soy whey growth medium via response surface methodology. *Lwt-Food Sci Tech*. 2010; 43(3): 563-567.

67. Mallikarjun Gouda KG, Gowda L, Rao AGA, Prakash V. Angiotensin I-converting enzyme inhibitory peptide derived from glycinin, the 11S globulin of soybean (glycine max). *J Agric Food Chem*. 2006; 54(13): 4568-4573.

68. Alok Chatterjee K,S.K. Whey protein hydrolysate - a potent hypertensive ingredient. *Indian Food Industry*. 2009; 28(5--6): 32-38.

69. Lee J, Yoo MA, Koo SH, Baek H, Lee HG. Antioxidant and ACE inhibitory activities of soybean hydrolysates: Effect of enzyme and degree of hydrolysis. *Food Sci. Biotechnol*. 2008; 17(4): 873-877.

70. XueYing M, JinRen N, WeiLing S, PengPeng H, Li F. Value-added utilization of yak milk casein for the production of angiotensin-I-converting enzyme inhibitory peptides. *Food Chem*. 2007; 103(4): 1282-1287.

71. Zhanmei Jiang, Bo Tian, Brodcrob, A.,Guicheng Huo. Production, analysis and *in vivo* evaluation of novel angiotensin-I-converting enzyme inhibitory peptides from bovine casein. *Food Chem*. 2010; 123(3): 779-786.

72. Fung W, Liong M. Evaluation of proteolytic and ACE-inhibitory activity of lactobacillus acidophilus in soy whey growth medium via response surface methodology. *LWT - Food Sci. Technol*. 2010; 43(3): 563-567.

73. Lo WMY, Li-Chan ECY. Angiotensin I converting enzyme inhibitory peptides from in vitro pepsin-pancreatin digestion of soy protein. *J Agric Food Chem*. 2005; 53(9): 3369-3376.

74. Lo WMY. Angiotensin I-converting enzyme inhibitory activity of soy protein digests in a dynamic model system simulating the upper gastrointestinal tract. *J Food Sci*. 2006; 71(3): S231.

75. Roufik S, Gauthier SF, Turgeon SL. Physicochemical characterization and in vitro digestibility of beta-lactoglobulin/beta-lg f142-148 complexes. *Int Dairy J*. 2007; 17(5): 471-480.

76. Mullally M, Meisel H, Fitzgerald R. Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine beta-lactoglobulin. *FEBS Lett*. 1997; 402(2-3): 99-101.

77. Quiros A, del Mar Contreras M, Ramos M, Amigo L, Recio I. Stability to gastrointestinal enzymes and structure-activity relationship of beta-casein-peptides with antihypertensive properties. *Peptides*. 2009; 30(10): 1848-1853.

78. Matoba, N., Doyama, N., Yamada, Y., Maruyama, N., Utsumi, S.,Yoshikawa, M. Design and production of genetically modified soybean protein with anti-hypertensive activity by incorporating potent analogue of ovokinin(2-7). *FEBS Lett*. 2001; 497(1): 50-54.

79. Roufik S, Gauthier S, Turgeon S. In vitro digestibility of bioactive peptides derived from bovine beta-lactoglobulin. *Int Dairy J*. 2006; 16(4): 294-302.

80. Kodera, T.,Nio, N. Identification of an angiotensin I-converting enzyme inhibitory peptides from protein hydrolysates by a soybean protease and the antihypertensive effects of hydrolysates in spontaneously hypertensive model rats. *J Food Sci*. 2006; 71(3):164-173.

81. Centeno JM, Burguete MC, Castello-Ruiz M, et al. Lactoferricin-related peptides with inhibitory effects on ACE-dependent vasoconstriction. *J Agric Food Chem*. 2006; 54(15): 5323-5329.

82. Hernández Ledesma B. ACE-inhibitory and radical-scavenging activity of peptides derived from β-lactoglobulin f (19-25). interactions with ascorbic acid. *J Agric Food Chem*. 2007; 55(9): 3392.

83. Gomez-Ruiz J, Recio I, Belloque J. ACE-inhibitory activity and structural properties of peptide asp-lys-ile-his-pro [beta-CN f(47-51)]. study of the peptide forms synthesized by different methods. *J Agric Food Chem*. 2004; 52(20): 6315-6319.

84. Dziuba J, Iwaniak A, Minkiewicz P. Computer-aided characteristics of proteins as potential precursors of bioactive peptides. *Polimery*. 2003; 48(1): 50-53.

85. Lehtinen R, Jauhiainen T, Kankuri E, et al. Effects of milk casein-derived tripeptides ile-pro-pro, val-pro-pro, and leu-pro-pro on enzymes processing vaso active precursors in vitro. *Arzneimittel-Forschung-Drug Research*. 2010; 60(4): 182-185.

86. Perpetuo EA, Juliano L, Lebrun I. Biochemical and pharmacological aspects of two bradykinin-potentiating peptides obtained from tryptic hydrolysis of casein. *J Protein Chem*. 2003; 22(7-8): 601-606.

87. Jauhiainen, T., Korpela, R., Vapaatalo, H., Turpeinen, A. M.,Kautiainen, H. Bioactive milk peptides and blood pressure. *Agro Food Industry hi-tech*. 2009; 20(2): 26-28.

88. Jauhiainen, T., Ronnback, M., Vapaatalo, H., Wuolle, K., Kautiainen, H., Groop, P. H.,Korpela, R. Long-term intervention with *lactobacillus helveticus* fermented milk reduces augmentation index in hypertensive subjects. *Eur J Clin Nutr*. 2010; 64(4): 424-431.

89. Turpeinen A, Kumpu M, Ronnback M, et al. Antihypertensive and cholesterol-lowering effects of a spread containing bioactive milk peptides and plant sterols. *J Hypertens*. 2009; 27: S272-S272.

90. Jauhiainen T, Vapaatalo H, Poussa T, Kyronpalo S, Rasmussen M, Korpela R. Lactobacillus helveticus fermented milk lowers blood pressure in hypertensive subjects in 24-h ambulatory blood pressure measurement. *Am J Hypertens* 2005; 18(12 Pt 1): 1600-1605.

91. Kamau, S. M., Cheison, S. C., Wei Chen, XiaoMing Liu,RongRong Lu. Alpha-lactalbumin: Its production technologies and bioactive peptides. *Comp Rev Food Sci Food Saf*. 2010; 9(2): 197-212.

92. Chatterton, D. E. W., Smithers, G., Roupas, P.,Brodkorb, A. Bioactivity of beta-lactoglobulin and alpha-lactalbumin - technological implications for processing. *Int Dairy J*. 2006; 16(11, Technological and Health Aspects of Bioactive Components of Milk.): 1229-1240.

93. Kanwar JR, Kanwar RK, Sun X, et al. Molecular and biotechnological advances in milk proteins in relation to human health. *Curr Protein Peptide Sci*. 2009; 10(4):308-338.
94. Hernandez-Ledesma B, Recio I, Amigo L. Beta-lactoglobulin as source of bioactive peptides. *Amino Acids*. 2008; 35(2): 257-265.

95. Murakami, M., Tonouchi, H., Takahashi, R., Kitazawa, H., Kawai, Y., Negishi, H.,Saito, T. Structural analysis of a new anti-hypertensive peptide (beta-lactosin B) isolated from a commercial whey product. *J Dairy Sci*. 2004; 87(7): 1967-1974.

96. Nurminen M, Sipola M, Kaarto H, et al. α-Lactorphin lowers blood pressure measured by radiotelemetry in normotensive and spontaneously hypertensive rats. *Life Sci*. 2000; 66(16): 1535-1543.

97. Pins JJ, Keenan JM. Effects of whey peptides on cardiovascular disease risk factors. *J Clin Hypertens (Greenwich)*. 2006; 8(11): 775-782.

98. Falsified data in "effects of whey peptides on cardiovascular disease risk factors" (J clin hypertens [greenwich] 2006 8(11):775-782)--JJ pins, JM keenan. *J Clin Hypertens*. 2008; 10(8): 631-631.

99. Lee Y, Skurk T, Hennig M, Hauner H. Effect of a milk drink supplemented with whey peptides on blood pressure in patients with mild hypertension. *Eur J Nutr*. 2007; 46(1): 21-27.

100. Geerlings A, Villar IC, Hidalgo Zarco F, et al. Identification and characterization of novel angiotensin-converting enzyme inhibitors obtained from goat milk. *J Dairy Sci*. 2006; 89(9): 3326-3335.

101. Miguel, M., Manso, M. A., LopezFandino, R., Alonso, M. J.,Salaices, M. Vascular effects and antihypertensive properties of kappa-casein macropeptide. *Int Dairy J*. 2007; 17(12): 1473-1477.

102. Jakala P, Pere E, Lehtinen R, Turpeinen A, Korpela R, Vapaatalo H. Cardiovascular activity of milk casein-derived tripeptides and plant sterols in spontaneously hypertensive rats. *J Physiol Pharmacol*. 2009; 60(4): 11-20.

103. Sanchez D, Kassan M, del Mar Contreras M, et al. Long-term intake of a milk casein hydrolysate attenuates the development of hypertension and involves cardiovascular benefits. *Pharmacol Res*. 2011; 63(5): 398-404.

104. Jakala, P., Jauhiainen, T., Korpela, R.,Vapaatalo, H. Milk protein-derived bioactive tripeptides ile-pro-pro and val-pro-pro protect endothelial function *in vitro* in hypertensive rats. *J Funct Foods*. 2009; 1(3): 266-273.

105. Jakala P, Turpeinen AM, Rajakari K, Korpela R, Vapaatalo H. Biological effects of casein-derived tripeptide powders are not affected by fermentation process. *Int Dairy J*. 2010; 20(5): 366-370.

106. Fuglsang A, Nilsson D, Nyborg NC. Characterization of new milk-derived inhibitors of angiotensin converting enzyme in vitro and in vivo. *J Enzyme Inhib Med Chem*. 2003; 18(5): 407-412.

107. Yamaguchi N, Kawaguchi K, Yamamoto N. Study of the mechanism of antihypertensive peptides VPP and IPP in spontaneously hypertensive rats by DNA microarray analysis. *Eur J Pharmacol*. 2009; 620(1-3): 71-77.

108. Tonouchi H, Suzuki M, Uchida M, Oda M. Antihypertensive effect of an angiotensin converting enzyme inhibitory peptide from enzyme modified cheese. *J Dairy Res*. 2008; 75(3): 284-290.

109. Inoue, K., Gotou, T., Kitajima, H., Mizuno, S., Nakazawa, T.,Yamamoto, N. Release of antihypertensive peptides in miso paste during its fermentation, by the addition of casein. *J Biosci Bioeng*. 2009; 108(2): 111-115.

110. del Mar Contreras M, Carron R, Jose Montero M, Ramos M, Recio I. Novel casein-derived peptides with antihypertensive activity. *Int Dairy J*. 2009; 19(10): 566-573.

111. Quiros A, Ramos M, Muguerza B, et al. Identification of novel antihypertensive peptides in milk fermented with enterococcus faecalis. *Int Dairy J*. 2007; 17(1): 33-41.

112. Miguel, M., GomezRuiz, J. A., Recio, I.,Aleixandre, A. Changes in arterial blood pressure after single oral administration of milk-casein-derived peptides in spontaneously hypertensive rats. *Mol Nutr Food Res*. 2010; 54 (10): 1422-1427.

113. RousseauRalliard, D., Goirand, F., Tardivel, S., Lucas, A., Algaron, F., Molle, D., Robert, V., Auchere, D., Boudier, J. F., Gaillard, J. L., Monnet, V., Tauzin, J.,Grynberg, A. Inhibitory effect of a_{S1}- and a_{S2}-casein hydrolysates on angiotensin I-converting enzyme in human endothelial cells *in vitro*, rat aortic tissue *ex vivo*, and renovascular hypertensive rats *in vivo*. *J Dairy Sci*. 2010; 93(7): 2906-2921.

114. Anadon A, Martinez MA, Ares I, et al. Acute and repeated dose (4 weeks) oral toxicity studies of two antihypertensive peptides, RYLGY and AYFYPEL, that correspond to fragments (90-94) and (143-149) from alpha(s1)-casein. *Food Chem Toxicol*. 2010;48 (7): 1836-1845.

115. Kurosawa MT, Nakamura Y, Yamamoto N, Yamada K, Iketani T. Effects of val-pro-pro and ile-pro-pro on nondipper patients: A preliminary study. *J Med Food*. 2011; 14(5): 538-542.

116. Zander, K. van der, Bots, M. L., Bak, A. A., Koning, M. M. G., Leeuw,P.W. Enzymatically hydrolyzed lactotripeptides do not lower blood pressure in mildly hypertensive subjects. *Am J Clin Nutr*. 2008; 88(6): 1697-1702.

117. Mierlo, L. A. van, Koning, M. M. G., Zander, K., van der,Draijer, R. Lactotripeptides do not lower ambulatory blood pressure in untreated whites: Results from 2 controlled multicenter crossover studies. *Am J Clin Nutr*. 2009; 89(2): 617-623.

118. Engberink MF, Schouten EG, Kok FJ, van Mierlo LAJ, Brouwer IA, Geleijnse JM. Lactotripeptides show no effect on human blood pressure - results from a double-blind randomized controlled trial. *Hypertension*. 2008; 51(2): 399-405

119. Cicero AFG, Rosticci M, Veronesi M, et al. Hemodynamic effects of lactotripeptides from casein hydrolysate in mediterranean normotensive subjects and patients with high-normal blood pressure: A randomized, double-blind, crossover clinical trial. *J Med Food*. 2010; 13(6): 1363-1368.

120. Seppo L, Jauhiainen T, Poussa T, Korpela R. A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. *Am J Clin Nutr*. 2003; 77(2): 326-330.

121. Mizushima S, Ohshige K, Watanabe J, et al. Randomized controlled trial of sour milk on blood pressure in borderline hypertensive men. *Am J Hypertens*. 2004; 17(8): 701-706.

122. Nakamura, T., Mizutani, J., Sasaki, K., Yamamoto, N.,Takazawa, K. Beneficial potential of casein hydrolysate containing val-pro-pro and ile-pro-pro on central blood pressure and hemodynamic index: A preliminary study. *J Med Food*. 2009; 12 (6): 1221-1226.

123. Ishida Y, Shibata Y, Fukuhara I, Yano Y, Takehara I, Kaneko K. Effect of an excess intake of casein hydrolysate containing val-pro-pro and ile-pro-pro in subjects with normal blood pressure, high-normal blood pressure, or mild hypertension. *Biosci Biotechnol Biochem*. 2011; 75 (3): 427-433.

124. Townsend RR, McFadden CB, Ford V, Cadee JA. A randomized, double-blind, placebo-controlled trial of casein protein hydrolysate (C12 peptide) in human essential hypertension. *Am J Hypertens*. 2004; 17 (11 Pt 1): 1056-1058.

125. Mizuno S, Matsuura K, Gotou T, et al. Antihypertensive effect of casein hydrolysate in a placebo-controlled study in subjects with high-normal blood pressure and mild hypertension. *Br J Nutr*. 2005; 94 (1): 84-91.

126. Pihlanto A, Virtanen T, Korhonen H. Angiotensin I converting enzyme (ACE) inhibitory activity and antihypertensive effect of fermented milk. *Int Dairy J*. 2010; 20(1): 3-10.

127. Ramchandran L, Shah NP. Yogurt can beneficially affect blood contributors of cardiovascular health status in hypertensive rats. *J Food Sci*. 2011; 76 (4): H131-H136.

128. Seppo L, Kerojoki O, Suomalainen T, Korpela R. The effect of a lactobacillus helveticus LBK-16 H fermented milk on hypertension - a pilot study on humans. *Milchwissenschaft-Milk Science International*. 2002; 57 (3): 124-127.

129. Xiao C. Health effects of soy protein and isoflavones in humans. *J Nutr*. 2008; 138 (6): 1244S-1249S.

130. Messina M. A brief historical overview of the past two decades of soy and isoflavones research. *J Nutr*. 2010; 140: 1350S-1354S.

131. Sacks FM, Lichtenstein A, Van Horn L, et al. Soy protein, isoflavones, and cardiovascular health: A summary of a statement for professionals from the american heart association nutrition committee. *Arterioscler Thromb Vasc Biol*. 2006; 26 (8): 1689-1692.

132. Wang W, De Mejia E. A new frontier in soy bioactive peptides that may prevent age-related chronic diseases. *Comp Rev Food Sci Food Saf*. 2005; 4(4): 63-78.

133. Chen J, Yang S, Suetsuna K, Chao JC. Soybean protein-derived hydrolysate affects blood pressure in spontaneously hypertensive rats. *J Food Biochem*. 2004; 28(1): 61-73.

134. Martin D, Williams JL, Breitkopf N, Eyster K. Pressor responsiveness to angiotensin in soy-fed spontaneously hypertensive rats. *Can J Physiol Pharmacol*. 2002; 80(12): 1180-1186.

135. Jianping W, Xiaolin D. Hypotensive and physiological effect of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats. *J Agric Food Chem*. 2001; 49(1): 501-506.

136. Yang H, Yang S, Chen S, Chen J. Soy protein hydrolysate ameliorates cardiovascular remodeling in rats with L-NAME-induced hypertension. *J Nutr Biochem*. 2008; 19(12): 833-839.

137. Palanisamy N, Viswanathan P, Ravichandran MK, Anuradha CV. Renoprotective and blood pressure-lowering effect of dietary soy protein via protein kinase C βII inhibition in a rat model of metabolic syndrome. *Can J Physiol Pharm*. 2010; 88(1): 28-37.

138. Yang H, Yang S, Chen J, Tzeng Y, Han B. Soyabean protein hydrolysate prevents the development of hypertension in spontaneously hypertensive rats. *Br J Nutr*. 2004; 92(3): 507-512.

139. Nevala R, Vaskonen T, Vehniinen J, Korpela R, Vapaatalo H. Soy based diet attenuates the development of hypertension when compared to casein based diet in spontaneously hypertensive rat. *Life Sci*. 2000; 66(2): 115-124.

140. Yang G, Shu X, Jin F, et al. Longitudinal study of soy food intake and blood pressure among middle-aged and elderly chinese women. *Am J Clin Nutr*. 2005; 81: 1012-1017.

141. Welty F, Lee K, Lew N, Zhou J. Effect of soy nuts on blood pressure and lipid levels in hypertensive, prehypertensive, and normotensive postmenopausal women. *Arch Intern Med*. 2007; 167(10): 1060-1067.

142. Washburn S, Burke GL, Morgan T, Anthony M. Effect of soy protein supplementation on serum lipoproteins, blood pressure, and menopausal symptoms in perimenopausal women. *Menopause*. 1999; 6(1): 7-13.

143. Rivas M, Garay RP, Escanero JF, Cia P,Jr, Cia P, Alda JO. Soy milk lowers blood pressure in men and women with mild to moderate essential hypertension. *J Nutr*. 2002; 132(7): 1900-1902.

144. Teede H, Giannopoulos D, Dalais F, Hodgson J, McGrath B. Randomised, controlled, cross-over trial of soy protein with isoflavones on blood pressure and arterial function in hypertensive subjects. *J Am Coll Nutr*. 2006; 25(6): 533-540.

145. Fang Hong, Luo Ming, Sheng Yi, Li Zhanxia, Wu Yongquan, Liu Chi. The antihypertensive effect of peptides: A novel alternative to drugs? *Peptides*. 2008; 29(6): 1062-1071.

146. Harris JA, Benedict FG, eds. *A biometric study of basal metabolism in man.* 279th ed. Washington, DC: Carnegie Institute of Washington; 1919.

147. Margatan, W., Ruud, K., Wang, Q., Markowski, T., and Ismail, B. Angiotensin converting enzyme inhibitory activity of soy protein subjected to selective hydrolysis and thermal processing. *J Agric Food Chem*. 2013; 61(14): 3460-3467.

148. Lee Y, Skurk T, Hennig M, Hauner H. Effect of a milk drink supplemented with whey peptides on blood pressure in patients with mild hypertension. *Eur J Nutr*. 2007; 46(1): 21-27.

149. Foltz, M., Meynen, E. E., Bianco, V., Platerink, C. van, Koning, T. M. M. G.,Kloek, J. Angiotensin converting enzyme inhibitory peptides from a lactotripeptide-enriched milk beverage are absorbed intact into the circulation. *J Nutr*. 2007; 137(4): 953-958.

150. Graudal NA, Galloe AM, Garred P. Effects of sodium restriction on blood pressure, renin, aldosterone, catecholamines, cholesterols, and triglyceride: A meta-analysis. *JAMA*. 1998; 279(17): 1383-1391.

151. Morimoto S, Abe R, Fukuhara A, Tanaka K, Yamamoto K. Effect of sodium restriction on plasma renin activity and renin granules in rat kidney. *Am J Physiol*. 1979; 237(5): F367-F371.