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Soy isoflavones and phytate: Effects on homocysteine, C-reactive protein, and iron status in postmenopausal women

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Soy isoflavones and phytate: Effects on homocysteine, C-reactive protein, and iron status in postmenopausal women

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GENERAL INTRODUCTION

Thesis Organization

This thesis includes a general introduction describing the objectives, hypothesis, specific aims, limitations of the study, and significance of this research project. This introduction is followed by a review of literature, my manuscript, and general conclusions. The review of literature and manuscript are followed by references for each section.

Objectives

This study was designed to determine whether soy protein components, isoflavones and phytate, would reduce the risk of cardiovascular disease (CVD) in postmenopausal women.

Hypothesis

Soy protein components will reduce the risk of CVD in postmenopausal women, specifically that isoflavones and/or phytate will reduce total homocysteine concentrations (tHcy) and C-reactive protein (CRP), and that phytate will reduce iron excess.

Specific Aims

1. To determine the contribution of CVD risk factors to tHcy and CRP concentrations.
2. To determine the independent effect of phytate and isoflavones from soy protein isolate (SPI) on CVD risk as reflected by tHcy, CRP, and iron indices.

Limitations

The limitations of this study include the small sample size, the use of soy protein isolate rather than soy foods, and the lack of a measure of body composition.

Significance of Study

Recently, tHcy and CRP have been designated as independent CVD risk factors. Soy protein contains a variety of components that may be responsible for the bioactive effects soy, including isoflavones. Isoflavones are compounds produced by plants with estrogenic effects that reduce tHcy, particularly in estrogen-deprived postmenopausal women. There is evidence that soy protein may reduce tHcy (Tonstad et al. 2002) and that these changes may not be due to isoflavone content (Jenkins et al. 2002).

Another component of soy protein, phytate, may also have some health benefits. Phytate reduces mineral absorption (Hurrell et al. 1992; Hurrell et al. 2003) and may help reduce iron excess in postmenopausal women. Iron excess in postmenopausal women is associated with increased risk of CVD (Sullivan 1981), and preventing iron excess with phytate may reduce risk of CVD.

Little research has been conducted to determine the effects of isoflavones on CRP, or the effects of phytate on tHcy and CRP. Studies with shorter-term intervention periods have not shown an effect of soy protein on CRP (Jenkins et al. 2002), but a 6 wk trial may show protective effects. This study will determine whether soy isoflavones or phytate have a cardioprotective effect by reducing tHcy and CRP concentrations in postmenopausal women.

REVIEW OF LITERATURE

Cardiovascular disease

Cardiovascular disease is the leading cause of death in the U.S. for both men and women. In 2001 alone, there were 700,142 deaths caused by CVD, making up 29% of total deaths in the U.S. (Centers for Disease Control and Prevention 2003). Awareness of CVD risk and prevention, as well as improvements in care, have resulted in more than a 50% decrease CVD-related deaths since 1950 (Centers for Disease Control and Prevention 2003). Although CVD mortality has decreased, observational studies show that the major decreases in mortality are not equivalent to the smaller changes in morbidity (Sytkowski et al. 1996). In the Framingham Heart Study, CVD mortality decreased by 59% in females and 53% in males, while 20-yr incidence of the disease decreased by only 21% and 6%, respectively (Sytkowski et al. 1996). The greater decrease in mortality may be related to better clinical care, including a better understanding of risk assessment. Traditional methods of assessing CVD risk have relied heavily on analysis of fasting blood lipids, including total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and the ratio of TC:HDL-C. However, lipids alone do not clearly demonstrate risk of CVD. More recently, other risk factors, including oxidative stress, elevated tHcy, elevated CRP, excess iron, and menopausal status in women, are now being used to better assess individual risk of CVD.

Risk Factors for Cardiovascular Disease

Dyslipidemia

Lipids are essential for membrane structure, energy storage, hormone synthesis, organ tissues, and vitamin absorption. The menopausal transition is associated with alterations in blood lipids and lipoproteins, including an increase in TC, triacylglycerols (TG), LDL-C, and TC/HDL-C and decrease in HDL-C (Akahoshi et al. 2001). These alterations are known as dyslipidemia or hypercholesterolemia, and are associated with CVD risk. Lipids and lipoprotein concentrations are strongly controlled by genetics (Vuorio et al. 2004), but lifestyle interventions can be successful in correcting dyslipidemia. Nutritional interventions to reduce dietary fat, particularly saturated fat, have been the traditional approach to reducing circulating cholesterol concentrations. Inclusion of high fiber foods, particularly soluble fiber, has also been quite effective in serum cholesterol reductions.

Oxidative Stress

There is a continual physiologic balance between oxidizing and reducing agents. Oxidizing agents, in the form of free radicals, are ingested along with food, and also produced during metabolic processes. Defenses against free radical damage consist of a combination of antioxidant enzymes, including catalase, glutathione peroxidase, glutathione reductase, and superoxide dismutase, as well as antioxidant molecules, including vitamins E and C, and specific body proteins. An imbalance in the system results in increased oxidative damage, referred to as oxidative stress. Oxidative stress increases with age (Packer 1995) and contributes to the development and progression of many chronic diseases, including renal failure, CVD, and neurodegenerative conditions (Packer 1995). Dietary intake of

antioxidants may improve antioxidant status, thereby reducing oxidative stress (Riso et al. 1999).

Hyperhomocysteinemia

Homocysteine (Hcy) is a metabolite produced in the demethylation of methionine. *S*-adenosylhomocysteine (SAH) is produced from hydrolysis of *S*-adenosylmethionine by methyltransferases, such as glycine *N*-methyltrasferase (GNMT; Figure 1) in the liver. Once the adenosyl group is removed, Hcy can either undergo remethylation to form methionine by methionine synthase, a vitamin B_{12} and folate dependent enzyme occurring in all tissues, or by betaine homocysteine methyltransferase in the liver. Homocysteine can also undergo condensation with serine to eventually produce cysteine by cystathionine β-synthase, a vitamin B_6 dependent enzyme. In addition, recent work demonstrates that in the liver, phosphatidylethanolamine N-methytransferase produces Hcy in the production of phosphatidylcholine, which may make a significant contribution to circulating tHcy concentrations (Noga et al. 2003). There is an inverse correlation of serum folate and vitamin B_{12} with tHcy concentrations (Tonstad et al. 2002). Deficiency of folate, vitamin B_{12}, and/or vitamin B_6 will result in impaired metabolism of Hcy and elevation of circulating concentrations, a condition called hyperhomocysteinemia (McKinley et al. 2001). In hyperhomocysteinemic individuals, treatment with folate reduces tHcy concentrations, and reverses the effects of Hcy on CVD risk (Brattström et al. 1985; Doshi et al. 2002; Usui et al. 1999; Verhaar et al. 1998).

Hyperhomocysteinemia is an independent, modifiable risk factor for CVD (Ridker et al. 1999). A prospective observational study of 587 coronary artery disease (CAD) patients revealed an association between tHcy concentrations and history of myocardial infarction,

and a very strong graded association with mortality due to CVD (Nygård et al. 1997). CVD patients from the Women's Health Study with concentrations of tHcy in the highest quartile (>13.26 μmol/L) were twice as likely to experience a future cardiovascular event as those in the lowest quartile (<9.54 μmol/L; Ridker et al. 1999). Hence, elevated tHcy concentration is now recognized as an independent risk factor for CVD.

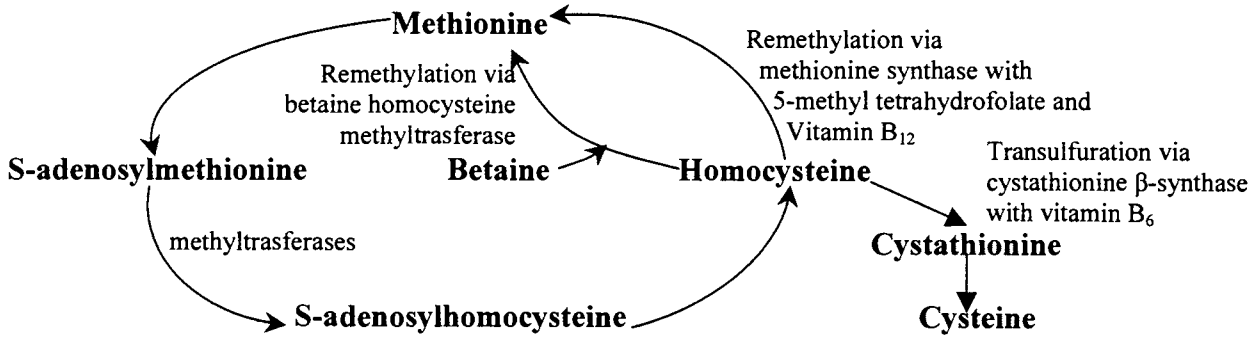

Figure 1 Hcy Metabolism

Lipid and lipoprotein concentrations are often used in clinical practice to evaluate risk of CVD, in part because they are inexpensive and easy to assess. However, lipids alone do not fully assess a patient's risk of CVD. For example, in a large observational study, patients diagnosed with CAD had significantly lower TC and LDL-C concentrations compared to the non-CAD controls, even after excluding those taking lipid-lowering medications (Foody et al. 2000). However, CAD patients had significantly higher tHcy compared to controls. Thus, tHcy may also be important in predicting CVD. For example, a 1% increase in TC relates to a 2% increase in CVD risk (Gensini et al. 1996), and a 5μmol/L or 50% increase in tHcy is associated with a 24% increase in risk of a CVD event (Ridker et al. 1999). Successfully reducing tHcy may be more readily achievable than decreasing TC or LDL-C,

and analyzing tHcy concentrations as a part of the CVD risk profile with usual clinical care may better diagnose and predict a cardiovascular event than lipids alone.

Epidemiological data from the National Health and Nutrition Examination Survey (NHANES) III reveals that circulating tHcy is positively correlated with male sex, age, alcohol consumption, smoking, serum creatinine, blood pressure, and body mass index (BMI), but is negatively correlated with vitamin and mineral consumption, serum folate, and serum vitamin B_{12} (Ganji et al. 2003). In a survey of older adults, fruit and vegetable intake was inversely associated with tHcy concentrations (Gao et al. 2004), suggesting that lower tHcy is associated with particular nutrients, such as folate or fiber. While elevations in tHcy are typically seen in older populations, elevated tHcy concentrations have also been observed in children, particularly in those with obesity and/or diabetes (Glowinska et al., 2003).

In addition to dietary and lifestyle factors, there is strong genetic variation in tHcy concentrations. Polymorphisms of genes for enzymes involved in the metabolism of Hcy may alter the factors that affect circulating tHcy concentrations. For instance, a common polymorphism is found on the C677T methyltetrahydrofolate reductase gene for the enzyme that produces 5-methyltetrahydrofolate, the form of folate required for remethylation of Hcy. This polymorphism potentially results in hyperhomocysteinemia, particularly in combination with marginal folate status. In homozygotes for this polymorphism, riboflavin status is inversely correlated with tHcy concentrations if folate status is compromised (Jacques et al. 2002). In these individuals, approximately 1-3% of the U.S. population, riboflavin status may be a major determinant in preventing hyperhomocysteinemia, while it does not appear to be a significant contributor in the general population.

The mechanism underlying Hcy's effect on CVD risk is not yet fully understood, but evidence suggests that Hcy may initiate damage to the vasculature by suppressing endothelial nitric oxide (NO; Li et al. 2002; Fu et al. 2002), stimulating lipid accumulation (Beauchamp et al. 2002; Li et al. 2002), and altering fibrinolytic factors (Nappo et al. 1999; Sauls et al. 2003; Tofler et al. 2002). Alterations in NO concentrations may increase oxidative damage to the endothelial cells and impair the NO-mediated vasodilation involved in the development of CVD. When endothelial cell cultures are treated with Hcy, NO production is reduced, perhaps by as much as 50% (Li et al. 2002). This reduction in NO may be due to the binding of NO to the sulfhydryl group of Hcy, increasing the proportion of bound-to-free NO and making NO unavailable (Fu et al. 2002). At higher concentrations NO acts as an antioxidant, while at low concentrations it acts as a pro-oxidant, although this mechanism is not fully understood (Rubbo et al. 1996; Heydrick et al. 2004). Elevated tHcy concentrations, perhaps through reductions in free NO, increase superoxide-induced lipid peroxidation dose-dependently in endothelial cell cultures (Heydrick et al. 2004) and reactive oxygen species in monocyte cell cultures (Zeng et al. 2003). Damage by Hcy may not just be due to oxidative damage, but it may also reduce antioxidant capacity. Hyperhomocysteinemia induced by folate deficiency in rats resulted in decreased hepatic antioxidant enzyme activity and increased hepatic liver peroxidation (Huang et al. 2001). This combination of increased oxidative agents and compromised antioxidant capacity in hyperhomocysteinemia leads to higher oxidative stress, thereby increasing the risk of CVD.

In addition to increasing oxidative stress, NO plays an important role in maintaining the elasticity of the vasculature. Under normal conditions the release of NO results in vasodilation, thereby reducing blood pressure. As previously discussed, NO is largely

unavailable with elevated tHcy concentrations. Systemic arterial compliance, a function of NO, is reduced by methionine-loading induced hyperhomocysteinemia in healthy adults (Nestel et al. 2003) by a mechanism independent of oxidative stress (Chao et al. 2000). Animal research explains this further in depth. Rats injected with both Hcy and NO preparations experience a compromised hypotensive response to vasodilators (Fu et al. 2002). The lack of action by NO in this experiment demonstrates that NO was not available to initiate a hypotensive effect, revealing that hyperhomocysteinemia interferes with NO-mediated vasodilation.

Accumulation of lipids, the classic characteristic of the atherosclerotic process, is accelerated by elevated tHcy concentrations. Treatment with Hcy increases stability of HMG-CoA reductase mRNA, thereby increasing expression of this enzyme which upregulates cholesterol synthesis, as well as increasing expression of caveolin-1, a cholesterol transport protein (Li et al 2002). These alterations in intracellular lipid metabolism result in increased total lipid content of endothelial cells. Although the mechanism for this effect remains to be determined, it may be due to increases in oxidative stress. Elevations in cholesterol are particularly harmful in atherosclerotic lesions, where cholesterol is taken up by macrophages, converting them to lipid-laden foam cells, thus contributing to the formation of the atherosclerotic plaque. There is evidence in cell culture that Hcy increases the conversion of normal macrophages into foam cells by stimulating uptake of lipids. Macrophages treated with superphysiologic concentrations of Hcy activate protein kinase C, which modulates factors increasing transcription of lipoprotein lipase mRNA (Beauchamp et al. 2002), thereby increasing lipid availability to circulating macrophages. These superphysiologic concentrations of tHcy are not observed in vivo, but

given the relationship between tHcy and hyperlipidemia, we might expect the same role of tHcy in the progression of atherosclerosis in humans.

Examination of subjects from the Framingham Heart Study revealed that subjects with the highest tHcy concentrations had significantly elevated clotting factors and plasma viscosity (Tofler et al. 2002), which would increase platelet aggregation in an atherosclerotic lesion. This association has been confirmed in animal studies showing that rabbits injected with Hcy to induce hyperhomocysteinemic concentrations have abnormal fibrinogen clotting that resembles the clots observed in thrombosis (Sauls et al. 2003). Such alteration of coagulation factors may be related to oxidative damage caused by hyperhomocysteinemia. A human study found that methionine-loading induced acute hyperhomocysteinemia resulted in an immediate increase in coagulation factors, including fibrinopeptide A, prothrombin fragments 1 and 2, and adhesion molecules (Nappo et al. 1999). However, providing antioxidant vitamins E and C with the methionine load prevented the rise in these factors, confirming that the effects of Hcy on coagulation factors are related to an increase in oxidative stress.

C-reactive protein

C-reactive protein is an acute phase protein produced in the liver in response to systemic inflammation. Circulating CRP is highly elevated during an acute infection, trauma, or stress. Some individuals may experience a sustained elevation of CRP, although not to the same degree as during infection. This long-term elevation of CRP is associated with increased risk of CVD. With a half-life of 18 to 20 hours for plasma CRP, it is an easily measurable and effective marker of CVD risk (Ridker 2003).

As we understand more about the initiation and progression of CVD, it is now recognized as an inflammatory disease. CRP is associated with several forms of vascular disease, including CAD, peripheral artery disease, and renal insufficiency (Stuveling et al. 2004). According to some authorities, measuring CRP alone can better predict a myocardial infarction than measuring TC or HDL-C (Ridker et al. 1998). The addition of CRP to the traditional diagnostic model improves the model's capability to predict CVD (Ridker et al. 2001). A large clinical trial demonstrated that elevated CRP *or* TC alone is associated with a 1.5-fold and a 2.3-fold increased risk of myocardial infarction, respectively (Ridker et al. 1998). In comparison, those with elevated CRP *and* TC have a five-fold increase in risk of myocardial infarction compared to those with low CRP and TC (Ridker et al. 1998). Although CRP is typically produced in the liver, analysis of human atherosclerotic lesions reveals that CRP and associated complement proteins are actually produced by the atherosclerotic lesion (Yasojima et al. 2001), further suggesting that CRP plays a role in atherosclerosis.

With both high and low levels of inflammation, CRP concentrations vary with lifestyle, nutrition, and genetic factors. Excess body weight has a strong effect on CRP concentrations, which can be predicted by BMI, waist circumference, and waist to hip ratio (Hak et al. 1999; Lear et al. 2003; Yudkin et al. 1999). Physical activity is also associated with reduced CRP, although this relationship may be indirectly related to body weight and adiposity (Pihl et al. 2003). In addition, CRP is strongly affected by dietary intake, particularly fiber, fat, and antioxidants. In a survey of older adults, fruit and vegetable intake was inversely associated with CRP concentrations (Gao et al. 2004), which could potentially be due to antioxidant and fiber content. NHANES III data reveal that CRP is inversely

related to dietary fiber intake and serum/plasma concentrations of retinol, retinyl esters, vitamin C, α and β-carotene, cryptoxanthin, lutein/zeazanthin, lycopene, and selenium, and positively associated with dietary saturated fat intake (Ford et al. 2003; King et al. 2003). Although dietary *trans* fatty acids are considered to have similar effects as saturated fats on CVD risk, there is no effect of *trans* fat on CRP concentrations (Lichtenstein et al. 2003). NHANES data suggest that fiber may also be protective against CRP; the odds ratio for elevated CRP with the highest quintile of fiber intake compared to the lowest is 0.49 (Ajani et al. 2004). Thus, consuming a diet rich in fruits and vegetables, which would be rich in antioxidants, high in fiber, and low in saturated fat, may reduce CRP concentrations. Use of a multivitamin/mineral supplement has also been shown to reduce CRP concentrations (Church et al. 2003), although it is not clear which specific nutrients are responsible for this effect. In a sample of adults taking a daily multivitamin supplement, only plasma B_6 and vitamin C concentrations were inversely correlated with CRP (Church et al. 2003). Genetics also play a role in CRP concentrations, as revealed by a study which demonstrated that CRP concentrations are moderately correlated between identical twins (Retterstol et al. 2003).

Recent studies have demonstrated that CRP is not only a predictor of CVD, but is a causative agent in atherogenesis. While the mechanism underlying this relationship has yet to be fully determined, it may be partially due to the effect of CRP on stimulating pro-inflammatory factors (Devaraj et al. 2004), inhibiting vasodilators (Venugopal et al. 2002; Verma et al. 2002; Venugopal et al. 2003), and causing endothelial dysfunction (Fichtlscherer et al. 2000; Venugopal et al. 2003; Wang et al. 2003; Yudkin et al. 1999).

CRP may increase the migration of monocytes to the atheroma, which would contribute to the formation of foam cells. Cell culture work demonstrates that incubation of

monocytes with CRP results in elevated monocyte chemoattractant protein-1 (MCP-1; Khreiss et al. 2004). Monocytes trafficked to the site of an atherosclerotic lesion may take up excess lipid, becoming foam cells, and be incorporated into the atheroma itself. Incubation of human endothelial cells with CRP increases secretion of IL-8, a cytokine that increases monocyte adhesion to the endothelium (Devaraj et al. 2004), thereby increasing the uptake of monocytes into the atheroma. Examination of human atherosclerotic lesions reveals that CRP infiltrates the tissue prior to a monocyte response (Torzewski et al. 2000). Moreover, monocytes have a receptor for CRP, which appears to be important in binding CRP and passing into an atherosclerotic lesion (Torzewski et al. 2000). This elevation may be related to CRP's suppression of NF-kappa B, which regulates the cytokine response, ultimately increasing IL-8 (Devaraj et al. 2004). Inhibition of IL-8 resulted in a 31% inhibition of the adhesion of monocytes to the endothelium (Devaraj et al. 2004), which may significantly reduce the development of an atheroma. Specifically, CRP has the greatest increase in the expression of complement protein CD11b, a phenotype involved in monocyte adhesion to the endothelium (Woollard et al. 2002). Although the complement protein increased in this study, the effects on monocyte adhesion to the endothelial cells were inconclusive, showing increased adhesion only when cell surface glycoproteins involved in cellular adhesion endothelial cells have been activated (Woollard et al. 2002). Further work needs to be done to understand whether CRP increases monocyte adhesion by complement proteins, or if it affects glycoproteins. The stimulatory effect of CRP on MCP and IL-8 and other adhesion factors may cause increased monocyte availability and incorporation into the atheroma.

Endothelial function is maintained by a careful system of regenerating old cells with new, and thus maintaining the elasticity of the vasculature. Endothelial progenitor cells are

important in providing new healthy cell growth to the endothelium. Endothelial progenitor cells migrate from bone marrow to blood vessels and contribute 5-26% to new endothelial cell growth (Murayama et al. 2002). The number of circulating endothelial progenitor cells is positively associated with endothelial function and inversely associated with risk of CVD (Hill et al. 2003). CVD risk factors, such as age, hypertension, and hyperlipidemia, are associated not only with reduced number of endothelial progenitor cells, but also with reduced capacity to migrate to a new site (Vasa et al. 2001). Potentially, damage to the endothelium in early stages of atherosclerosis could be repaired if there were a sufficient supply of endothelial progenitor cells available to heal the injury and prevent inflammation and lipid accumulation. At the site of injury, exogenous NO stimulates endothelial progenitor cells to form microtubules, a necessary step in the regeneration of the endothelium (Verma et al. 2004). Recently it has been demonstrated that the detrimental effects of CRP on NO generation (Venugopal et al. 2002, Verma et al. 2002, Venugopal et al. 2003) interfere with the ability of endothelial progenitor cells to form microtubules, thereby decreasing angiogenesis (Verma et al. 2004). This interference endothelial health with may be one cause of the increased risk of CVD with elevated CRP.

Endothelial function can be measured by vasoreactivity, or the ability of the vasculature to expand and contract to accommodate changes in blood flow. Elevated CRP concentrations are associated with endothelial dysfunction (Yudkin et al. 1999) and serve as an independent predictor of blunted endothelial vasodilatory capacity (Fichtlscherer et al. 2000). This may be partially due to the relationship between CRP and angiotensin II, a powerful vasoconstrictor that, through action of its receptors (AT_1-R and AT_2-R), also has effects on inflammation and vasoprotection, respectively (Wang et al. 2003). Maintenance of

the angiotensin II system is imperative to healthy endothelial function. Elevations in CRP may cause a disturbance in this system, thereby interfering with endothelial function, and increasing risk of CVD. Cell culture models have demonstrated that incubation of vascular smooth muscle cells with a high dose of CRP (50 μg/mL) results in a 225% increase in mRNA expression for AT_1-R, but no change in mRNA stability, receptor affinity, or expression of AT_2-R (Wang et al. 2003). Increased expression of AT_1-R would stimulate an inflammatory response, as seen in atherogenesis, and a lack of stimulation of AT_2-R would have no increase in vascular protection to compensate. In response to an injury to the endothelium, angiotensin II stimulates migration of vascular smooth muscle cells to the site of injury, potentially by increasing ROS and vascular smooth muscle cell proliferation. However, incubation with CRP over exaggerated the effect of angiotensin II on vascular smooth muscle cell migration and proliferation (Wang et al. 2003), which would lead to thickening of the arterial wall. Support for these cell culture results comes from the Rotterdam Study that found an association between elevated CRP and increased carotid intima-media thickness (van der Meer et al. 2002).

In addition to the vasoconstrictive response to CRP, it also suppresses vasodilators, which are important in maintaining vasoreactivity. Similar to the effects of tHcy, CRP attenuates NO synthesis by decreasing stability of the mRNA of endothelial NO synthase (eNOS) and thus reducing production of NO (Venugopal et al. 2002, Verma et al. 2002; Verma et al. 2004). Decreased enzymatic activity results in reduced concentrations of NO and cyclic guanosine monophosphate (cGMP), a second messenger of NO (Verma et al. 2002). Conversely, elevated CRP increases activity of inducible NO synthase (iNOS; Venugopal et al. 2003) and concentrations of superoxide anion (Venugopal et al. 2003). The

latter reacts with NO produced by iNOS to form peroxynitrite (ONOO-), which can cause nitration of proteins. Nitration of the tyrosine residue of prostaglandin I synthase could reduce its activity and result in decreased prostacyclin (PGI_2) production. PGI_2 is a beneficial compound with vasodilatory and antiplatelet effects. Incubation of human aortic endothelial cells with CRP results in a dose-dependent decrease in PGI_2 metabolites (Venugopal et al. 2003), suggesting that this may occur in humans. Moreover, scavengers of ONOO- reverse this decrease (Venugopal et al. 2003), confirming the role of superoxides and iNOS in this process to decrease vasodilation. Thus, CRP increases risk for CVD because it not only suppresses vasodilation, and stimulates response to vasoconstrictors, but it also stimulates the accumulation of monocytes and vascular smooth muscle cells to cause damage to the endothelium.

Iron Excess

Considering that iron deficiency is the most common nutritional deficiency in the world, research has focused on approaches to alleviate the deficiency. Those particularly at risk of iron deficiency include infants and young children, adolescents, pregnant women, and women of childbearing age. Iron deficiency is the result of dietary iron with low bioavailability, increased iron needs due to growth, or losses from menstrual bleeding or parasites. Iron deficiency affects 30% of the world's population and decreases cognitive performance, intellectual function, and physical capacity (Swanson 2003).

On the other hand, some populations are at risk of excess body iron, including those with genetic hemochromatosis or with a chronic high iron intake. Genetic hemochromatosis is a condition that increases the absorption of iron, with a frequency for homozygotes of 5/1000 and for heterozygotes of 1/10 in those of northern European origin (Tuomainen et al.

1999). Loss of function of the *Hfe* protein in these individuals results in unregulated iron absorption. In this disorder, there is no mechanism to suppress iron absorption even when iron stores are sufficient to meet body demands. With increased iron absorption, iron stores are elevated, causing iron overload. Another group of minor concern for iron overload is those who chronically consume very high amounts of iron, such as the African Bantu tribe who store food and beverages in iron-containing vessels, allowing iron to pass into the food from the containers (Fairbanks 1998).

Results of a Finnish study drew attention to the possibility of the role of excess iron stores (ferritin > 200 μg/L) in increased CVD risk of males and postmenopausal females (Salonen et al. 1992). Although not clear, iron excess in men and postmenopausal women is of concern due to the epidemiological evidence of its relationship with CVD (Sullivan 1981). Iron stores increase with age in both men and women, and the risk of CVD is higher in men than in women until after menopause, when women's risk of CVD rises to that of men (Sullivan 1981). Premenopausal women are not typically at risk for iron excess because of monthly iron losses with menstruation and circulating estrogen concentrations. However, postmenopausal women do not experience monthly blood losses, and hormone deficiency may have an effect on iron status. Postmenopausal women receiving hormone therapy have significantly lower serum ferritin and greater total iron binding capacity than similar women not receiving hormone therapy (Penckofer et al. 2000). The women receiving hormone therapy also had significantly higher circulating estradiol concentrations, suggesting that estrogen concentrations may be related to iron status. Whether due to iron losses or hormones, the risk for elevation of iron is similar in postmenopausal women to that of men.

Serum ferritin concentrations in women are clearly elevated with each decade of life beyond 40 years, and women have an odds ratio of 1.54 for developing CAD with each 100 μg/L increase in ferritin (Kiechl et al. 1994). A large sample of men (n=2443) showed a significant relationship between serum ferritin and carotid plaque formation, particularly when LDL-C concentrations were taken into account (Wolff et al. 2004). While much research is needed to understand the relationship between iron and CVD, potential mechanisms may include the effects if iron on lipid metabolism and oxidative stress.

The elevation in body iron may increase risk for CVD by altering lipids. Studies with pre- and postmenopausal women have shown that serum ferritin is positively correlated with TC and LDL-C (Berge et al. 1994; Penckofer et al. 2000). Kiechl et al. (1994) demonstrated a stronger effect of ferritin on CVD risk in those with elevated TC concentrations. However, this does not clearly indicate a causative effect since both ferritin and lipids are increased with age, and may just be common in those who have the highest CVD risk due to other causes. Closer investigation in animal models suggests that hypercholesterolemia may alter iron metabolism by decreasing circulating iron, but increasing iron sequestered in the liver (Dabbagh et al. 1997; Turbino-Ribeiro et al. 2003). Interestingly, iron overload-induced animals fed a hypercholesterolemic diet have reduced TC concentrations compared to animals without iron overload (Dabbagh et al. 1997; Turbino-Ribeiro et al. 2003). This hypocholesterolemic effect of iron overload is an indirect effect most likely due to liver damage caused by the elevation in iron stores, and does not suggest that iron overload should be used as a means to reduce TC. Rather the effect seems quite the opposite; there is now evidence of iron accumulation in atherosclerotic lesions, an area also rich in cholesterol (Stadler et al. 2004). Further analysis of atherosclerotic lesions in rabbits reveals that iron

concentrations are associated with the depth of the lesion, and that receiving iron chelation therapy slowed progression (Minqin et al. 2003). The combination of hypercholesterolemia as well as excess iron may have a synergistic effect in elevating risk of CVD.

An alternative explanation for the association of excess iron and CVD is the prooxidant property of iron in generating free radicals (Halliwell 1986). Iron is essential in the human diet and is needed for many important physiological functions when bound to hemoglobin, myoglobin, cytochromes, several enzymes, and nonheme iron proteins. The bound iron is transported in circulation by transferrin, while excess iron is stored as ferritin and hemosiderin. A small amount of body iron is also found as free or non-transferrin bound iron, which is bound to low-molecular-weight ligands such as ATP, ADP, GTP, and citrate. The non-transferrin bound iron pool is small in healthy people but can be slightly elevated in people at risk for iron overload (Crichton et al. 2002). Based on the involvement of iron in the Fenton reaction under in vitro conditions, the low-molecular-weight iron complexes may presumably react with H_2O_2 resulting in the formation of hydroxyl radicals (Luo et al. 1996).

Fenton reaction (Luo et al. 1996)

$$Fe^{3+} + O_2\bullet^- \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO\bullet$$

Hydroxyl radicals are the most reactive ROS and are known to react with cellular constituents, ncluding amino acid residues and purine/pyrimidine bases in DNA, as well as to attack cell membrane lipids causing lipid peroxidation. Due to participation in the Fenton reaction, iron is considered a pro-oxidant, but this reaction requires free iron not associated with proteins (hemoglobin, myoglobin, transferrin, ferritin, enzymes, and hemosiderin). Given the small amount of catalytic iron in the body, the Fenton reaction is unlikely to occur

under normal conditions. Some research has shown that iron is unlikely to catalyze oxidative damage in healthy individuals, showing no association between iron status and LDL oxidation, a marker of oxidative damage to lipids (Derstine et al. 2003). In a moderately iron overloaded rabbit model, a hypercholesterolemic diet did not result in an increase in markers of lipid peroxidation, or changes antioxidant status, whereas was actually a protective effect of iron overload on the formation of atherosclerotic lesions (Dabbagh et al. 1997). However, there is some evidence of the presence of free iron and copper in human atherosclerotic lesions (Swain et al. 1995) and in the liver of an iron overload animal model (Britton et al. 1990). Animal work reveals that iron overload increases ROS within the arterial vessel wall itself (Day et al. 2003), which could contribute to endothelial dysfunction, and development of atherosclerosis. Lipid peroxidation caused by treatment of intact rabbit hearts with oxidizers, such as H_2O_2, is greater when also treated with iron (Lesnefsky et al. 1992). This reaction is not isolated to the vasculature; there is evidence that iron chelation therapy inhibits lipid peroxidation stimulated by ultraviolet light in the epidermis (Seité et al. 2004).

This research suggests that at least in some instances, protein bound iron is available to participate in production of ROS and oxidative damage. There is also evidence that O_2^- may stimulate the release of iron from ferritin (Oteiza et al. 1995). Hence, in a situation of high oxidative stress, there may be increased free iron available to participate in the generation of hydroxyl radicals, creating a further increase in oxidative stress. As discussed earlier, lipid-laden atherosclerotic lesions are also rich in iron (Stadler et al. 2004). This may be a link between the relationship of hypercholesterolemia, a high oxidative stress condition, and iron in increasing risk of CVD (Araujo et al. 1995; Kiechl et al. 1994).

Another possibility is that variations in pH in an atherosclerotic lesion may increase iron release. The transferrin-iron association is strong at physiological pH of 7.4. Due to the lower pH at an atherosclerotic lesion, it is possible that iron may be released from transferrin. In vitro work has demonstrated that activity of lipoprotein lipase in lipolysis reduces physiological pH from 7.4 to 7.0, causing iron release from transferrin, resulting in LDL oxidation (Balagopalakrishna et al. 1999). Further work is required to determine if an alteration in pH does indeed occur in vivo.

Given the possibility that excess iron may increase oxidative stress, it is important to determine whether iron supplementation is harmful. Thus far, in a sample with low to normal ferritin stores, iron supplementation did not affect LDL oxidation (Binkoski et al. 2004). Nonetheless, it may be prudent to advise older men and postmenopausal women consuming sufficient dietary iron to avoid taking iron supplements, unless there is indication of iron deficiency, to prevent the contribution of iron excess to CVD risk.

Why is Menopause Associated with Increased Risk of Cardiovascular Disease?

The incidence of CVD is much lower in premenopausal women compared to men, thus premenopausal women are considered to be protected from CVD. However, during the menopausal transition, women's risk of CVD rises by 3.4 times (Witteman et al. 1996), rising to that of men. Menopause is a transitional phase in women's lives, where loss of ovarian function is marked by cessation of menstruation and reproductive capability (World Health Organization 1981). This period of life results in changes in behavior, hormone concentrations, and disease risk.

During the menopausal transition, women may make behavioral changes, such as dietary habits. Compared to their premenopausal counterparts, postmenopausal women consume less total and saturated fat and more fiber, potassium, and antioxidants, but there is no difference in total energy intake (Massé et al. 2004). This change in dietary habits may reflect increased awareness of CVD risk. However, these overall improvements in dietary habits of menopausal women are not necessarily effective in reducing body weight, as shown in a study finding no difference in body weight or BMI of pre- and postmenopausal women (Massé et al. 2004).

Menopause is associated with changes in a variety of CVD risk factors. This may be related to the alterations in circulating hormones, primarily the loss of 17 β-estradiol, the most potent form of estrogen, as well as an increase in estrone. Menopause is also associated with an increase in androgens, such as testosterone, which has also been related to the increased risk of CVD (Guthrie et al. 2004). However, most research has focused on the effects of estrogens, which may modulate cholesterol, protect from oxidative damage, modulate tHcy concentrations, and maintain endothelial function. Menopause has a negative effect on many markers of CVD risk, such as development of dyslipidemia. After menopause, women often experience an increase in TC, LDL-C, and TG concentrations. Estrogen therapy has been effective in lowering LDL-C, although it may increase TG. In the estrogen-deficient state, it appears that androgen concentrations control 87% of the variability in VLDL-TG (van Beek et al. 2004), illustrating the importance of increased androgens in relation to TG.

Estrogen also increases the protective antioxidant mechanisms to quench free radicals and prevent oxidative damage (Meng et al. 1999). Oxidation of LDL-C results in a

lipoprotein that is more readily taken up by endothelial cells, contributing to the progression of atherosclerosis. Oxidation of HDL reduces the efficiency of this lipoprotein to remove cholesterol from circulation. Estrogen may reduce the oxidation of lipids, thereby reducing risk of CVD. Treatment of LDL exposed to an oxidizing agent with estrogen reduces oxidation of LDL in vitro (Meng et al. 1999). Although there is continuing debate on the mechanism of the protective effect of estrogen on LDL and HDL oxidation, it is possible that estrogens actually may be incorporated into the surface of the lipoprotein. Although only trace amounts of estrogens are detected in either of these lipoproteins in plasma, incubation with high concentrations of free or esterified estrogens results in incorporation into either LDL or HDL (Meng et al. 1999).

Postmenopausal tHcy concentrations are 7% higher than in premenopausal women (Hak et al. 2000). Estrogen stimulates the cystathionine β-synthase system to convert Hcy to glutathione, potentially reducing tHcy concentrations and increasing antioxidant defenses to protect the endothelium (Dimitrova et al. 2002). Endothelial function is decreased in women who undergo surgical menopause via the removal of the ovaries, as indicated by decreases flow-mediated dilation (Ohmichi et al. 2003). Contractile responses are diminished in arteries of ovariectomized rats, related to decreases in NO production (Chataigneau et al. 2004). Menopause affects many factors of CVD risk, making it all the more important to understand how to treat menopause-related risk factors.

Many women use a combination of estrogen and progesterone called hormone therapy to treat menopausal symptoms. Women who have had their uterus removed may choose to use estrogen therapy without progesterone. Originally researchers believed that hormone therapy also protects against osteoporosis and CVD. Recent evidence indicates that

while hormone therapy may help reduce bone loss and fractures (Writing Group for WHI 2002), it may actually increase the risk of CAD, stroke, venous or pulmonary embolism (Pradhan et al. 2002), and invasive breast cancer (Writing Group for WHI 2002). Women receiving oral hormone therapy typically experience an increase in TG and CRP (Pradhan et al. 2002; Sanada et al. 2004; Yilmazer et al. 2003). Changing oral estrogen therapy users to a transdermal form of estradiol decreases TG concentrations (Sanada et al. 2004). Transdermal estrogen therapy users do not typically experience the rise in CRP concentrations that women do who receive oral administration (Yilmazer et al. 2003). Hence, it is probable that the form of administration may alter the impact of estrogen therapy on CVD risk.

Estrogen administration may reduce TC and LDL-C and increase HDL-C (Samsioe et al. 2002) in some, but not all studies. Oral doses also effectively reduce Lp(a), a risk factor for CVD (Hemelaar et al. 2003). There is a similar reported effect of combined hormone therapy on lipids (Pornel et al. 2002, Writing Group for WHI 2002) in that both do not only reduce circulating cholesterol, but have been shown to reduce hepatic free and esterified cholesterol in cynomologus monkeys (Register et al. 2002), suggesting that estrogens actually decrease hepatic synthesis of cholesterol. In contrast, other studies have reported no effect of oral or transdermal estrogen therapy on lipids (Hashimoto et al. 2002) or on LDL-C oxidation (Hermenegildo et al. 2002).

While sterod hormones modulate TC and LDL-C and thus alter CVD risk, estrogen may also be effective in modulating other factors of cardiovascular health. Although estrogen therapy is effective in reducing heart rate and systolic and diastolic blood pressure (Christ et al. 2002), combined hormone therapy has been shown to increase systolic blood pressure by 1.0 mmHg (Writing Group for WHI 2002). Either transdermal or oral

administration of hormone therapy reduces tHcy concentrations by 9-14% (Chiantera et al. 2003). Long term use of hormone therapy is associated with an improvement in flow-mediated dilation, a measure of endothelial function (Hashimoto et al. 2002). Progesterone treatment without estrogen appears to be beneficial in restoring NO-mediated vascular tone (Chataigneau et al. 2004), indicating that combined hormone therapy may be preferable to estrogen alone in maximizing benefits to the vasculature. While menopausal women experience an increase in intimal-medial thickness, a contributor to vascular disease, women receiving hormone therapy do not experience this same thickening (Hashimoto et al. 2002). Despite this evidence that hormone therapy may protect the vasculature, clinical trials have shown no effect on prevention of peripheral vascular disease (Hsia et al. 2004).

The decision of the Women's Health Initiative Data and Safety Monitoring Board to discontinue the estrogen and progestin group in 2002 based on increased risk of CVD and breast cancer (Writing Group for WHI 2002), and the more recent decision to terminate the estrogen only arm in 2004, has brought to light the drawbacks of steroid hormone use. Results of this trial revealed that combined hormone therapy is associated with a 22% increase in incidence of a CVD event, and more specifically increased incidence of stroke (41%) and venous thromboembolism (20%; Writing Group for WHI 2002). These women also had a 22% increase in incidence of invasivebreast cancer (Writing Group for WHI 2002). Although steroid hormone use is associated with substantial risk, it may also carry some benefits, including a possible hypocholesterolemic effect. It is possible that improved pharmacology will introduce improved hormone therapy formulae in the future that may minimize the side effects to offer a safe option for menopausal women.

Nutritional Approaches to Reducing Cardiovascular Disease Risk

There are many successful approaches to reducing risk for CVD, many of which are nutritional interventions. Just a few of these include diets low in fat, but high in fiber, omega-3 fatty acids, and plant sterols. In addition to dietary changes, physical activity may be beneficial in reducing risk of CVD. Incorporation of high fiber foods, particularly soluble fiber, into a healthy diet significantly reduces LDL-C and fasting blood glucose concentrations in healthy individuals (Aller et al. 2004). In hyperinsulinemic men, high fiber cereals have also been effective in improving insulin responses (Wolever et al. 2004). Intake of high fiber foods is also associated with reduced CRP (King et al. 2003) and tHcy concentrations (Gao et al. 2004), demonstrating that fiber may have a beneficial effect on many aspects of CVD risk. Omega-3 fatty acids (n-3 polyunsaturated fatty acids) may be effective in reducing hypertension, insulin response, and lipids (Aguilera et al. 2004; Thomas et al. 2004). In metabolic syndrome-induced rats, administration of omega-3 fatty acids effectively reduced blood pressure, insulin, TG, and TC (Aguilera et al. 2004). In humans, supplementation with omega-3 fatty acids also increases HDL-C (Thomas et al. 2004). Omega-3 fatty acids are obtained from the diet by inclusion of fish and flax seed, or can also be taken in supplemental form to improve lipids. Plant sterols have also proven to be effective in reducing lipids. Inclusion of 1.6 g/d of plant sterols resulted in 10% reductions in LDL-C in hyperlipidemic subjects consuming a normal diet (Thomsen et al. 2004). Higher doses of plant sterols (2.7 g/d) in hypercholesterolemic males elicited marked improvements in lipids, reducing TC by 9%, LDL-C by 15%, and TC:HDL-C by 9% (Matvienko et al. 2002). Plant sterols have recently been incorporated into commercial food products, such as Minute Maid's "Heart Wise" orange juice to reduce cholesterol absorption when taken with a

high fat, high cholesterol meal, such as breakfast sausages or bacon. Physical activity has also been shown to be highly effective in reducing risk of CVD. A meta-analysis of thirty physical activity studies revealed that a minimum of 1 hr/wk of walking in women dose-dependently decreased CVD, CHD, and stroke risk (Oguma et al. 2004). The benefits of physical activity are most evident in very active individuals such as athletes, who have low circulating cholesterol, possibly due to increased concentrations of HDL-C, contributing to their reduced risk of CVD (Olchawa et al. 2004). A combination of all of the discussed nutritional and behavioral approaches as part of a healthy lifestyle may prove to be most effective in reducing CVD risk.

Soy Protein

Recently soy protein has gained attention due to its ability to affect many of the CVD risk factors. The FDA has approved a food label claiming that 25 g of soy protein each day may help to prevent CVD. While the typical American does not consume 25 g of soy protein per day, there is growing interest in soy components, especially soy isoflavones for the estrogen-like benefits in postmenopausal women. Many recent studies have attempted to determine the validity of this claim. However, detecting the physiological effects of adding soy to the diet is a complicated matter, because soy contains many components, such as isoflavones, saponins, β-conglycinin, or phytate, which could be responsible for the beneficial effect. To complicate matters further, the processing techniques of soy foods can greatly alter their composition, and may impact various bioactive effects. Many clinical trials have chosen soy protein isolate (SPI) or textured vegetable protein for dietary interventions because they are both easily incorporated into foods. Others have used soymilk, protein bars, or tofu products. It is hard to compare the effects of different soy

products because processing methods used to prepare the foods may alter their bioactive compounds.

Inclusion of SPI into the diet may have beneficial cardioprotective effects, particularly in those who have already begun to develop CVD, such as with hypercholesterolemia. Early work in rats fed a hypercholesterolemic diet revealed that animals receiving soy protein did not experience the increase in TC, LDL-C, or VLDL-C that was observed in those receiving casein (Sirtori et al. 1984). A more recent human trial comparing the effects of consuming 30-50 g/d of SPI compared to equal doses of casein for 12 weeks found that the SPI group experienced a greater reduction in TC, LDL-C, and tHcy compared to the casein group (Tonstad et al. 2002). This reduction of TC, LDL-C, apolipoprotein B100, and tHcy was also demonstrated in type II diabetic patients who received 50 g/d of SPI for six weeks compared to a casein-based placebo group (Hermansen et al. 2001). These and other protein interventions show that soy is as effective as hormone therapy in reducing cholesterol (Chiechi et al. 2002) and atherosclerosis (Clarkson et al. 2001), suggesting that it is a safer option for hyperlipidemic postmenopausal women. The results of 10 human soy protein trials were recently summarized in a meta-analysis which revealed that while soy protein consumption reduces LDL-C and increases HDL-C, there is not a dose-response relationship (Weggemans and Trautwein 2003). Other studies have shown no beneficial effects of soy. One study revealed no effect of SPI on CRP in a 4-week intervention (Jenkins et al. 2002), and another study showed no effect on lipids or lipoproteins in a 6-month human trial (Dent et al. 2001). This discrepancy in results may be partially related to the control protein used in these studies. In work published by Dent et al. (2001), no effect of 40 g/d of SPI was found on lipids when compared to a whey protein

control. However, in studies that used casein protein as the control, SPI had a hypocholesterolemic effect (Hermansen et al. 2001; Jenkins et al. 2002; Sirtori et al. 1984; Tonstad et al. 2002). The casein protein control in these studies actually had a mild *hyper*cholesterolemic effect, suggesting that casein is an inappropriate control. Hence, when comparing the cholesterol-lowering effects of soy to the cholesterol-raising casein control, the difference is more dramatic, and the effects of soy become statistically significant. Similarly, a casein control is also inappropriate to determine changes in tHcy with SPI because of differences in the amino acid profiles. Casein has a higher methionine:cysteine ratio compared to soy protein, which is associated with increases in tHcy concentrations in pigs (Shoveller et al. 2004). Hence, human studies comparing the effects of SPI to casein on tHcy may overestimate reductions in tHcy concentrations. Although improvements in study design of clinical trials may change the extent of the benefit of soy on lipids and tHcy, most studies have agreed that there is at least a modest trend in reducing cholesterol and tHcy. In order to better determine whether soy can truly reduce cholesterol and tHcy concentrations, future studies should use a non-casein control.

The reduction in cholesterol appears to be due to an up regulation of LDL receptors that remove cholesterol from circulation. This was clearly demonstrated in a crossover human study comparing the effects of a soy-based to an animal protein-based diet in hypercholesterolemic patients (Lovati et al. 1987). The soy-based diet resulted in 13-18% reductions in TC and LDL-C, and a significant increase in LDL receptor activity in mononuclear cells. This was confirmed in a more recent 6-month study where hyperlipidemic, postmenopausal women on the National Cholesterol Education Program Step 1 diet, which is low in fat and cholesterol, received 40 g/d of protein either as SPI or casein.

The SPI group, regardless of isoflavone content, experienced an increase in circulating HDL-C concentration and LDL receptor mRNA concentration in mononuclear cells, resulting in a reduction in non-HDL-C concentrations (Baum et al. 1998), compared to the casein group. Despite no change in TC, these results indicate that consumption of soy protein may act to help correct dyslipidemia at the transcriptional level, although researchers are still attempting to determine which components are responsible for this effect. If soy protein improves lipid profiles, soy foods may be useful as part of intervention diets for those at risk of CVD.

While it is accepted that soy protein may be beneficial in reducing risk of CVD, it is not clear the how much protein is required to exhibit the protective effect. A 12 wk feeding trial comparing the effects of 30 g vs. 50 g of SPI each day found a protective effect of soy on lipids and tHcy, but there was no difference between the higher and lower dose of protein (Tonstad et al. 2002). Sometimes incorporating even one serving of a soy food into a usual diet is difficult. For example, in a clinical trial, compliance was low when subjects were asked to include just one soy food in their diet everyday, even when receiving dietary counseling (Chiechi et al. 2002). Considering that it would take one cup of soy milk, a half of cup of tofu, and 8 ounces of soy yogurt every day (United Soybean Board 2004), or a similar combination of soy foods, to satisfy the FDA's suggestion of 25 g of soy protein per day for cardiovascular health, it is unlikely that a large portion of the general population will make such drastic dietary changes. As an alternative, many people have turned to supplemental forms of soy components instead of modifying their diets.

Isoflavones

Many researchers believe that the isoflavone content of soy may be the most beneficial component of soy. The major soy isoflavones are genistein, daidzein, and

glycitein. Midlife women from the Framingham Heart Study who consumed high quantities of isoflavones in their diets had lower TG concentrations and a reduced risk of metabolic disease, based on measurement of lipids, blood pressure, and waist-to-hip ratio (de Kleijn et al. 2002).

Oxidative damage to lipids, proteins, and DNA leads to increased uptake of oxidized LDL by macrophages, inflammation, and possibly mutations in DNA leading to CVD, cancer, and neurodegenerative diseases (Reddy and Clark 2004). Soy isoflavones may help reduce oxidative stress to prevent CVD (Djuric et al. 2001; Meng et al. 1999; Wiseman et al. 2001). In a unique project comparing the effects of isoflavones in men and women, supplementation with soy isoflavones (100 mg/d for men and 50 mg/d for women) reduced oxidative damage to DNA within three weeks (Djuric et al. 2001). Interestingly, females (22-56 y) responded more quickly to treatment, showing the highest plasma concentrations of genistein and daidzein during the second week of treatment, compared to the males' response by the third week. Similarly, there was an appreciable reduction in DNA damage within the first week in women, whereas this effect was not noted in men until the third week. However, this study used a very small sample size (men n=6, women n=6), did not compare men and women at the same intake of isoflavones, and did not use a control group. Another clinical trial observed that soy protein with high isoflavone content reduced oxidative damage to lipids and LDL, while soy protein with low isoflavone content had no effect (Wiseman et al. 2000). Further investigation in rabbits revealed that providing a soy isoflavone extract also reduced the concentration of oxidized LDL in foam cells within atherosclerotic lesions, which would slow or reverse the process of atherosclerosis (Yamakoshi et al. 2000). The mechanism of this relationship may involve incorporation of

the isoflavones into LDL (Meng et al. 1999) or scavenging of lipid peroxyl radicals (Patel et al. 2001). Isoflavones may be esterified with fatty acids and incorporated into the membrane of LDL, making LDL more resistant to oxidative damage (Meng et al. 1999). There is evidence that genistein may be the most potent antioxidant of the major soy isoflavones (Patel et al. 2001; Wei et al. 1995) and the protection to LDL offered by genistein and daidzein may be greater when they are esterified with oleic acid (Meng et al. 1999). Isoflavones may scavenge peroxyl radicals, generating a phenoxyl radical which is reduced by other antioxidants, such as ascorbate under in vitro conditions (Patel et al. 2001). It is difficult to extrapolate these results to physiological conditions because in this study, pure, undigested, and unaltered isoflavones were applied to the cells in very high concentrations, but it may provide an possible explanation for the benefits noted in clinical trials. However, not all studies have found a beneficial effect of soy isoflavones on reducing oxidative stress. Regardless of isoflavone content, total antioxidant status improved in perimenopausal women consuming 40 g/d of soy protein (Swain et al. 2002), suggesting that the effect of soy is due to a component other than isoflavones. Further work is required to understand the antioxidant properties of isoflavones alone.

Soy isoflavones have also received great attention for their hypocholesterolemic properties. Ovariectomized Golden Syrian hamsters experience a rise in TC and atherosclerotic lesions; yet, administration with soy isoflavones dose-dependently reduced TC and fatty streaks (Lucas et al. 2003). Similar work in rabbits found a reduction in atherosclerotic lesions with isoflavone administration (Yamakoshi et al. 2000). However, human trials have shown that isoflavones may not be completely responsible for the hypocholesterolemic effects of soy (Baum et al. 1998; Jenkins et al. 2002). An interesting

trial reported that consumption of soy foods (50 g/d) with high and low isoflavone content for 4 weeks in hyperlipidemic men and postmenopausal women (Jenkins et al. 2002) lowered TC, LDL-C, HDL-C, apo B, , and apo A-I, as well as CAD risk compared to the dairy-based group. In both groups a decrease in tHcy was observed, but only the low isoflavone group was significant. These results support an earlier trial where hyperlipidemic postmenopausal women receiving a low-fat diet, with either moderate or high isoflavone content SPI, had similar improvements in lipids compared to those on a casein-based diet (Baum et al. 1998). Therefore, it is apparent that isoflavones are not solely responsible for the beneficial effect of soy protein.

Saponins

Saponins are steroid compounds that are also associated with soy and other plant proteins, and are reported to exert a hypocholesterolemic effect (Matsuura 2001). Saponins may decrease the activity of pancreatic lipase (Han et al. 2000) and may bind bile acids (Sidhu et al. 1986) to decrease lipid and cholesterol absorption in the gut, resulting in a reduction of circulating TG and LDL-C. However, like isoflavones, saponins are soluble in alcohol and are co-extracted from soy protein with isoflavones. Hence, trials designed to test the benefit of soy isoflavones per se using an isoflavone extract should be viewed with caution because the extracts also contain saponins.

β-conglycinin

Further investigation of the hypocholesterolemic effect of soy suggests that the β-conglycinin (7S globulin) subunit of soy protein may be partially responsible. Inclusion of the 7S globulin alone in the diet reduces TC and TG to the same extent as the complete SPI (Aoyama et al. 2001). It appears that the 7S globulin upregulates LDL-receptors on

hepatocytes, causing more LDL-C removal from circulation with sequestering in the liver. In a mouse model, feeding 7S globulin reduced the development of atherosclerosis compared to animals fed a diet with isoflavones or casein (Adams et al. 2004). More specifically, the α′ subunit may be the bioactive portion of the 7S globulin. Hepatocyte cell cultures incubated with a textured vegetable protein product naturally rich in 7S globulin increased LDL uptake and degradation by 73%, while a strain of soy with 7S globulin missing the α′ subunit had no effect (Manzoni et al. 1998). Synthesized peptides sharing the same sequence as the α′ subunit also elicit a response in hepatocytes to increase LDL receptor expression (Lovati et al. 2000). Oral administration of the α′ subunit of 7S globulin in rats reduced plasma TC and TG, and upregulated β-VLDL receptors (Duranti et al. 2004). Further work has demonstrated that the α′ subunit acts within the cytosol of hepatocytes to upregulate LDL receptors, indicating that the mechanism is not via direct influence on DNA (Manzoni et al. 2003). Thus, in order to maximize the protective benefits of soy, it is important to preserve the 7S globulin with intact α′ subunit during processing. While processing does partially denature soy protein, the 7S globulin degradation products in textured vegetable protein upregulate LDL receptors in hepatocytes to the same extent as isolated, intact 7S globulin (Manzoni et al. 1998). These results suggest that processing may not diminish the effects of the 7S protein.

Phytate

Phytate, or inositol hexaphosphate (IP_6), is another component of soy that has received remarkably less attention. Although phytate is present in soy protein, it is also found in other plant products, such as cereals, legumes, seeds, and nuts. While the phytate area has received less research than isoflavones, we do know that IP_6 is found in human

plasma and must therefore either be produced in vivo or absorbed from the diet. Phytate may protect against CVD by reducing platelet aggregation (Vucenik et al. 1999), oxidative stress (Porres et al. 1999), and circulating cholesterol (Jariwalla et al. 1990; Koba et al. 2003). Phytate in the gut chelates divalent cations, including calcium, iron, and zinc, thereby reducing mineral absorption (Jariwalla et al. 1990). In humans, reduction of phytate in SPI increased iron absorption by up to 5-fold compared to a native SPI (Hurrell et al. 1992), with similar results reported when phytate is reduced in cereals (Hurrell et al. 2003). Reducing mineral absorption may be beneficial in reducing oxidative stress, since iron, copper, and zinc participate in free radical generation. Phytate administration dose-dependently reduced hydroxyl radical production in a rat ischemic heart reperfusion model, a situation of high oxidative stress (Rao et al. 1991), and decreased lipid peroxidation in the colon of pigs (Porres et al. 1999). However, neither of these studies indicate that dietary phytate can be absorbed into the bloodstream to exert a systemic effect in reducing oxidative stress.

In addition to acting as antioxidant, phytate may also affect blood lipids. Phytate treatment reduced TC by 32% and TG by 64% in rats (Jariwalla et al. 1990). Phytate depletion compromises the hypocholesterolemic effect of SPI, which is restored with the addition of phytate, due in part to reduction of hepatic cholesterol concentrations (Koba et al. 2003) and presumably production and export. The reduction in hepatic lipids with phytate parallels decreased activity of lipogenic enzymes that produce NADPH (glucose-6-phosphate dehydrogenase and malic enzymes) in sucrose-fed rats treated with 0.5% phytate, a quantity that may be obtained from the diet (Katayama 1995). In contrast, work by Koba et al. (2003) showed no relationship between phytate and fatty acid synthesis or phospholipid content of hepatocytes. A high zinc/copper ratio is associated with hypercholesterolemia (Potter 1995),

thus zinc chelation by phytate may reduce the ratio, thereby lowering cholesterol (Jariwalla et al. 1990).

The mineral chelating properties of phytate may be a concern with regard to increasing the incidence of mineral deficiency, particularly in nutritionally compromised individuals. However, a soy-based diet including phytate did not diminish mineral status in rats provided with a wide variety of dietary mineral sources (Koba et al. 2003). In individuals with iron excess, such as postmenopausal women, phytate consumption could potentially reduce dietary iron absorption to control iron stores and prevent oxidative stress thereby reducing risk of CVD.

Although phytate is absorbed in rodents, its absorption in humans is not clear at this time. Grases et al. (2000) have demonstrated that urinary phytate excretion reflects large changes in dietary intake of phytate. Although only a small amount of phytate is absorbed (Grases et al. 2000), it has a potential health benefit beyond its action in the gut. The ability of phytate to chelate divalent metal cations may also be beneficial to kidney stone-formers. Phytate binds calcium in the renal tubules, making it unavailable for the formation of calcium-oxalate crystals, suggesting that phytic acid absorbed from the diet may also have an effect in reducing the formation of calcium stones in susceptible individuals (Vucenik and Shamsuddin 2003). Although there is no direct evidence, phytate could potentially have the same metal-chelating effects in circulation to bind minerals and reduce oxidative stress in an atherosclerotic region. While the metal chelating properties of phytate may be beneficial for individuals at risk for excesses of particular minerals, a high-phytate diet may have an unfavorable effect on reducing mineral absorption in individuals at risk for mineral

deficiencies. As a precaution in some individuals susceptible to mineral deficiencies, it may be prudent to advise high-phytate consumers to consider a multimineral supplement.

Conclusion

Postmenopausal women are at increased risk of CVD, partially related to increases in circulating lipids and lipoproteins, oxidative stress, tHcy, CRP, and iron excess. Regular consumption of soy foods may have a cardioprotective effect on these CVD risk factors. Although soy has many components that may be responsible for this protection, it is difficult to separate the specific effects of each compound. Therefore, to derive the maximum benefit from soy, nutritionists recommend consuming the whole soy protein with all of its native components.

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SOY ISOFLAVONES AND PHYTATE: EFFECTS ON HOMOCYSTEINE, C-REACTIVE PROTEIN, AND IRON STATUS IN POSTMENOPAUSAL WOMEN

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Abstract

Background

Menopause increases the risk of cardiovascular disease (CVD), partially due to increases in total homocysteine (tHcy), C-reactive protein (CRP), and excess body iron.

Objectives

To identify contributing factors to tHcy and CRP and to determine the independent effect of the soy protein components, isoflavones and phytate, on CVD risk in postmenopausal women.

Design

In a double-blind 6 wk study (n=55), free-living postmenopausal women (47-72 y) were randomly assigned to one of four soy protein isolate (40 g/day) treatment groups: 1) native phytate/native isoflavone (NP/NI, n=14); 2) native phytate/low isoflavone (NP/LI, n=13); 3) low phytate/native isoflavone (LP/NI, n=14); 4) low phytate/low isoflavone (LP/LI, n=14). Assessment of CVD risk factors included: tHcy, CRP, iron indices, and body mass index (BMI).

Results

At baseline, BMI was correlated positively with tHcy (P=0.003) and CRP (P<0.0001). Analysis of variance showed that treatment reduced plasma tHcy (P<0.04) and serum ferritin (P=0.06), but had no effect on CRP. Contrast coding to test the independent effect of phytate and isoflavones (NP vs. LP and NI vs. LI) further indicated that only the phytate-containing treatments significantly decreased tHcy (P=0.02) and serum ferritin (P=0.03), whereas isoflavone treatments had no effect.

Conclusions

Our results suggest that phytate-rich foods may protect postmenopausal women from CVD by reducing tHcy and excess iron. Given the contribution of BMI to tHcy and CRP, maintaining healthy body weight may also be important in reducing these specific CVD risk factors.

Key words

Postmenopausal women, homocysteine, C-reactive protein, iron, cardiovascular disease, soy protein, isoflavones, phytate

Introduction

Cardiovascular disease (CVD) is the leading cause of death in the U.S. (Centers for Disease Control and Prevention 2003). The incidence of CVD is lower in premenopausal women compared to men; however, the risk of CVD rises 3.4 times after menopause (Witteman et al. 1996). This increase in risk may be partially related to increases in total homocysteine (tHcy; Ridker et al. 1999), C-reactive protein (CRP; Ridker et al. 1998), and excess body iron (Sullivan 1981).

Elevated tHcy concentrations are an independent, modifiable risk factor for CVD (Nygård et al. 1997; Ridker et al. 1999). Patients with CVD from the Women's Health Study with concentrations of tHcy in the highest quartile (>13.26 μmol/L) were twice as likely to experience a future cardiovascular event as those in the lowest quartile (<9.54 μmol/L; Ridker et al. 1999). Premenopausal women typically have lower tHcy concentrations than men, but after menopause tHcy increases 7% (Hak et al. 2000), more comparable to similarly-aged men. Although there are a number of potential mechanisms explaining the impact of tHcy on CVD risk, there is strong evidence to indicate that hyperhomocysteinemia suppresses production of nitric oxide (NO; Fu et al. 2002), which is an important vasodilator and antioxidant, indirectly causing damage to the vasculature (Heydrick et al. 2004; Nestel et al. 2003; Zeng et al. 2003). More in depth studies are required to understand how tHcy concentrations may be involved in the initiation and progression of CVD.

Elevated CRP, a marker of acute inflammation, has recently emerged as a reliable predictor of CVD (Ridker et al. 1998) and is associated with a 1.5-fold increased risk of myocardial infarction (Ridker et al. 1998). Many physicians have prescribed hormone therapy to postmenopausal women under the assumption that correcting hormonal deficiency would reduce risk of CVD. To the contrary, studies have shown that hormone therapy in postmenopausal women results in a short-term rise in CRP (Sanada et al. 2004; Yilmazer et al. 2003). CRP may stimulate the incorporation of monocytes into atherosclerotic lesions (Khreiss et al. 2004; Devaraj et al. 2004) and contribute to endothelial dysfunction (Venugopal et al. 2003; Verma et al. 2004).

Iron stores increase with age in both men and women, paralleling the rise in CVD risk (Sullivan 1981). Results of a Finnish study associated excess iron stores (ferritin > 200

μg/L) in men with CVD (Salonen et al. 1992). In women, serum ferritin concentrations are clearly elevated with each decade of life beyond 40 years, with each 100 μg/L increase resulting in an odds ratio of 1.54 for developing coronary artery disease (CAD; Kiechl et al. 1994). Excess iron acts as a prooxidant (Day et al. 2003; Halliwell 1986), thereby causing oxidative stress. In addition, excess iron may adversely alter lipid metabolism (Berge et al. 1994).

Recently the FDA has approved a food label claim that 25 g/d of soy protein may help prevent CVD (Food and Drug Administration 2002). Dietary soy protein is associated with reduced risk of CAD, possibly due to reductions in lipids and tHcy (Hermansen et al. 2001; Jenkins et al. 2002b; Tonstad et al. 2002). Since soy has many components, including isoflavones, saponins, β-conglycinin, and phytate, that may be responsible for its cardioprotective effect, it is difficult to distinguish the specific effects. Soy isoflavones may reduce TC (Lucas et al. 2003), but effects on tHcy (Jenkins et al. 2002b) and CRP (Jenkins et al. 2002a) are not clear. Phytate in soy also decreases iron absorption (Jariwalla et al. 1990; Reddy et al. 1996), which may potentially reduce iron stores. Due to the oxidative damage associated with iron (Porres et al. 1999; Rao et al. 1991), phytate may be beneficial in reducing risk of CVD in individuals prone to excess iron.

This study was designed to determine the independent effects of soy protein components, isoflavones and phytate, on tHcy, CRP, and iron status in postmenopausal women. We hypothesized that the isoflavone-containing soy protein would reduce tHcy and CRP, whereas the phytate-containing soy protein would reduce iron status in this group at-risk for excess iron.

Subjects and Methods

Study design

Fifty-five healthy postmenopausal women (47-72 years of age) participated in a randomized, double-blind, 6 wk soy protein study. Subjects were randomly assigned to receive one of four soy protein isolate (40 g/d) treatments: low phytate/low isoflavone (LP/LI, $n = 14$); native phytate/low isoflavone (NP/LI, $n = 13$); low phytate/native isoflavone (LP/NI, $n = 14$); or native phytate/native isoflavone (NP/NI, $n = 14$). The soy protein isolate treatments were prepared and donated by the Solae Company (St. Louis, MO). Isoflavones were removed by alcohol extraction and phytate was removed by enzyme hydrolysis (**Table 1**). According to the Solae Company, the isoflavone content of the native isoflavone treatments was higher than the normal SPI isoflavone content, hence the term "native" is used instead of "normal".

Subject selection

A telephone screening questionnaire was used to recruit nonsmoking, postmenopausal women with no history of chronic disease, cholesterol-lowering drug use, hysterectomy, use of oral hormonal therapy within the last year or topical hormones within the last six months, and who were willing to avoid using nutritional supplements or consuming isoflavone- or phytate-rich foods during the treatment. A list of these foods with specific instructions was provided to women at baseline to ensure compliance. Fifty-five women completed the study, in addition to two subjects who began the study, but discontinued due to gastrointestinal discomfort with the protein treatment. Subjects were provided with recipes to incorporate the SPI treatment into meals. The study protocol, consent form, and subject-related materials

were approved by Iowa State University Human Subjects Review Committee (Institutional Review Board; IRB ID# 02-351).

Data collection

At baseline subjects were assessed for typical dietary intake using a food frequency questionnaire from Block Dietary Data Systems (Berkeley, CA). In addition, interviewers administered health and medical history (Alekel et al. 2000), nutrition history (Alekel et al. 2000), and soy food intake (Kirk et al. 1999) questionnaires. Fasted blood and 24-hour urine samples were collected between 7:00 and 8:00 am at baseline and at 6 wk for analysis of CVD risk factors and markers of iron status. Blood and urine samples were stored at -80°C and at -20°C, respectively, until analyses.

Sample analysis

Concentrations of tHcy were determined using a method adapted from Araki et al. (1987) and Ubbink et al. (1991). Plasma samples were derivatized with ammonium 7-flourobenzo-2-oxa-1,3-diazole-4-sulphonate obtained from Sigma (St. Louis, MO), and tHcy samples were measured in duplicate using high pressure liquid chromatography and a fluorescence detector. Serum concentrations of CRP were determined using a high sensitivity ELISA kit from Alpco Diagnostics (Windham, NH). Serum ferritin was measured using an ELISA kit from Ramco Laboratories (Houston, TX). Blood samples were analyzed by a certified clinical laboratory (Quest Diagnostics, St. Louis, MO) to determine iron status: hemoglobin, hematocrit, serum iron, total iron-binding capacity (TIBC), and transferrin saturation.

Statistical analysis

Statistical analyses were performed using SAS (version 8.0; Cary, NC) with results considered statistically significant at $P \leq 0.05$. The descriptive statistics included means (min-max) for age, BMI, height, weight, and dietary factors. Pearson correlation analysis was performed to determine the relationship between tHcy and CRP and other CVD risk factors (lipids/lipoproteins, oxidative stress indices, serum ferritin, plasma folate, plasma B_{12}, and BMI) at baseline. Multiple regression with stepwise selection was used to determine the effect of these contributors to baseline tHcy and CRP concentrations. To determine the effects of treatment on CVD risk factors, the changes (6 wk – baseline) in tHcy, CRP, and iron indices were compared among the treatments using analysis of variance (ANOVA). Contrast coding (LP vs NP and LI vs NI) was used to further determine the individual effects of phytate and isoflavones on tHcy, CRP, and iron indices.

Results

Compliance

We provided each subject with a fixed number of treatment protein packets, with instruction to return the unconsumed packets at the end of the study for compliance determination. Only four subjects returned unused daily supplies (1, 1.5, 2, or 4 days), while two subjects requested additional daily supplies (1.5 or 2 days). Fortunately, these subjects were dispersed across treatment groups; thus, this should not have significantly impacted overall results. Additionally, random sampling of urinary isoflavone excretion in four subjects from each treatment (16 altogether) at baseline and 6 wk, respectively, confirmed compliance (LP/NI and NP/NI: 23 and 29 μmol/L; LP/LI and NP/LI: 1.6 and 2.8 μmol/L).

Subject Characteristics

Women in all treatment groups were similar in age, height, weight, and BMI (**Table 2**). There were no significant differences in dietary intake among the treatment groups at baseline. Although the mean dietary intakes of folate and vitamin B_6 met the 1998 Dietary Reference Intakes (DRIs; Standing Committee on the Scientific Evaluation of Dietary Reference Intakes) in each treatment group, there were a number of individuals in each treatment who did not meet the DRIs for these nutrients, as follows: in the LP/LI group, 10 and 5 women, respectively, for folate and vitamin B_6; in the NP/LI group, 6 and 1 women, respectively, for folate and vitamin B_6; in the LP/NI group, 9 and 5 women, respectively, for folate and vitamin B_6; in the NP/NI group, 10 and 7 women, respectively, for folate and vitamin B_6. The food frequency questionnaire did not provide vitamin B_{12} results; however, values of plasma folate and B_{12} for all women were within normal ranges. We did not assess circulating vitamin B_6 concentrations. The subjects had been postmenopausal for a mean of 6.4 years. Thirteen subjects reported having been diagnosed with iron deficiency in their lifetime, and only two reported current regular use of iron supplements.

Factors contributing to tHcy and CRP

Given that tHcy and CRP are independent risk factors of CVD, we determined the factors contributing to their circulating concentrations. Oxidative stress indicators (Barwick et al. 2004) and blood lipids results (Clark et al. 2004) are published elsewhere. Pearson correlation coefficients between tHcy and CRP with CVD risk factors are shown in **Table 3**. Circulating tHcy and CRP were correlated positively with each other (P=0.01) and other CVD risk factors. BMI was correlated positively with both tHcy (P=0.003) and CRP (P<0.0001), whereas HDL-C was correlated negatively with CRP (P=0.02). A marginal

relationship was found between tHcy and plasma B_{12} (P=0.09), but not with plasma folate. Multiple regression analysis showed that LDL-C and BMI were the only contributors to tHcy concentrations (**Table 4**). BMI and LDL-C, respectively, accounted for 13.9% (P=0.01) and 4.9% (P=0.10) of the variance in tHcy concentrations, whereas, CRP was affected only by BMI.

Effect of treatment

During treatment, subjects experienced a decrease in BMI across all treatment groups, likely due to the fact that they were consuming the soy protein as a meal replacement. However, the reduction was not significant in any treatment group. Concentrations of tHcy, CRP, and iron status indicators at baseline and 6 wk are presented in **Table 5**. At 6 wk, all three treatments containing either phytate and/or isoflavones significantly reduced tHcy compared to baseline (NP/LI 23.2%, NP/NI 8.1%, LP/NI 12.7%) compared to an insignificant change with LP/LI. ANOVA indicated that treatment had a significant effect only on change in tHcy (F=2.87, P<0.04), and a moderate effect on ferritin (F=2.62, P=0.06), TIBC (F=2.6, P=0.07), and transferrin saturation (F=2.63, P=0.06), but no effect on CRP or serum Fe concentrations. Tukey's multiple comparison test revealed that the reductions in tHcy and transferrin saturation in the NP/LI treatment were significantly different from LP/LI. Contrast coding further showed a clear effect of phytate on reducing tHcy (1.17 μmol/L in NP vs. 0.33 μmol/L in LP) and ferritin (15.29 in NP vs. 0.84 μg/L in LP), regardless of isoflavone content. However, neither of the soy components had an effect on CRP.

Discussion

As expected, we observed positive correlations between BMI and the CVD risk factors tHcy and CRP in support of previous findings (Ganji et al. 2003; Lear et al. 2003), emphasizing the impact of obesity on CVD risk. In our subjects, overweight individuals (BMI $\geq$25 kg/m^2; n=31) had 1.0 μmol/L higher tHcy and 1.0 mg/L higher CRP compared to normal weight individuals (BMI <25 kg/m^2; n=24). Our findings indicate that tHcy and CRP are partially dictated by BMI; thus, controlling BMI may have an added benefit in reducing CVD risk.

Based on the association of LDL-C with CVD risk factors, it was not surprising that LDL-C was correlated positively with baseline tHcy values. In hyperlipidemic individuals, the risk of CVD is higher with elevated compared to normal concentrations of CRP and tHcy (Ridker et al. 2001). Similarly, in our study, subjects who had LDL-C >140 mg/dL had 2.1 kg/m^2 higher BMI, 0.7 μmol/L higher tHcy, and 0.6 mg/L higher CRP compared to subjects with LDL-C $\leq$140 mg/dL. Although previous studies have shown that excess iron and elevated TC (Kiechl et al. 1994) and LDL-C (Wolff et al. 2004) have an additive effect on increasing CVD risk, we found no difference in serum ferritin between subjects with LDL-C >140 mg/dL vs. $\leq$140 mg/dL. The above studies included subjects with very high iron stores; however, in our study the group mean serum ferritin values ranged from 59 - 78 μg/L, which are considered normal.

Since we recruited healthy women without chronic diseases, it was not surprising that only 3 subjects had elevated CRP (>3 mg/L) and none had elevated tHcy (>10 μmol/L). Baseline values of plasma tHcy in all participants were low in our study (mean of 6.3 $\pm$ 1.4 μmol/L) compared to other studies (7-15 μmol/L) for this age group (Jenkins et al. 2002;

Ridker et al. 2001; Tonstad et al. 2002). However, this discrepancy may have been due to the method we used to analyze tHcy. Other researchers using similar methods have reported mean plasma tHcy concentrations for healthy females (19-39 years) of 5.7 ± 1.2 μmol/L (Araki et al. 1987). In addition, typical *serum* tHcy concentrations of 7 ± 2 μmol/L have been reported for midlife males and females, which are generally 10-30% higher than *plasma* concentrations (Foody et al. 2000). Although the plasma tHcy values appear low in our study, they agree with the above studies using the same method for plasma samples. Given that the effect of treatment was determined based on change in tHcy rather than absolute values, this variation from most of the literature may not be important.

The strength of this study lies in the fact that we did not use a casein control. Studies which have used a casein control have noted an increase in tHcy in the control group (Jenkins et al. 2002; Tonstad et al. 2002). Casein has a higher methionine:cysteine ratio compared to soy protein, which is associated with increases in tHcy concentrations in pigs (Shoveller et al. 2004). Thus, any effect the soy treatment may have had on lowering tHcy is more dramatic compared to those on casein control. Instead, we used a soy protein with low phytate and low isoflavone content (LP/LI) as a control. Using one type of protein provided a more apt comparison between the independent effect of isoflavones and phytate on tHcy, CRP, and iron status. Although it remains controversial, isoflavones in soy protein have been thought to exert a protective effect on tHcy concentrations (Tonstad et al. 2002). Further work revealed that while soy protein reduces tHcy, it may not be due to the isoflavone content (Jenkins et al. 2002). Our study supports these results, showing the changes in tHcy concentrations were similar between NI and LI treatments (mean reductions were 0.65 vs.

0.84 μmol/L, respectively), indicating that a factor other than the isoflavone content of soy may affect tHcy.

Phytate has a well-documented effect on reducing iron absorption in feeding trials (Hurrell et al. 1992; Reddy et al. 1996). Although reductions in iron stores may not be desirable for those at-risk of iron deficiency, the effect of phytate on reducing iron stores may be beneficial in postmenopausal women who are at-risk for excess iron. Consistent with previous studies, phytate moderately reduced iron stores in our subjects, even after only 6 wk of treatment. Even in the low phytate-containing treatments, there was a reduction in ferritin concentrations. This may be due to the low levels of phytate still found in the treatment. Previous work has shown that phytate must be reduced to less than 0.3mg/g protein to remove the inhibitory effect on iron absorption (Reddy et al. 1992). Even in our LP treatments, there was still 5.5 mg phytate/g protein present, which may have reduced iron absorption, thereby reducing iron status, although not to the same extent as the NP treatments. In addition, as expected, there was no effect of isoflavone treatments on iron indices.

Although tHcy concentrations were reduced with all treatments, the change was significantly different between the NP vs. LP groups, suggesting that consumption of phytate-rich foods may be beneficial in reducing tHcy. Phytate may have a more dramatic effect on further reducing tHcy concentrations in hyperhomocysteinemic individuals. Further studies are needed to determine the effectiveness of phytate in correcting hyperhomocysteinemia. This relationship has not been previously reported in the literature, and there is no clear mechanism for this effect. However, based on the relationship between tHcy and folate, we speculate that the effects on tHcy may be due to increased availability of

intracellular folate. It is evident that folate is important in Hcy metabolism because its deficiency results in hyperhomocysteinemia (McKinley et al. 2001), but this may be reversible with folate administration (Brattström et al. 1985). Iron status may not be directly related to tHcy, but may have an indirect effect through folate catabolism. Increased ferritin parallels the increase in folate catabolism in animal studies (Suh et al. 2000; Zhu et al. 1995), depriving folate needed for the removal of Hcy from circulation. Insertion and overexpression of the rat gene for heavy chain ferritin in Chinese hamster ovarian cells stimulated folate turnover and a 40% decrease in intracellular folate concentration (Suh et al. 2000). Elevations in heavy chain ferritin may reflect alterations in the iron regulatory pool (Picard et al. 1996), influencing activity of regulatory enzymes in the folate pathway, thereby altering folate availability to recycle Hcy to methionine (Girgis et al. 1997; Oppenheim et al. 2001). Increases in ferritin in postmenopausal women may not cause folate deficiency; however, it is possible that despite normal plasma folate, there is an effect of ferritin on intracellular folate concentrations. This would explain observations that providing folate to individuals with normal circulating folate concentrations can reduce tHcy concentrations (Wilcken et al. 1998). In a similar situation, oral contraceptive use is associated with increased ferritin (Task Force et al. 1998), but not necessarily with folate deficiency (Lussana et al. 2003). However, in rats receiving oral contraceptive treatments, red cell folate was decreased, leading to moderate hyperhomocysteinemia (Durand et al. 1997). In our study, the phytate content in the soy protein reduced iron stores and potentially spared folate for remethylation of Hcy, thereby lowering plasma tHcy concentrations. Although in our study treatment had no effect on plasma folate, we did not analyze intracellular folate

concentrations. Further investigation is required to understand the effects of phytate on red cell folate and tHcy concentrations.

Previous research has found no effect of dietary soy protein or isoflavones on CRP concentrations (Jenkins et al. 2002). Our study confirms these results, showing no effect of soy protein components on CRP concentrations in postmenopausal women. However, our findings may be partially related to the fact that only three subjects had elevated CRP (>3 mg/dL) at baseline, and it is possible that soy protein may only have an impact on reducing inflammation in those with elevated CRP concentrations. Future studies are needed to determine the effects of soy protein on CRP in individuals with high levels of inflammation.

In conclusion, BMI is a strong predictor of CVD risk factors, particularly tHcy and CRP. Thus, maintaining a healthy BMI through diet and physical activity may help control CVD risk. Isoflavones had no protective effect on CVD risk factors in our study, whereas phytate significantly reduced tHcy and iron stores. Consuming soy protein or other phytate-rich foods may prevent menopause-associated rises in tHcy and iron excess, thereby reducing CVD risk in postmenopausal women.

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MBR and DLA designed the study and secured funding. LNH and HME recruited and interviewed women and collected samples and data. MBR and LNH analyzed the data, whereas MBR, LNH, and DLA reviewed the analyses. LNH prepared the first draft of the manuscript and MBR, DLA, and KLS provided advice and consultation on the final draft. Authors had no conflict of interest.

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Table 1 Content of 40 g of soy protein isolates

Treatment	Phytate (g)	Isoflavones (aglycone, mg)	Iron (mg)	Calcium (mg)
LP/LI	0.22	1.2	5.9	504
NP/LI	0.64	1.2	5.7	510
LP/NI	0.22	85.8	59	488
NP/NI	0.78	84.6	5.7	480

LP/LI= low phytate/low isoflavone; NP/LI= normal phytate/low isoflavone; LP/NI= low phytate/normal isoflavone; NP/NI= normal phytate/normal isoflavone

Table 2 Baseline subject characteristics

	LP/LI (n=14)	**NP/LI (n=13)**	**LP/NI (n=14)**	**NP/NI (n=14)**
Age (y)	56 [49-70]	59 [53-69]	58 [47-72]	60 [50-70]
Height (m)	1.66 [1.54-1.77]	1.62 [1.49-1.75]	1.65 [1.56-1.76	1.65 [1.56-1.74]
Weight (kg)	71 [58.2-93.6]	72.6 [59.0-96.8]	72.7 [55.2-92.5]	69.2 [52.3-92.4]
BMI (kg/m^2)	25.9 [18.6-32.9]	27.9 [21.2-34.0	26.5 [19.8-32.3]	25.3 [21.4-33.8]
Daily Dietary Intake*				
Energy (kcal)	1702 [515-2559]	1766 [763-2708]	1769 [877-2827]	1647 [1144-2595]
Protein (g)	65 [22-104]	68 [30-108]	71 [31-122]	63 [39-103]
Carbohydrate (g)	217 [53-412]	239 [119-405]	213 [85-339]	204 [146-328]
Fat (g)	66 [25-96]	64 [22-113]	75 [36-139]	67 [30-105]
Saturated fat (g)	21 [7-37]	18 [6-30]	21 [12-38]	18 [9-26]
Folate (μg)	343 [114-654]	423 [208-617]	394 [234-810]	355 [251-582]
Vitamin B_6 (mg)	1.7 [0.7-2.8]	2.0 [1.1-2.8]	1.7 [0.8-3.1]	1.6 [1.0-2.5]
Iron (mg)	11.7 [4.5-20.3]	13.7 [7.0-22.1]	12.7 [5.6-22.2]	11.8 [8.5-19.4]

Mean [min-max]
LP/LI= low phytate/low isoflavone; NP/LI= normal phytate/low isoflavone; LP/NI= low phytate/normal isoflavone; NP/NI= normal phytate/normal isoflavone
Body mass index (BMI)
* Dietary intake assessed using Block Dietary Data Systems Food Frequency Questionnaire
No significant differences were found among the treatments for any variables.

Table 3 Relationship* between baseline total homocysteine (tHcy) and C-reactive protein (CRP) and cardiovascular disease risk factors

CVD Risk Factors	tHcy	CRP
tHcy		0.35 (*0.01*)
Ferritin	-0.04	0.14
BMI	0.39 (*0.003*)	0.55 (*<0.0001*)
HDL-C	0.20	-0.30 (*0.02*)
LDL-C	0.27 (*0.04*)	0.07
TG	0.07	0.24 (*0.08*)
oxLDL	0.30 (*0.02*)	0.08
PGF	0.16	0.16
PC	-0.07	-0.04
Folate	-0.22	-0.01
Vitamin B_{12}	-0.23 (*0.09*)	-0.05

*Pearson correlation coefficient [r (*P*-value)]
Body mass index (BMI)
Lipid/lipoprotein indices: high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triacylglycerols (TG; values published in Clark et al. 2004)
Oxidative stress indices: 8-iso-prostaglandin F2α (PGF), protein carbonyls (PC), oxidized LDL (oxLDL; values published in Barwick et al. 2004)

Table 4 Regression analysis: Contributors to plasma homocysteine at baseline
Overall model r^2=19.9, adjusted r^2=18.8 (F=6.48, P=0.003)

Independent variable	Variable estimate	Percentage variance	*P*
Intercept	1.3569		0.33
BMI	0.1349	13.9	0.006
LDL-C	0.0096	4.9	0.097

Body mass index (BMI), low density-lipoprotein cholesterol (LDL-C)

Table 5 Effect of treatment on cardiovascular disease risk factors and iron status

	Treatment group			
	LP/LI (n=14)	NP/LI (n=13)	LP/NI (n=14)	NP/NI (n=14)
CVD risk factors				
Homocysteine (μmol/L)				
0 wk	5.8 ± 1.5	6.9 ± 1.2	6.2 ± 1.8	6.3 ± 1.1
6 wk	5.6 ± 1.4	5.3 ± 1.9	5.7 ± 1.5	5.5 ± 1.3
change	-0.2 ± 0.4[a]	-1.3 ± 0.3*[b]	-0.5 ± 0.1*[ab]	-0.8 ± 0.3*[ab]
C-reactive protein (mg/L)				
0 wk	1.9 ± 1.4	1.7 ± 1.3	1.4 ± 0.9	1.2 ± 0.9
6 wk	2.4 ± 2.1	1.7 ± 1.7	1.6 ± 1.3	1.5 ± 1.3
change	0.4 ± 0.3	0.0 ± 0.2	0.1 ± 0.3	0.3 ± 0.2
Iron indices				
Serum iron (μmol/L)				
0 wk	76.9 ± 27.3	95.5 ± 31.6	90.6 ± 20.1	83.1 ± 32.7
6 wk	86.1 ± 41.7	78.5 ± 20.6	89.4 ± 22.3	75.1 ± 24.1
change	9.1 ± 9.8[a]	-17.0 ± 8.8[b]	-1.2 ± 6.1[ab]	-8.1 ± 6.6*[ab]
Transferrin saturation (%)				
0 wk	23.4 ± 9.5	29.7 ± 11.1	28.6 ± 6.6	25.7 ± 11.9
6 wk	23.6 ± 11.8	21.3 ± 6.2	25.9 ± 6.0	21.5 ± 9.4
change	0.3 ± 2.4[a]	-8.4 ± 2.8*[b]	-2.7 ± 1.5[ab]	-4.2 ± 2.0[ab]
Serum ferritin (μg/L)				
0 wk	38.6 (2.1-322.0)	46.8 (4.9-203.8)	42.9 (13.4-167.0)	44.7(3.0-250.7)
6 wk	38.2 (1.0-342.7)	44.0 (4.8-166.8)	38.8 (7.1-207.6)	38.0 (1.0- 171.9)
change	-0.5 ± 2.2[a]	-23.2 ± 8.3*[b]	-1.2 ± 7.6[a]	-7.9 ± 6.1[ab]

Means ± SEM; change = 6wk - 0 wk; for variables that are not normally distributed, the median and range are presented

LP/LI= low phytate/low isoflavone; NP/LI= normal phytate/low isoflavone; LP/NI= low phytate/normal isoflavone; NP/NI= normal phytate/normal isoflavone

*Change is significantly different based on t-test (P<0.05)

Values sharing the same letters are not significantly different from each other based on ANOVA.

GENERAL CONCLUSIONS

In conclusion, the results of our study indicate that BMI affects both tHcy and CRP concentrations in postmenopausal women. Hence, maintaining a healthy BMI may help to prevent elevations in these two CVD risk factors. In addition, we found that soy protein treatment had no effect on CRP, regardless of isoflavone or phytate content. The phytate-containing treatments significantly reduced iron indices as well as tHcy concentrations. While there is no known mechanism explaining the effects of phytate on tHcy, we speculate that the reductions in iron stores with phytate-containing soy protein may have increased intracellular availability of folate, allowing greater recycling of Hcy to methionine, thereby reducing plasma tHcy concentrations. A limitation to this study is that we did not measure intracellular folate, and thus have no data to directly support this theory. However, our results suggest that consumption of phytate-rich foods may prevent increases in tHcy and iron stores, thereby reducing risk of CVD in postmenopausal women.