HIGH-DENSITY LIPOPROTEIN-ASSOCIATED 17β-ESTRADIOL FATTY ACYL ESTERS AND PHYTOESTROGENS: IMPACT ON REVERSE CHOLESTEROL TRANSPORT AND WOMEN’S CARDIOVASCULAR HEALTH

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ACADEMIC DISSERTATION

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Helsinki, Finland 2009
Dedicated to my beloved children:
JACK and HANK
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## Abbreviations

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABCA1</td>
<td>adenosine triphosphate-binding cassette transporter A1</td>
</tr>
<tr>
<td>ABCG1</td>
<td>adenosine triphosphate-binding cassette transporter G1</td>
</tr>
<tr>
<td>AcLDL</td>
<td>acetylated low-density lipoprotein</td>
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<tr>
<td>ApoA-I</td>
<td>apolipoprotein A-I</td>
</tr>
<tr>
<td>ApoB</td>
<td>apolipoprotein B</td>
</tr>
<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
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<tr>
<td>ACAT</td>
<td>acyl-coenzyme A: acyltransferase</td>
</tr>
<tr>
<td>BLT1</td>
<td>blocking lipid transport-1</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CETP</td>
<td>cholesterol ester transfer protein</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
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<tr>
<td>Ct</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DPM</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>E1</td>
<td>estrone</td>
</tr>
<tr>
<td>E2</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>E3</td>
<td>estriol</td>
</tr>
<tr>
<td>E2-FAE</td>
<td>17β-estradiol fatty acyl ester</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen response element</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration of the USA</td>
</tr>
<tr>
<td>Fu5AH</td>
<td>rat hepatocellular carcinoma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>hA-I^Tg</td>
<td>human apolipoprotein A-I transgenic</td>
</tr>
<tr>
<td>HepG2</td>
<td>human hepatocellular carcinoma</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HL</td>
<td>hepatic lipase</td>
</tr>
<tr>
<td>HSL</td>
<td>hormone sensitive lipase</td>
</tr>
<tr>
<td>ICI</td>
<td>ICI 182780; fulvestrant</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate-density lipoprotein</td>
</tr>
<tr>
<td>LCAT</td>
<td>lecithin:cholesterol acyltransferase</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LXR</td>
<td>liver X receptor</td>
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<tr>
<td>MEM</td>
<td>minimal essential media</td>
</tr>
<tr>
<td>PLTP</td>
<td>phospholipid transfer protein</td>
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<tr>
<td>RCT</td>
<td>reverse cholesterol transport</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute media</td>
</tr>
<tr>
<td>SHBG</td>
<td>sex hormone binding globulin</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SR-BI</td>
<td>scavenger receptor class B, type I</td>
</tr>
<tr>
<td>THP-1</td>
<td>human acute monocytic leukemia cell line</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
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Abstract

Reverse cholesterol transport (RCT) is an important function of high-density lipoproteins (HDL) in the protection of atherosclerosis. RCT is the process by which HDL stimulates cholesterol removal from peripheral cells and transports it to the liver for excretion. Premenopausal women have a reduced risk for atherosclerosis compared to age-matched men and there exists a positive correlation for serum 17β-estradiol (E₂) and HDL levels in premenopausal women supporting the role of E₂ in atherosclerosis prevention. In premenopausal women, E₂ associates with HDL as E₂ fatty acyl esters. Discovery of the cellular targets, metabolism, and assessment of the macrophage cholesterol efflux potential of these HDL-associated E₂ fatty acyl esters were the major objectives of this thesis (study I, III, and IV). Soy phytoestrogens, which are related to E₂ in both structure and function, have been proposed to be protective against atherosclerosis but the evidence to support these claims is conflicting. Therefore, another objective of this thesis was to assess the ability of serum from postmenopausal women, treated with isoflavone supplements (compared to placebo), to promote macrophage cholesterol efflux (study II). The scope of this thesis was to cover the roles that HDL-associated E₂ fatty acyl esters have in the cellular aspects of RCT and to determine if soy isoflavones can also influence RCT mechanisms. SR-BI was a pivotal cellular receptor, responsible for hepatic and macrophage uptake and macrophage cholesterol efflux potential of HDL-associated E₂ fatty acyl esters. Functional SR-BI was also critical for proper LCAT esterification activity which could impact HDL-associated E₂ fatty acyl ester assembly and its function. In hepatic cells, LDL receptors also contributed to HDL-associated E₂ fatty acyl esters uptake and in macrophage cells, estrogen receptors (ERs) were necessary for both HDL-associated E₂ ester-specific uptake and cholesterol efflux potential. HDL-containing E₂ fatty acyl esters (E₂-FAE) stimulated enhanced cholesterol efflux compared to male HDL (which are deficient in E₂) demonstrating the importance of the E₂ ester in this process. To support this, premenopausal female HDL, which naturally contains E₂, showed greater macrophage cholesterol efflux compared to males. Additionally, hepatic and macrophage cells hydrolyzed the HDL-associated E₂ fatty acyl ester into unesterified E₂. This could have important biological ramifications because E₂, not the esterified form, has potent cellular effects which may influence RCT mechanisms. Lastly, soy isoflavone supplementation in postmenopausal women did not modulate ABCA1-specific macrophage cholesterol efflux.
but did increase production of plasma pre-β HDL levels, a subclass of HDL. Therefore, the impact of isoflavones on RCT and cardiovascular health needs to be further investigated. Taken as a whole, HDL-associated E₂ fatty acyl esters from premenopausal women and soy phytoestrogen treatment in postmenopausal women may be important factors that increase the efficiency of RCT through cellular lipoprotein-related processes and may have direct implications on the cardiovascular health of women.
1. Introduction

Globally, cardiovascular disease (CVD) is a major cause of mortality (The World Health Report 2004, World Health Organization). CVD, a clinical manifestation of atherosclerosis, is caused by a combination of inflammation and disorders in lipid metabolism (Barter & Rye, 2007, Glass & Witztum, 2001). Healthy, premenopausal women have a low incidence of atherosclerosis compared to men of the same age and only upon the onset of menopause do these women develop the disease’s characteristics (Mendelsohn & Karas, 2005, Kannel et al., 1976, Collins et al., 2007, Maxwell, 1998, Mosca et al., 1997). Endogenous estrogens, especially 17β-estradiol (E2) - the most biologically active estrogen (Brook & Marshall, 2001), have been suggested to provide this protection based on observational and experimental investigations (Mendelsohn & Karas, 1999). High-density lipoproteins (HDL) also defend against atherosclerosis through many different processes. The mechanism of reverse cholesterol transport (RCT) is thought to be the most potent of the atheroprotective functions of HDL (Barter & Rye, 2007). HDL transports excess cholesterol, removed from macrophage cells, to the liver for excretion (Schaefer et al., 2007). Premenopausal women have naturally elevated HDL levels and a positive correlation between plasma E2 and HDL-cholesterol levels exists (Lamon-Fava et al., 2005). These women also have enhanced hepatic cholesterol efflux to HDL compared to men (Catalano et al., 2008).

HDL functions as a transporter not only of cholesterol but of lipophilic compounds and hormones, such as E2 (Tikkanen et al., 2002, Schaefer et al., 2007). This association between HDL and E2 warrants attention because these E2-containing HDL particles exist naturally in female plasma, with E2, in a fatty acyl ester form (Larner et al., 1987, Vihma et al., 2003). This combination raises the possibility that this may increase the efficiency of RCT. E2 needs to be esterified in order to be efficiently packaged into the HDL and plasma lecithin:cholesterol acyltransferase (LCAT), accomplishes this (Höckerstedt et al., 2004, Schaefer et al., 2007)). In endothelial cells, HDL-containing E2 interacts with the scavenger receptor class B, type I (SR-BI), a receptor that can bind HDL and either selectively internalize the cholesterol ester but leave the remnant intact or can efflux cellular cholesterol, and can trigger enhanced endothelial nitric oxide synthase production compared to HDL lacking E2 (Rigotti et al., 2003, Gong et al., 2003).
Since HDL-containing $E_2$ is more potent than HDL alone and interacts with SR-BI, a major HDL receptor involved in the RCT pathway, the idea that this may also enhance RCT-related mechanisms was generated. No previous investigation has specifically probed this question and studies III and IV of this thesis explore these issues. SR-BI deficiency in mice was also investigated (study I) and has relevance to the HDL-associated $E_2$-RCT relationship because any effect of SR-BI ablation on plasma enzymes, such as LCAT, would have consequences on HDL-associated $E_2$ ester entero-hepatic metabolism and RCT.

The impact of soy phytoestrogens, plant-derived compounds with polyphenolic structure and estrogenic function (Cornwell et al., 2004), on RCT is unclear. In terms of cardiovascular health, isoflavones may or may not be effective in providing benefits. Soy is a common source of protein in Asian countries and these countries have low incidences of heart disease (Adlercreutz & Mazur, 1997, Keinan-Boker et al., 2002, Tham et al., 1998). There are conflicting investigations either supporting or denying the cardiovascular protection offered by soy because in clinical trials or experimentally, phytoestrogens alone or with the protein component of soy show inconclusive evidence for either (Sacks et al., 2006a, Clarkson, 2002, Kokubo et al., 2007, Wagner et al., 2003, Yamakoshi et al., 2000). The potential of phytoestrogens to modulate RCT, specifically macrophage adenosine triphosphate binding cassette A-1 (ABCA1)-mediated cholesterol efflux to serum, in postmenopausal women could provide insight into this dilemma and is the subject of study II.

The ultimate objective of this thesis is to systematically, provide evidence for a connection between a CVD protective mechanism, specifically RCT, in women, and HDL-associated $E_2$ fatty acyl esters or soy phytoestrogens.
2. Review of the literature

2.1. Lipoprotein metabolism

2.1.1. Overview

Lipoproteins are specialized transporters of hydrophobic lipids in circulation. These carriers serve to provide energy and structural components to tissues. Lipoproteins are composed of a core of hydrophobic lipids, cholesterol esters and triglycerides, surrounded by an amphipathic phospholipid monolayer. In this monolayer exist apoproteins, providing structural scaffolds to the particle, and unesterified cholesterol (Garrett & Grisham, 1999). Lipoproteins are classified based on their densities and from lightest to densest are: chylomicrons, very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Each lipoprotein class has a distinct biological role. Chylomicrons and VLDL mainly transport energy-rich triglycerides to adipose or muscle tissue, LDL functions to circulate cholesterol from the liver to extra-hepatic tissues, sometimes called forward cholesterol transport. HDL mediates the removal of cholesterol from extra-hepatic tissues back to the liver for excretion from the body - a process called reverse cholesterol transport (RCT) (Barter & Rye, 2007, Rader et al., 2008). These are the most basic functions of these lipoproteins. Lipoprotein classes have additional functions that are not listed here.

2.1.2. Development of atherosclerosis

Cardiovascular disease (CVD) is a leading cause of death in western societies and creeping as a global killer (The World Health Report 2004, World Health Organization). Atherosclerosis, a major cause of cardiovascular disease, is characterized by a localized inflammatory response and accumulation of excess cholesterol in the arterial intima (Glass & Witztum, 2001, Barter & Rye, 2007). In humans, the development of atherosclerosis
occurs over many decades. Atherosclerosis is triggered first by lipid deposition and subsequent leukocyte migration to areas of the coronary arteries that have points of sheer stress, such as arterial branch points (Libby et al., 2002). Lipids are major players in atherogenesis because retention of LDL by matrices in the arterial intima causes the modification and oxidation, of the lipids and apolipoproteins in these particles (Glass & Witztum, 2001, Libby et al., 2002, Tabas et al., 2007). In the plasma compartment, LDL is protected from oxidation. However, retention in the intima enhances the LDL oxidation potential (Glass & Witztum, 2001). This triggers an inflammatory response, the release of cellular adhesion molecules, chemokines, and proinflammatory cytokines causing a mass recruitment of monocytes to the sites where LDL retention occurs. Lacking a feedback inhibition mechanism for cholesterol accumulation, macrophage uptake of modified LDL by scavenger receptor A and CD-36 steadily occurs and the phenotypic lipid-laden macrophage foam cells become apparent (Vainio & Ikonen, 2003). Within these cells, cholesterol esterification by acyl coenzyme A: cholesterol acyltransferase (ACAT) ensues. This process leads to a cellular aggregation in the arterial intima. Eventually, these foam cells become necrotic, which augments the inflammatory responses to these sites. A complex lesion or fibrous plaque develops by the calcification and the capping by newly synthesized smooth muscle cells. This occludes the arterial lumen diameter and causes ischemia to the heart and peripheral cells. In addition, the plaque may become vulnerable ultimately leading to rupture and causing myocardial infarction or stroke.

2.1.3. High-density lipoprotein - structure and metabolism

An inverse correlation exists between serum HDL cholesterol levels and the risk for CVD (Barter & Rye, 2007). Experimental and epidemiological investigations demonstrate that for every 1 mg/dl⁻¹ (0.025 mmol/l⁻¹) raise in HDL cholesterol values, there is a 2-3% decrease in CVD risk. HDL has many functions which enable it to combat progression of atherosclerosis such as improving endothelial function, providing antioxidant protection, protecting against endothelial damage, and by its anti-inflammatory properties. RCT, mediated by HDL, is the most investigated and perhaps the most pivotal of these protective functions (Barter & Rye, 2007).
HDL exists in circulation in different sizes with varying amounts and types of phospholipids and apolipoproteins. HDL subclasses, from smallest to largest size, are preβ-HDL, HDL\(_3\), and HDL\(_2\). Preβ-HDL comprises 2-5% of the total plasma HDL and the remainder consists of α-migrating particles (Fielding & Fielding, 1995). The α and β nomenclature for lipoproteins originates from their detected mobility on agarose gels. HDL is able to pass freely between the plasma compartment and extracellular spaces of the blood vessel, unlike LDL, in which case apolipoprotein B (apoB) gets retained by heparin-binding sites of apoB to proteoglycans. Apolipoproteins in HDL are exchangeable with other lipoproteins. This makes HDL a dynamic player in lipid metabolism (Schaefer et al., 2007). The average composition of HDL is approximately 50% protein (as mass %), 25% phospholipid, and 20% cholesterol (mainly cholesterol ester) and 2-5% triglyceride. Apolipoprotein A-I (apoA-I) is the major apolipoprotein of HDL. HDL also has other apolipoproteins such as A-II, A-IV, A-V, C-I, C-II, C-III, D, E, J, and M.

2.1.4. High-density lipoprotein and reverse cholesterol transport

The mechanism of RCT and HDL metabolism have been thoroughly described using mouse models (Rader et al., 2008) and is outlined below and illustrated in Figure 1: HDL biogenesis occurs by hepatic and intestinal cells and is marked by the secretion of lipid-poor apoA-I with a secretion rate of 12 mg kg\(^{-1}\) day\(^{-1}\) and a plasma residence time of 4 to 5 days (Schaefer et al., 2007). This newly secreted apoA-I becomes partially lipidated by ATP binding cassette transporter A1 (ABCA1), a cell membrane-associated protein, at these sites (Schaefer et al., 2007). If there is a rare defect in apoA-I secretion, marked by human HDL cholesterol levels of less than 0.13 mmol per liter, a high risk for CVD occurs (Schaefer et al., 2007). ApoA-I binding to the macrophage surface may trigger neutral cholesterol ester hydrolase activity which liberates the cholesterol from its fatty acyl ester stores (Zhao \textit{et al.}, 2007). Efflux of free cholesterol and phospholipids, from macrophage cells to this poorly lipidated apoA-I, generates a nascent, discoidal, preβ-migrating HDL and marks the initial stages of RCT. In Tangier disease, a disease marked by a mutated and dysfunctional ABCA1 gene, preβ-HDL cannot be lipidated and patients have cholesterol accumulation in peripheral cells (tissues), including macrophages, and a high
Figure 1: HDL metabolism and RCT. Reprinted with permission from Wiley-VCH Verlag GmbH & Co. (Swift et al., 2007)
risk for CVD (Schaefer et al., 2007). Cholesterol efflux to HDL occurs by ABCA1, ABCG1, scavenger receptor class B, type I (SR-BI) or by passive diffusion through the plasma membrane and active cholesterol efflux is dependent upon the HDL size and composition (Figures 1 and 2). The small, discoidal preβ-HDL then acquires more phospholipids and cholesterol is effluxed by macrophage ABCA1 after which lecithin-cholesterol acyltransferase (LCAT) esterifies free cholesterol and small HDL$_3$-type particles are generated. ApoE-cholesterol complexes also fuse with apoA-I to form HDL$_3$ particles. LCAT further esterifies free cholesterol in the HDL$_3$ particle creating a larger, cholesterol ester enriched, HDL$_2$ particle. At the HDL surface, LCAT removes a fatty acid from the sn-2 position of phosphatidylcholine, present in HDL, to free cholesterol creating cholesterol ester and lysophosphatidylcholine (Schaefer et al., 2007). The cholesterol ester is partitioned into the core of HDL and LCAT is recycled for another round of esterification. Cholesterol ester transfer protein (CETP) causes an exchange between the HDL$_2$ cholesterol ester component with VLDL/LDL-derived triglycerides. LDL is then catabolized majorily via a hepatic LDL receptor pathway.

Another route in RCT is HDL$_2$ binding to hepatic SR-BI. SR-BI selectively removes the cholesterol esters from HDL, and causes hepatic cholesterol internalization. The remnant apoA-I particles, which have escaped internalization and degradation, are either recycled for another round of HDL assembly or catabolized by kidney receptors (Schaefer et al., 2007). The SR-BI pathway in RCT is the major one in rodents, such as mice, but its role in humans is not well known.
2.2. Estrogens

2.2.1. Structure and metabolism of estrogens

Estrogens are a class of steroid hormones known for their effects on the secondary sex characteristics in women, on the menstrual cycle, and reproduction (Brook & Marshall, 2001). Endogenously, they are found at the highest levels in pregnancy and at lower levels in premenopausal women, and at much lower levels in men and postmenopausal women (Brook & Marshall, 2001). They are derived from cholesterol and have a similar hydrocarbon ring structure (see Figure 4). The three main types of estrogens are estrone (E₁), estradiol (E₂), and estriol (E₃) (Brook & Marshall, 2001).

The ovaries, placenta (during pregnancy), adrenal glands, and adipose tissue are sites of synthesis for estrogens (MacDonald et al., 1979, Brook & Marshall, 2001). E₂ and E₁ are the most biologically active. After stimulation by follicle stimulating hormone and luteinizing hormone, the theca interna cells of the ovaries synthesize estrogens from cholesterol converting it into androstenedione. Conversion of androstenedione or testosterone by aromatase is mediated at the ovarian granulosa cells (Brook & Marshall, 2001). Estrogens, unconjugated to sulfates or glucuronides, are transported in circulation majorily by sex hormone binding globulin (SHBG), and to a lesser extent by albumin, or lipoproteins (Anderson, 1974, Hochberg, 1998).

During the menstrual cycle, serum E₂ values for premenopausal women range from 367 pmol l⁻¹ in the follicular phase to 2200 pmol l⁻¹ at ovulation (Mendelsohn & Karas, 1999). During pregnancy, E₂ values can be as high as 70,000 pmol l⁻¹. Postmenopausal women and men have values ranging from 18 to 74 pmol l⁻¹ (Mendelsohn & Karas, 1999).

2.2.2. Cellular effects of estrogens—role of estrogen receptors

The nuclear superfamily of ligand-activated DNA transcription factors includes estrogen receptors (ER) and these exist in two subtypes, α and β (Dahlman-Wright et al., 2006). In order to be activated, estrogen bound ERs must dimerize and bind to specific DNA binding
elements (ERE). The dimerization can include either an ERα/ERα, ERβ/β, or ERα/β configuration (Li et al., 2004). Complex arrays of coactivator and corepressor proteins interact with these receptors to coordinate cell-specific effects. There also exist cellular extranuclear plasma membrane ERs (Hammes & Levin, 2007). The ER α and β isotypes and a G-protein coupled receptor, GPR-30, reside there. GPR-30 binds with high affinity but low binding capacity to E2. Both forms of extranuclear ERs, ER or GPR-30, induce rapid cellular signaling transduction mechanisms. These modulate cellular gene transcription (Figure 3) and also nontranscriptional functions in cells (Hammes & Levin, 2007).

Expression of nuclear ERα and ERβ vary from cell type. Based on mouse tissue ER expression, ERα is predominantly expressed in the endometrium, breast cancer cells, ovarian stroma cells, and in the hypothalamus. ERβ is expressed highly in the kidney, brain, bone, heart, lungs, intestinal mucosa, prostate, and endothelial cells (Couse et al., 1997). Importantly, the ratio of ER isotypes in different tissues has been shown to influence the development of certain diseases (Bakas et al., 2008).

Figure 3: Scheme of estrogen and estrogen receptor cellular genomic effects.
2.3.3. Endogenous estradiol effects on CVD development

Binding of estrogens to ERs is critical for their function. In order to enter the cell, estrogen can diffuse passively across the plasma membrane into the cytosol. Unactivated ERs are mostly present in the cytosol, and a minority is present in the nucleus (Htun et al., 1999). Following ligand binding, the ER-estrogen complex enters the nucleus, and binds to estrogen response elements (Figure 3). E$_2$ binds to both types of ERs equally, E$_1$ binds to ER$\alpha$, preferentially, and E$_3$ and genistein, a phytoestrogen, prefers ER$\beta$ (Htun et al., 1999, Kuiper et al., 1997). Based on a large number of observational, intervention, and experimental studies, estrogen has been linked to mechanisms that are protective against the development of atherosclerosis. To support this, premenopausal women, in general, have a reduced risk for CVD compared to men of a similar age (Kannel et al., 1976, Maxwell, 1998, Collins et al., 2007, Jousilahti et al., 1999, Mosca et al., 1997). However, this risk rises upon the onset of menopause and among postmenopausal women CVD is the leading cause of death (Mendelsohn & Karas, 2005). At least 8.6 million women worldwide die of CVD every year (The World Health Report 2004, World Health Organization). The areas of estrogen protection are broad: influencing circulating coagulation, fibrinolytic, and vasoreactive proteins; improving endothelial function; providing antioxidant protection to LDL; increasing HDL biosynthesis and by modulating serum lipid levels (Mendelsohn & Karas, 1999). It has been suggested that estrogenic effects aimed directly at the vessel wall provide the greatest amount of protection compared to their other cardioprotective effects (Mendelsohn & Karas, 1999). E$_2$ interacts with ER$\alpha$ which causes an upregulation of prostacyclin, an anti-inflammatory cytokine, provoking a defense against atherosclerosis. Estrogens improve endothelial function by increasing endothelial nitric oxide levels and promote vasodilation (Sader & Celermajer, 2002). Vasodilation and blood pressure fluctuate in premenopausal women based on their phase in the menstrual cycle and premenopausal women have a lower blood pressure compared to postmenopausal women (Dubey et al., 2002, Sader & Celermajer, 2002). Antioxidant protection provided by estrogens are free radical scavenging (Prokai et al., 2003), chelating of ions (Pinchuk & Lichtenberg, 2002), and stabilizing the conformation of apoB-100 (Brunelli et al., 2000), the major apolipoprotein of LDL, to be resistant to oxidation.
In addition to the effects aimed at the vessel wall, another important parameter of cardiovascular health is serum lipid levels. Premenopausal women have higher HDL, lower LDL, and lower triglyceride concentrations compared to age-matched men and postmenopausal women (Mendelsohn & Karas, 1999). Also, a positive correlation exists for serum levels of endogenous $E_2$ and HDL levels in premenopausal women (Lamon-Fava et al., 2005). Liver X receptor $\alpha$ (LXR$\alpha$), in macrophages and adipose tissue (Kramer & Wray, 2002, Lundholm et al., 2004), and apoA-I gene regulation are influenced by $E_2$ and these may be critical regulators of serum lipid levels in women (Lamon-Fava & Micherone, 2004). LXR is a sterol responsive nuclear transcription factor that binds to oxysterols and modulates many genes in RCT, such as ABCA1, and affects overall triglyceride levels (Repa & Mangelsdorf, 2002).

2.2.4. Estradiol fatty acyl esters and lipoprotein-associated estradiol fatty acyl esters

$E_2$ fatty acyl esters are found in various tissues and in circulation (Larner et al., 1992, Larner & Hochberg, 1985). The presence of these has implicated the $E_2$ fatty acyl ester as a storage form of $E_2$ which may be mobilized for future utilization. They have an extended half-life and protection from metabolic clearance compared to unesterified $E_2$ (Larner et al., 1985b, Larner & Hochberg, 1985, Vázquez-Alcántara et al., 1989). $E_2$ fatty acyl esters are synthesized and accumulate in tissues, such as adipose and breast tissue, and these may be important reservoirs of $E_2$ (Larner et al., 1992, Larner et al., 1985a, Badeau et al., 2007). $E_2$ esters are also produced and stored in spleen, lung, uterus, and liver (Larner et al., 1992). The esterification of $E_2$ occurs at the C-17 position in the steroidal D ring by various long-chain fatty acids (Kanji et al., 1999). Intracellular enzymes necessary for the esterification of $E_2$ esters in humans remain unclear.

The function of $E_2$ fatty acyl esters also is uncertain. Since esterified $E_2$ is not found in urine, the metabolic end-stage of estrogens, it is thought that $E_2$ esters must first be hydrolyzed to become biologically active (Larner et al., 1985b, Schatz & Hochberg, 1981, Janoko & Hochberg, 1983). Functionally, $E_2$ fatty acyl esters may induce breast cancer because of a local concentration of $E_2$ in these tissues (Larner et al., 1985a, Mills et
Candidacy enzymes for liberation of \( \text{E}_2 \) from its esterified state are hormone-sensitive lipase (HSL) and a yet unnamed \( \text{E}_2 \) ester-cleaving enzyme found in breast cyst fluid (Lee et al., 1988, Banerjee et al., 1991, Mills et al., 2008). HSL is found in adipose tissue, heart, adrenal cortex, breast tissue, corpus luteum, macrophages, skeletal muscle, testis, and the \( \beta \)-cells of the pancreas (Kraemer & Shen, 2002). It has specificity for different steroidal molecules such as cholesterol and steroid fatty acyl esters and also mono-, di-, and triglycerides (Kraemer & Shen, 2002, Yeaman, 2004). Cellular hydrolysis of \( \text{E}_2 \) fatty acyl esters is critical for estrogenic effects.

\( \text{E}_2 \) fatty acyl esters exist also in humans both in circulation and in ovarian follicular fluid. These exclusively associate with lipoproteins for solubilization. The biological function of these lipoprotein-associated \( \text{E}_2 \) fatty acyl esters is unclear. Whether these specific \( \text{E}_2 \) fatty acyl esters are shuttled to targets of lipoprotein metabolism remains unclear (Larner et al., 1987, Leszczynski & Schafer, 1990, Helisten et al., 2001, Meng et al., 1999). \( \text{E}_2 \) fatty acyl esters comprise the majority of total \( \text{E}_2 \) in lipoproteins.

![Diagram of Estradiol (E2) and Cholesterol Fatty Acyl Esters](image)

**Figure 4:** \( \text{E}_2 \) and cholesterol fatty acyl esterification by LCAT. \( \text{E}_2 \) and cholesterol have similar hydrocarbon structures and LCAT partitions these into HDL. \( \text{E}_2 \) ester accumulation in HDL is markedly lower compared to cholesterol ester. Illustration by RM Badeau 2009.

\( \text{E}_2 \) esterification at the C-17 position at its D ring occurs by a LCAT mediated mechanism and HDL\(_3\) is the primary platform for this reaction (Kanji et al., 1999, Höckerstedt et al., 2002) as depicted in Figure
4. CETP is able to mediate the transfer of HDL-associated E\textsubscript{2} esters to LDL (Helisten et al., 2001). The metabolic stage when E\textsubscript{2} associates with HDL\textsubscript{2} is unknown.

The relationship between lipoprotein-associated E\textsubscript{2} fatty acyl esters and atherosclerosis is not fully understood but there is evidence suggesting that the presence of E\textsubscript{2} in lipoproteins protects against lipoprotein oxidation (Meng et al., 1999, Shwaery et al., 1997, Abplanalp et al., 2000, Höckerstedt et al., 2004). In vitro preparations of HDL-associated E\textsubscript{2} have demonstrated that HDL-associated E\textsubscript{2} can boost endothelial nitric oxide synthase (eNOS) activity compared to HDL. In the same investigation, premenopausal female HDL had a greater aortic vasodilatory effect compared to age-matched males (Gong et al., 2003). Taken together, these support the cardioprotective potential of HDL-associated E\textsubscript{2} esters.

At the cellular level, lipoprotein receptors interact with HDL-associated E\textsubscript{2} fatty acyl esters. In vitro, HDL particles containing E\textsubscript{2} interact with endothelial and osteoblastic cell SR-BI receptors (Gong et al., 2003, Brodeur et al., 2008). The uptake of E\textsubscript{2} by endothelial cells is undocumented, but SR-BI does modulate E\textsubscript{2} uptake in osteoblastic cells (Brodeur et al., 2008). The critical points demonstrated, in these two studies, are that HDL can transport E\textsubscript{2} to cellular HDL receptors, for example, SR-BI, and at these sites, HDL-derived E\textsubscript{2} can mediate estrogen-specific functions such as vasorelaxation. HDL-associated estrogen fatty acyl esters and their uptake by cellular receptors are currently undocumented.

2.3. Phytoestrogens and isoflavones

2.3.1. Definition, structure and metabolism

Phytoestrogens are naturally found plant-derived, non-steroidal compounds that can exert estrogenic effects (Cornwell et al., 2004). These were first identified in the 1940’s when farmers noticed that sheep, which grazed on phytoestrogen-rich red clover, were experiencing fertility problems (Cornwell et al., 2004). Isoflavones, a class of phytoestrogens, exist in soybeans, fruits, vegetables, and other plants (Cornwell et al., 2004). Genistein (4’,5,7-trihydroxyisoflavone), daidzein (4’,7-dihydroxyisoflavone) and
glycitein (4’7-di-hydroxy-6-methoxyisoflavone) are common isoflavones (Cornwell et al., 2004) having some structural resemblance to E2 (Figure 5). They are capable of binding to ERs, as either agonists or antagonists (Tempfer et al., 2007) which make them possible Selective Estrogen Receptor Modulators (SERMs) (Mikkola & Clarkson, 2002). Both genistein and daidzein bind with greater affinity to ERβ than ERα and genistein, perhaps the most biologically potent isoflavone (Cornwell et al., 2004), has one-third the effectiveness of E2 to stimulate ERβ-mediated, estrogen-dependent reporter plasmids in vitro (Kuiper et al., 1998).

Genistein is naturally conjugated to glycosides which are biologically inert and these sugars need to be hydrolyzed by the intestinal enzymes, becoming active aglycones, to become absorbed and exist in circulation (Setchell et al., 2002b, Setchell, 1998, Chen et al., 2003). After ingestion, genistein and daidzein reach the maximum concentrations in serum after 5-6 hours and are excreted through urine within 24 hours (Nielsen & Williamson, 2007, Setchell et al., 2003). To a lesser extent, plasma proteins are able to bind isoflavones (Lapcik et al., 1998, Shelnutt et al., 2002) and in vitro evidence has demonstrated that genistein can associate with lipoproteins (Kaamanen et al., 2003). Genistein can also be fatty acyl esterified in lipoproteins in vitro (Kaamanen et al., 2003). Genistein fatty acyl esters are found at very low concentrations in plasma from non-human primates after oral or subcutaneous administration of genistein (Badeau et al., 2005).

Equol (7-hydroxy-3(4’hydroxyphenyl)-chroman) is also classified as an isoflavone. It has a unique property compared to other isoflavones in that it is endogenously produced, in its S-isomer form, in humans, from the intestinal metabolism of daidzein by bacteria (Setchell et al., 2002a, Setchell et al., 2003, Setchell et al., 2005). Metabolism of equol in humans is limited and approximately 30% of individuals can produce it. An equol producer or a non-producer can be determined by the plasma equol levels, after consumption of
isoflavones, of >83 nmol l\(^{-1}\) or <40 nmol l\(^{-1}\), respectively or an urinary equol level of >1000 nmol l\(^{-1}\) (Setchell et al., 2002a). Factors that affect these values in equol producers are: antibiotic use (negatively), consumption of fat (negatively), non-starch polysaccharides (positively), and probiotics (positively) (Rowland et al., 2000, Fooks & Gibson, 2002, Setchell et al., 2002a). Functionally, equol can bind to ER\(\beta\) but the cellular effects equol exerts through ER\(\beta\) are unknown (Setchell et al., 2005).

2.3.2. Dietary supplementation of phytoestrogens

Soy is popular and consumed largely in eastern Asian societies. On average, daily dietary intake of isoflavones, in the form of soy, by these populations can be from 20 to 80 milligrams per day, while peoples in North America or Europe consume minimal amounts (0.5 to 3 milligrams per day) (Setchell et al., 2002a, Adlercreutz & Mazur, 1997, Tham et al., 1998, Keinan-Boker et al., 2002). Cultural reasons may account for this discrepancy. Intake of fifty milligrams of isoflavones can yield approximately 1 to 4 \(\mu\)mol l\(^{-1}\) per liter of these in circulation and withdrawal of this intake can rapidly reduce these concentrations (Bhathena & Velasquez, 2002). Isoflavones can be isolated from different tissues and their biological activities at these locations are uncertain (Setchell, 2000).

For 5000 years, soybeans have been regular staples of the Chinese diet and components to many of the drugs used in that culture. In fact, in 2853 B.C., Emperor Shennong of China declared that soybeans, rice, wheat, barley, and millet were the five sacred plants (www.soya.be). Currently, soybeans, or soy, are considered to be a source of complete dietary protein (www.fda.gov/Fdac/features/2000/300_soy.html), polyunsaturated fatty acids, dietary fiber, vitamins, and minerals, and exist in many processed foods (Sacks et al., 2006b). Isoflavones are present in soy. Soy-based food products are available commercially in Europe and North America and some examples include: soy milk, tofu, soy bars, and in foods designed for vegetarians (United-Soybean-Board, 2007). Based on the low incidence of cardiovascular disease and cancers of the breast, prostate, and endometrium, in countries which consume large quantities of soy (Tikkanen & Adlercreutz, 2000, Valachovicova et al., 2004, Sacks et al., 2006b), the scientific support for these health effects are being sought and if found, could boost the
popularity of soy in Western countries. This evidence, currently, is conflicting and unclear.

2.3.3. Soy, isoflavones, and development of atherosclerosis

In 1999, the Food and Drug Administration of the USA (FDA) claimed that 25 grams of soy protein daily was protective against coronary heart disease based on meta-analysis reports (Erdman, 2000). This caused much hype and hope for a natural alternative to the current hormone therapies, for the food industry, and for health conscious individuals. Unfortunately, the FDA reversed this claim as contrary evidence accumulated (Sacks et al., 2006b). Current opinion among some experts is that any protective effect soy has on the cardiovascular system is minimal (van der Schouw et al., 2005, Hall et al., 2005, Kreijkamp-Kaspers et al., 2004, Clarkson, 2002, Sacks et al., 2006b). In light of this, there are other investigations that still support the cardioprotective nature of soy. The key to delineate this unsettled evidence may be in the gender specific, cultural, and also age-dependent parameters. In a 12-year follow-up study assessing 40,000 Japanese subjects, the greatest reduction in heart disease risk was found in women, especially, post-menopausal women who consumed soy (Kokubo et al., 2007). Chinese women, consuming soy also showed lower risk for CVD (Zhang et al., 2003). Supporting these studies are cross-sectional analyses of about 1000 postmenopausal women who underwent isoflavone dietary supplementation (de Kleijn et al., 2002). Additionally, an investigation has linked a reduction of body mass index and fasting insulin concentrations, and elevated HDL values in postmenopausal women consuming isoflavones (Bhatena & Velasquez, 2002). Another explanation for this complexity may be the type, dose, and duration of soy administration to these women (Dewell et al., 2006, Clarkson, 2002).

Since it is uncertain which component of soy may provide the best protection against atherosclerosis, the question arises: what part of soy is important for a positive effect - the isoflavones, the protein, or both? Isoflavones can be extracted from soy protein by alcohol based treatments (Anthony et al., 1996). The most common clinical parameters to assess soy’s modulation on cardiovascular health are plasma lipids, lipoproteins, and apolipoprotein values. Investigations assessing these parameters in postmenopausal women treated with isoflavone supplements in humans show conflicting outcomes (Taku et al.,
Confoundingly, soy protein and isoﬂavones together are also critical for the cardioprotective effects of soy (Anderson et al., 1995, Allen et al., 2007). Experimentally, non-human primates administered soy isoﬂavones with the protein demonstrated decrease in atherosclerotic plaque development and an antiatherogenic effect on plasma lipids (Clarkson et al., 2001, Anthony et al., 1996, Greaves et al., 1999, Wagner et al., 2003). In line with this, rabbit models of atherosclerosis show that soy isoﬂavones together with the protein also reduce aortic plaque development and defend against LDL oxidation (Castiglioni et al., 2003). However, there is evidence that in the same animal model, these effects can be achieved upon isoﬂavone treatment only, in the absence of soy protein (Yamakoshi et al., 2000). In light of all these conﬂicting results, the question remains: does soy have any effect on cardiovascular health and if so, which components of soy could provide this protection? A neglected but important key to unlocking this mystery may lie in equol’s effect on plasma lipid proﬁles, which remains unknown (Setchell et al., 2002a). These investigations did not divide the subjects into equol and non-equol producers and this may or may not be critical for understanding further isoﬂavone’s role in CVD protection.

Isoﬂavones can inﬂuence plasma lipids, have antiproliferative effects on vascular endothelial and smooth muscle cells, and thrombus formation, and improve endothelial function (Anthony et al., 1998, Tikkanen & Adlercreutz, 2000, Tham et al., 1998). Their greatest antiatherosclerotic property may be by providing antioxidant protection to lipoproteins (Tikkanen et al., 1998, Kanazawa et al., 1995, Wiseman et al., 2000). Genistein is antiproliferative by inhibiting certain tyrosine kinases (Adlercreutz & Mazur, 1997) and endothelial function is improved by enhancing aortic vasodilation (Cruz et al., 2006, Mäkelä et al., 1999). In terms of RCT and modulation of lipoprotein metabolism, genistein can increase hepatic apoAI gene expression, leading to potentially elevated plasma HDL levels (Lamon-Fava & Micherone, 2004). It also decreases de novo cholesterol biosynthesis and increases cellular LDL receptor expression by effecting 3-hydroxy-3-methylglutaryl CoA reductase (Borradaile et al., 2002, Kirk et al., 1998). Exposure of genistein to macrophage cells inhibits ABCA1-mediated cholesterol efﬂux (Tang et al., 2004). Also isoﬂavone rich serum from postmenopausal women does not promote enhanced SR-BI mediated cellular cholesterol efﬂux (Törmälä et al., 2006). Further investigations into the relationship between isoﬂavones and RCT processes are
warranted to decipher possible cellular machinery that could be modulated for protection against atherosclerosis.
3. Aims of the study

1. To determine the effect of SR-BI deletion on plasma enzymes, specifically LCAT, involved in HDL metabolism (I)

2. To assess the difference in serum lipids and of macrophage ABCA1 cholesterol efflux potential to serum from postmenopausal women before and after dietary phytoestrogen supplementation (II)

3. To systematically evaluate HDL-associated E₂ fatty acyl ester hepatic uptake and hydrolysis and the specific cellular lipoprotein receptors involved. (III)

4. To study macrophage cholesterol efflux potential and cellular E₂ ester internalization and hydrolysis stimulated by HDL-associated E₂ fatty acyl esters (IV)

5. To test whether gender differences exist in HDL-mediated macrophage cholesterol efflux potential (IV)
4. Materials and methods

Study I:

Mice
Wild-type (SR-BI\textsuperscript{+/+}), heterozygous (SR-BI\textsuperscript{+/−}), and homozygous (SR-BI\textsuperscript{−/−}) progeny were generated by cross-breeding SR-BI\textsuperscript{+/−} mice. The genetic background for the SR-BI mice was 129/C57BL/6. Founder human apoA-I transgenic mice (hAI-Tg) were generated by established protocols (Rubin et al., 1991). Human apoA-I transgenic mice were purchased from Charles River Laboratories® (Wilmington, MA). SR-BI\textsuperscript{+/+} (both genders) and SR-BI\textsuperscript{−/−} (male) mice were mated with hAI-Tg mice (both genders) to create SR-BI, hAI\textsuperscript{Tg} progeny. At three weeks of age, the mice were weaned and genotyped. Mice were housed in the animal facility at the Wake Forest University School of Health Sciences. All procedures were approved by the animal care and use committee (ACUC) of Wake Forest University School of Health Sciences.

Determination of plasma lipid concentrations
For lipid plasma measurements and whole plasma LCAT cholesterol esterification assays, mice were bled at between 8-12 weeks of age. Plasma lipid concentrations were determined by using the following commercial assays: total plasma cholesterol (Roche®), free cholesterol (Wako®), triglyceride (Roche®), and phospholipid (Wako®) (Carr et al., 1993).

Human ApoA-I quantitation
Human apoA-I concentration was determined by an enzyme linked immunosorbent assay protocol as described previously (Koritnik & Rudel, 1983).

Plasma LCAT, HL, or PLTP activity assays
LCAT cholesterol esterification activity, HL, or PLTP activities were determined as reported previously (Parks et al., 1999, Lee et al., 2005). Exogenous LCAT activity in plasma was determined by using recombinant HDL particles containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, [\textsuperscript{3}H]cholesterol, and human apoA-I were synthesized.
using a cholate dialysis method and used as substrates to measure plasma LCAT activity. Five microliters of plasma were incubated with a saturating substrate concentration of recombinant HDL cholesterol (3 µM) for 2 h at 37°C, after which conversion of radiolabeled FC to EC was determined. PLTP activity was measured with a commercially available fluorescent assay kit (Cardiovascular Target, New York, NY) according to the manufacturer’s instructions. HL activity in plasma was determined using Triton X-100-stabilized triolein as substrate. Plasma HL activity was measured by incubating 20 µl of plasma (source of enzyme), 265 µl of substrate, and 95 µl of 4 M NaCl (to inhibit lipoprotein lipase activity) for 30 min at 37°C. The reactions were stopped by adding CHCl₃/methanol/heptanes solvent mixture. K₂CO₃ was added and the phases were separated by centrifugation. The free fatty acid released was determined by [³H] radiolabel quantification in the aqueous phase.

**LCAT mRNA abundance**

LCAT mRNA gene expression was assessed in the different genotypes of mice by real-time quantitative PCR. Livers were harvested from hAI-Tg, SR-BI +/+, +/-, -/- and non-transgenic LCAT-/- mice that had been fasted for 4 hours and were between 8-12 weeks of age (except LCAT-/-). Total hepatic RNA was isolated. Quantification of mouse hepatic LCAT mRNA and mouse hepatic GAPDH mRNA was conducted using the ABIPrism® 7700 sequence detection system and SYBR®-green technology according the manufacturer’s instructions (Applied Biosystems®). Three mouse livers, from each genotype, were used for analysis. Reaction tubes, containing an identical master mix and cDNA, were assayed in triplicate for each mouse liver to obtain three C_t values. Five micromolar of primer was used in the reaction as well as 50 ng/µL of template cDNA. Calculation of the level of mRNA expression was performed by first calculating the threshold cycle (C_t) value. For each individual sample, the C_t value for the LCAT gene was subtracted from that of GAPDH. After normalization of the mouse LCAT expression to GAPDH, hA-I-Tg SR-BI +/- or hA-I-Tg SR-BI -/- LCAT C_t values were subtracted, respectively, from the hA-I-Tg SR-BI +/- value. The fold change in gene expression in the hAI-Tg, SR-BI +/-, -/- and the non-transgenic LCAT-/-, respectively, from the wild-type genotype was calculated using the method described previously (Livak & Schmittgen, 2001). A one way analysis of variance (non-parametric test) was performed using C_t values linearized by the
equation $2^{\Delta Ct}$ to determine if there was any difference in LCAT gene expression among the genotypes.

**HDL phospholipid species determination**

Lipids from plasma from $hAI^{Tg}$ SR-BI$^{+/+}$ or $hAI^{Tg}$ SR-BI$^{-/-}$ were extracted and phospholipid classes were identified by TLC and quantitated by phosphorus assay (Parks & Gebre, 1991).

**LCAT protein expression**

Plasma samples (0.2 µl) from either $hA-I^{Tg}$ SR-BI$^{+/+}$ or $hA-I^{Tg}$ SR-BI$^{-/-}$ mice, respectively, were applied to 4-16% SDS-PAGE followed by Western blot analysis with specific antimouse LCAT antibody.

**Study II:**

**Subjects and study design**

The study protocol was first designed and conducted (Nikander et al., 2004, Nikander et al., 2003). Briefly, the study protocol was approved by the Institutional Review Board and the Ethics Committee. With a written consent from the participants, 56 postmenopausal women volunteered for treatment with isolated isoflavones (soy phytoestrogens) or placebo tablets for 3 months. The study was conducted as a randomized cross-over trial, and two months washout period separated the treatments. Women were given daily 114 mg of soy-isoﬂavone tablets (comprised of 58% glycistein, 36% daidzein, and 6% genistein). Overnight fasting blood samples were obtained and serum isolated by centrifugation and stored at -70°C until assayed. For this study, 15 equol-producing (equol levels exceeding 5 times baseline after soy isoflavone consumption) and 15 non-equol producing women were selected.

**Measurement of ABCA1-dependent cholesterol efflux**

J774 mouse macrophages were cultured and ABCA1-dependent cholesterol efflux was determined as described (Yancey et al., 2004). ABCA1 activity was upregulated by addition of 0.3 mM cpt-cAMP (Sigma-Aldrich Finland Oy, Helsinki, Finland).
Macrophage cells, in triplicate wells, were incubated for 15 hours in 0.2% BSA in RPMI containing 2 μg/ml of the ACAT inhibitor in the presence or absence of cAMP. Before the cholesterol efflux experiments, cells were washed once with 1 ml of 1% BSA in MEM and once with MEM. Serum samples (1%, v/v) were incubated with triplicate sets of control and cAMP treated cells for 4 hours at 37°C. As an interassay control, a standard of 1% (v/v) pooled normolipidemic human serum was also included in each assay. The efflux process was stopped by placing the cell plates on ice. Lipids were extracted as previously described (Mikkola et al., 2003). Efflux values were corrected against the value obtained in cells incubated with RPMI and no added serum as an acceptor (non-specific efflux).

**Serum isoflavone, lipid, and apolipoprotein analysis**

Serum isoflavone (daidzein, genistein, and equol) concentrations, serum lipids, and apolipoproteins were analyzed with standard methods as reported previously (Nikander et al., 2004).

**Quantification of preβ-HDL**

Total preβ-HDL concentrations were measured using a previously validated crossed immunoelectrophoresis method (van Haperen et al., 2000).

**Study III:**

**Incubation of [3H]E₂ or [3H]-cholesterol with serum.**

Normolipemic pooled sera from male and female donors were obtained from the Finnish Red Cross (Helsinki, Finland) and frozen at -80°C in 2 ml aliquots. Radiolabeled estrogen, [2,4,6,7-3H(N)]E₂ (Perkin-Elmer Life Sciences, Boston, MA) or radiolabeled cholesterol [1,2,3-3H(N)] cholesterol (Perkin-Elmer Life Sciences, Boston, MA) was added to 10 ml of thawed pooled human serum. The mixtures were incubated for 24 hours at 37°C.

**Isolation of HDL and LDL.**

Each serum sample containing either [3H]E₂ or [3H]-cholesterol was adjusted to a density of 1.063 g/ml and centrifuged Beckman (Palo Alto, CA) Tabletop 1000 ultracentrifuge at
100,000 rpm for 3 hours at 10°C (Havel et al., 1955). After removal of the top layer containing apoB containing lipoproteins, the density of the bottom fraction was adjusted to 1.21 g ml⁻¹ and centrifuged at 100,000 rpm, in the same Tabletop 1000 ultracentrifuge for 18 hours at 10°C. Total HDL in the top layer was removed and recentrifuged (washed) under the same conditions. LDL was isolated at density 1.019 < d < 1.063 g ml⁻¹. Densities were adjusted with solid KBr.

**Purification of HDL and LDL.**

Ultracentrifugally isolated HDL or LDL from serum was further purified by size-exclusion chromatography on Sephadex G-25 (column dimensions, 2 x 20 cm or 1 x 20 cm: GE Healthcare Life Sciences, Uppsala, Sweden). The applied sample volume was 2 ml. Phosphate-buffered saline (PBS), pH 7.4, was used as elution buffer. HDL and LDL eluted in the column void volume (V₀) (Höckerstedt et al., 2004). Protein concentration was determined with the Bio-Rad protein assay kit (Bio-Rad, Hercules, California) based on the protocol by Lowry et al. (Lowry et al., 1951) using BSA as a standard. The fractions containing HDL and LDL were pooled for further analysis.

**Purification of esterified and unesterified [³H]E₂**

The radioactivity associated with the pooled HDL fractions obtained after the Sephadex G-25 step was further analyzed by LH-20 hydrophobic chromatography (Vihma et al., 2003) as follows: lipids were extracted four times with ethylacetate/diethylether (1:1, v/v) (2.5 x sample volume). The water phase was quickly frozen, followed by removal of the organic layer and its evaporation to dryness under N₂. The dry residues were dissolved in 0.3 ml hexane/chloroform (1:1, v/v). To separate [³H]E₂-FAE from the unesterified [³H]E₂, samples (in 0.6 ml hexane/chloroform, 1:1 v/v) were applied on a Sephadex LH-20 column (0.5 x 5 cm; GE Healthcare Bio-Sciences, Uppsala, Sweden) and eluted with 8 ml of the same solvent at room temperature collecting 1 ml fractions. [³H]E₂-FAE eluted first, after which the elution was continued with 7 ml of methanol to elute the unesterified [³H] E₂. All fractions were evaporated to dryness under N₂ and dissolved in 0.5 ml methanol. The radioactivity was counted in each fraction. Following incubation of [³H]E₂ in the presence of serum, the isolated and purified HDL fraction contained approximately 83% of [³H]E₂-FAE and 17% free [³H]E₂.
Cell culture

Rat Fu5AH hepatoma cells were maintained in phenol-red free MEM (Invitrogen, Scotland, U.K.) supplemented with penicillin, streptomycin, L-glutamine, and 5% fetal bovine serum. The intracellular and medium radioactivity after incubation with $[^3H]E_2$ containing HDL was analyzed as described in the previous paragraph.

Measurement of $[^3H]E_2$ or $[^3H]cholesterol$ cellular uptake

Cells were plated on 6-well plates for parallel analysis of both $[^3H]E_2$ or $[^3H]cholesterol$ at the same time point. In 6-well culture dishes, total HDL (80µg as protein), in MEM, containing either about 3000 dpm of $[^3H]E_2$ or about 89000 dpm of $[^3H]cholesterol$ was incubated with the cells for 0, 0.5, 4, or 24 hours at 37°C. At each time point, media from three wells was removed from the plate and combined in a separate tube. The cell monolayer was washed twice with phosphate buffered saline (pH 7.4). Intracellular lipids were extracted by treating the cell monolayer with isopropanol for 1 hour at room temperature and the lipid extracts from the three wells were combined in a separate tube. Media and intracellular $[^3H]$ associated radioactivity was determined by scintillation counting and the amount of either the $[^3H]E_2$ or $[^3H]cholesterol$ cellular uptake was expressed as a percentage of the total radioactivity [(intracellular $[^3H]$ dpm) / (total $[^3H]$ dpm (intracellular $[^3H]$ dpm + media $[^3H]$ dpm) x 100)]. For SR-BI mediated uptake analysis, cells were pretreated or non-treated (control) with BLT1 (ChemBridge, San Diego, CA), a chemical inhibitor of SR-BI, and then BLT1 or media (in control wells) was removed and new media containing HDL was added. In order to determine the intracellular $E_2$ fatty acyl ester and free $E_2$ distribution, cells were plated on 15-cm plates and incubated with HDL-$[^3H]E_2$ at 37°C. Media and intracellular lipid substances were collected as described above. The intracellular $E_2$- fatty acyl ester to free $E_2$ ratio was determined by LH-20 chromatography as described above.
Separation of apoE and non-apoE HDL-[^3]H]E\textsubscript{2} particles by heparin-Sepharose affinity chromatography

ApoE–containing HDL-[^3]H]E\textsubscript{2} was separated based on the protocol by Skinner (Skinner, 1992). ApoE mass was quantified using a human ApoE enzyme linked immunosorbent assay, as reported (Siggins \textit{et al.}, 2003).

\textbf{Study IV:}

\textbf{Human plasma lipoproteins}

Total HDL (1.063-1.21 \text{g} \text{ml}^{-1}) was isolated from pooled plasma from either 4 normolipidemic premenopausal women, or 4 age-matched male volunteers, respectively, by sequential ultracentrifugation using solid KBr for density adjustments (Havel \textit{et al.}, 1955). Informed oral consent was granted by the volunteers. All women were premenopausal, non-pregnant, reproductively mature individuals with no reported use of contraceptive drugs. The phase of their menstrual cycle was unknown to the investigators. LDL (1.019-1.063 \text{g} \text{ml}^{-1}) was obtained from pooled plasma, from males and females, received from the Finnish Red Cross and isolated by ultracentrifugation.

\textbf{Labeling of lipoproteins}

LDL was acetylated using acetic anhydride and radiolabeled by incubations with \([1\alpha,2\alpha(n)-[^3]H]\)-cholesterol oleate (Amersham Biosciences, Buckinghamshire, U.K.) (Vikstedt \textit{et al.}, 2007, Goldstein \textit{et al.}, 1979). \[^3\text{H}\]cholesterol oleate acetyl LDL (acLDL) preparations had specific activities ranging from 2 to 3 \times 10^4 \text{cpm per \mu g LDL protein}. For experiments using total HDL with \[^3\text{H}]\text{E}_2, 10 \text{ml of male serum was incubated for 24 hours at 37}^\circ\text{C with 2} \times 10^6 \text{cpm per ml of } [2,4,6,7-[^3]H-N]17\beta\text{-estradiol (Perkin-Elmer Life Sciences, Boston, MA) and total HDL, containing both \[^3\text{H}\]estradiol and \[^3\text{H}\]estradiol fatty acyl esters, was isolated as described above. Isolated HDL-associated \[^3\text{H}\]E}_2 contained approximately 100 \text{cpm per \mu g total HDL protein.}
**Cell culture**

THP-1 cells (American Type Culture Collection, Monassas, VA) were maintained in phenol-red free RPMI 1640 medium supplemented with 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin, 2 mM L-glutamine, and 10% (v/v) fetal bovine serum. To differentiate cells into macrophages, cells were plated onto 24-well plates at a density of 1.5 x 10⁶ cells per well and 100 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO) was added to the cell media at day 0. At day 3, the cells were washed with PBS, pH 7.4, twice and then loaded with 25 µg of acLDL (as total protein) in media containing lipoprotein deficient bovine serum (5%, v/v) (Vikstedt et al., 2007). At day 5, the cells were washed with PBS twice and incubated with the experimental samples for 16 hours. For experiments using the estrogen receptor antagonist, ICI 182,780 (AstraZeneca, Cheshire, UK) and/or the SR-BI inhibitor, BLT1 (ChemBridge, San Diego, CA), cells were pretreated for 90 minutes with 1 µM of ICI 182780 or 10 µM of BLT1 in serum-free media at 37°C and then the experimental samples were incubated with the cells without removing the inhibitors (Wakeling & Bowler, 1992, Matveev et al., 2001). For experiments using HDL-associated [³H]E₂, 360 µg of total HDL was added to differentiated, non-radiolabeled acLDL loaded, macrophages and incubated as outlined above. At the end of the incubation period, the cellular media was removed and put into a tube. Cells were washed twice with PBS and then 0.2 M NaOH was added to the cell monolayer to lyse the cells. Radioactivity was measured in the media and cellular fractions by scintillation counting. Percentage cholesterol efflux was expressed as the percentage of media [³H]cholesterol from the total amount of [³H]cholesterol (intracellular and media [³H]cholesterol). Total macrophage protein was then determined using the 0.2 M NaOH treated cell extracts (Vikstedt et al., 2007).

**Preparation of E₂ fatty acyl ester containing HDL particles**

E₂ 17β-monoo-oleate was loaded into male HDL or BSA by using a Celite 545 AW (Sigma-Aldrich, St. Louis, MO) transfer system (Meng et al., 1999, Avigan, 1959). Celite 545 (33 mg) and 3 µmol ml⁻¹ E₂ oleate was added to 1 ml of chloroform, mixed, and the chloroform was evaporated under N₂. HDL (1 mg HDL protein) or 1 mg BSA was added to the Celite dispersion, mixed, and incubated at 37°C for 24 hours. After this Celite beads were pelleted with low-speed centrifugation. The supernatant was removed and applied to a
Sephadex G-25 chromatographic column to remove unbound E$_2$ oleate. As a control, HDL, BSA, or RPMI cell media lacking E$_2$ were processed in the same way. The purity of the E$_2$ oleate was analyzed prior to the Celite incubations by TLC.

Quantification of E$_2$ in lipoprotein and serum fractions
Free (non-esterified) and esterified E$_2$ in the samples were determined by an established method. Briefly, an internal standard, [³H]E$_2$-3,17β-dioleate, was added to each sample and then lipids were extracted four times with 2.5 volumes of diethyl ether-ethyl acetate (1:1, vol/vol) and evaporated to dryness under N$_2$. Sephadex LH-20 chromatography (Pharmacia Biotech, Uppsala, Sweden) was used to isolate E$_2$ fractions as described for Study III. E$_2$ in either the ester or free fractions were quantitated by time-resolved fluoroimmunoassay. Instruments and related reagents were obtained from Wallac Oy (PerkinElmer), Turku, Finland. The results were adjusted to reflect the recovery based on the internal standard and dilution factors used.

Apolipoprotein A-I analysis
ApoA-I was quantified by enzyme linked immunosorbent assay as described (Kärkkäinen et al., 2002).
5. Results

5.1. Plasma LCAT activity, protein, and hepatic mRNA expression in human apoA-I transgenic, SR-BI knockout mice (hA-I^Tg SR-BI^-/-) mice (Study I)

The impact of SR-BI expression on plasma factors, such as LCAT, involved in RCT, were investigated using mice with varying levels of SR-BI expression in a human apoA-I transgenic background. Plasma lipid characterization and measurements of three plasma factors in hA-I^Tg mice expressing different gene levels of SR-BI were conducted (Table 1). LCAT activity in hA-I^Tg SR-BI^-/- was reduced by 75% compared to hA-I^Tg SR-BI^+/+ mice. Correspondingly, the esterified to total cholesterol percentages (EC/TC) were significantly reduced in the hA-I^Tg SR-BI^-/- mice and plasma sphingomyelin to phosphatidylcholine ratios were significantly higher in hA-I^Tg SR-BI^-/- compared to the wild type. HL and PLTP activities were not affected upon SR-BI deletion. No differences

<table>
<thead>
<tr>
<th>Genotype (hA-I^Tg)</th>
<th>Total plasma cholesterol (mg/dl)</th>
<th>Free Cholesterol (FC) (mg/dl)</th>
<th>Esterified Cholesterol (EC) (mg/dl)</th>
<th>EC/TC ratio (%)</th>
<th>SM/Phosphatidyl-choline ratio (%)</th>
<th>LCAT (nmol/ml plasma/h cholesterol ester formed)</th>
<th>HL (µmol fatty acid released/ml plasma)</th>
<th>PLTP (arbitrary units)</th>
<th>Human apoAI (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR-BI^+/+</td>
<td>193±6</td>
<td>43±12</td>
<td>146±58</td>
<td>78±4</td>
<td>0.080±0.010</td>
<td>30.5±7.9</td>
<td>12±2</td>
<td>14294±3611</td>
<td>399±172</td>
</tr>
<tr>
<td>SR-BI^+/−</td>
<td>287±62</td>
<td>72±21</td>
<td>235±50</td>
<td>78±3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SR-BI^-/-</td>
<td>823±159</td>
<td>239±106</td>
<td>573±147</td>
<td>69±11</td>
<td>0.247±0.081</td>
<td>8.2±4.5</td>
<td>15±3</td>
<td>16445±4679</td>
<td>440±178</td>
</tr>
</tbody>
</table>

Table 1: Plasma cholesterol, cholesterol to phosphatidylcholine ratios, apoA-I, LCAT, HL, PLTP characterization among hA-I^Tg SR-BI mice. Mice were 8-12 weeks of age and chow-fed. SM=Sphingomyelin. ND=not determined. Mean ± S.D. are shown and for all the parameters, 7 or greater individual mice were characterized. Values in each column with a different subscript letter are significantly different at P ≤ 0.05. Erratum in original publication-esterified cholesterol-phosphatidylcholine ratio should read sphingomyelin-phosphatidylcholine ratio.

in hepatic LCAT mRNA and plasma protein levels differences between hA-I^Tg SR-BI^+/+ and hA-I^Tg SR-BI^-/- mice were observed (Figure 6 a and b).
5.2. Soy isoflavone supplementation in postmenopausal women and macrophage cholesterol efflux (Study II)

5.2.1. Serum lipid and lipoprotein profiles before and after isoflavonoid treatment

This investigation was a branch study stemming from previous studies (Nikander et al., 2004, Nikander et al., 2003) in which a randomized, crossover trial was carried out. Postmenopausal women, average age of 54, consumed isoflavone tablets, containing 114 milligrams of isoflavones, or a placebo daily for three months with a two-month washout period. The tablets consisted of 58% glycine, 36% daidzein, and 6% genistein. The mean serum isoflavone concentrations (nmol/L) and respective standard deviations were:
genistein, baseline, 9.0 ± 16, treatment, 436.3 ± 281; daidzein, baseline, 3.7 ± 5, treatment, 1133.2 ± 757, equol, baseline, 3.1 ± 3, treatment, 53.4 ± 94) (Nikander et al., 2004). From the original study population (Nikander et al., 2004, Nikander et al., 2003) only 15 equol producing and 15 equol non-producing subjects were chosen for the current study and changes in serum lipid and lipoprotein parameters between isoflavonoid and placebo groups, from baseline and three-months, were reported in all 30 women. Upon isoflavonoid treatment, preβ-HDL values, as the percentage of total apoA-I, demonstrated a significant increase from baseline values with no significant changes in the placebo group (for treatment group: baseline, 12.7% and three months, 15.0%). No other parameters measured in either group, had significant changes.

5.2.2. ABCA1 dependent macrophage cholesterol efflux potential of serum from equol producing and non-equol producing postmenopausal women

J774 mouse macrophages were [3H]cholesterol labeled and incubated with serum from 30 postmenopausal women who consumed isoflavone tablets or placebo at baseline and after three-months. Macrophages were treated with or without cAMP to determine specific ABCA1-dependent cholesterol efflux. Cellular ABCA1 protein expression is upregulated upon cAMP treatment and ABCA1-dependent cholesterol efflux can be addressed using this technique (Yancey et al., 2004, Bortnick et al., 2000). No significant differences were detected among the groups (Figure 7).

To determine whether differences in ABCA1 dependent macrophage cholesterol efflux potential exists between equol producing and non-equol producing postmenopausal women, postmenopausal women’s sera from isoflavone or placebo were tested. Furthermore, a non-significant reduction in this efflux was observed after three-months of isoflavone treatment in the non-equol producing group.
5.3. **HDL-associated E\(_2\) fatty acyl ester uptake by hepatoma cells (Study III)**

5.3.1. **Comparison of the kinetics between HDL-associated E\(_2\) fatty acyl esters and HDL-cholesterol uptake by hepatoma cells**

Fu5AH rat hepatoma cells were used because these cells express high levels of SR-BI (Zimetti et al., 2006), a candidate lipoprotein receptor for cellular HDL-associated E\(_2\) fatty acyl uptake. To assess HDL-associated E\(_2\) ester uptake in Fu5AH cells, total HDL was prepared containing either \(^{3}\text{H}\)E\(_2\)-FAE or \(^{3}\text{H}\)cholesterol (majority of which was cholesterol esters (Francone et al., 1990)). Incubation of HDL-containing \(^{3}\text{H}\)E\(_2\) esters with Fu5AH cells for 0.5, 1, 4, and 24 hours and the \(^{3}\text{H}\)E\(_2\) in the cellular fraction was determined (Figure 8 a). A rapid exponential increase existed for the internalization of E\(_2\) with a plateau reached by 4 hours. To compare HDL-associated E\(_2\) fatty acyl ester internalization with HDL-associated cholesterol uptake, HDL-associated \(^{3}\text{H}\)cholesterol was incubated with Fu5AH cells exactly as HDL-associated \(^{3}\text{H}\)E\(_2\) was. The early incubation kinetics are depicted in Figure 8 b. The curve for HDL-associated
$[^3]H$cholesterol was linear in contrast to the HDL-associated $[^3]H$E$_2$ curve and did not plateau by 4 hours.

5.3.2. SR-BI and LDL receptor dependent uptake of HDL-associated E$_2$ fatty acyl ester by hepatoma cells

Chemical inhibition of cellular SR-BI function was accomplished by using BLT1 (Nieland et al., 2002). This inhibitor reduced cellular HDL-associated E$_2$ ester uptake (Figure 9 a). 80% of the $[^3]H$E$_2$ label was localized in apoE-rich HDL (data not shown). The LDL receptor was also studied since apoE binds to it (Windler et al., 1980). Competition for binding to the LDL receptor was conducted by addition of increasing amounts of unlabeled LDL to the cell culture which contained HDL enriched with E$_2$ fatty acyl esters (Figure 9 b). To test the contribution of SR-BI and LDL receptors, specifically, BLT1 was added to the LDL receptor competition incubation and the amount of internalized $[^3]H$E$_2$ was determined (Figure 10 b). Significant reductions in the cellular uptake of HDL-associated $[^3]H$E$_2$ fatty acyl esters were found only in incubations containing both BLT1 and LDL (open circles). LDL alone, although non-significantly, reduced the overall uptake of HDL-associated $[^3]H$E$_2$-FAE approximately 20 to 40 percent.
In Fu5AH cells the maximum amount of HDL-derived $[^3]H$E$_2$-FAE were hydrolyzed to unesterified or free E$_2$ after the 24 hour incubation (Figure 10). The initial HDL preparation containing $[^3]H$E$_2$-FAE is shown also (Figure 10, preincubation control HDL).
5.4. Macrophage cholesterol efflux potential to HDL-particles containing E₂ fatty acyl esters and their internalization (Study IV)

5.4.1. SR-BI and estrogen receptor play a role in HDL-associated E₂ fatty acyl ester mediated macrophage cholesterol efflux potential

In order to specifically address the potential importance of the HDL and associated E₂ fatty acyl esters for stimulation of macrophage cholesterol efflux, E₂ mono-oleate was loaded into male HDL or bovine serum albumin (BSA) by a Celite 545 transfer system (Meng et al., 1999) and then HDL-associated E₂ oleate was quantified by a combination of LH-20 chromatography and time resolved fluoroimmunoassay methods. HDL-associated E₂ oleate preparations had 20.4 pmol E₂ mg HDL protein⁻¹ present in the fatty acyl ester form. Human THP-1 macrophages were loaded with [³H]cholesterol oleate labeled, acetylated LDL and thereafter HDL- or BSA-associated E₂ oleate was incubated with the cells and [³H]cholesterol efflux percentage was determined (Figure 11 a). HDL particles with E₂ oleate (male HDL + E₂-oleate) displayed the greatest cholesterol efflux compared to non-E₂ oleate containing male HDL (Figure 11 a). Cholesterol efflux in the presence of to BSA-associated E₂ oleate was significantly higher compared to BSA (Figure 11 a). ICI 182780, an ER antagonist, has been used in previous investigations assessing ER effects in cells and on specific HDL-associated E₂ mediated mechanisms (Gong et al., 2003, Booth et al., 2003, Lamon-Fava & Micherone, 2004, Wakeling & Bowler, 1992). Pretreatment of cells with either BLT1 or BLT1 together with ICI showed a significant reduction in HDL-associated E₂ oleate mediated macrophage cholesterol efflux potential (Figure 11 b). No effect on macrophage cholesterol efflux potential was present in the male HDL lacking E₂ oleate (Figure 11 b).
Macrophage SR-BI and estrogen receptor dependent uptake of HDL-associated E₂ fatty acyl esters

Male serum was incubated with [³H]E₂ for 24 hours at 37°C and isolated total HDL contained 91% [³H]E₂-FAE and 9% unesterified [³H]E₂. HDL-associated [³H]E₂-FAE was incubated with non-radiolabeled acLDL loaded THP-1 macrophages for 16 hours and cellular lipids were extracted and [³H]E₂ ester and free E₂ distribution was determined by LH-20 chromatography (Figure 12). These cells were also pretreated with BLT1, ICI, or both in the presence of HDL-[³H]E₂-FAE and the intracellular [³H]E₂ distribution was measured. Cells internalized HDL-associated [³H]E₂ ester and 71% of the total [³H]E₂ radioactivity was in the ester fraction and approximately 30% were unesterified. Upon inhibitor treatments, the intracellular total [³H]E₂ radioactivity was reduced between 50% (ICI treatment) and 75% (BLT1 or BLT1 and ICI treatments) compared to incubations in the absence of these inhibitors. The distribution of [³H]E₂-FAE to free [³H]E₂ did not differ between the incubations with or without these inhibitors.

5.4.2. Macrophage SR-BI and estrogen receptor dependent uptake of HDL-associated E₂ fatty acyl esters

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5.5. Gender specific macrophage cholesterol efflux potential to HDL (Study IV)

In order to determine whether premenopausal female serum or isolated female HDL can mediate greater macrophage cholesterol efflux potential compared to male serum or HDL, THP-1 macrophages, loaded with acLDL and were incubated in the presence of serum or HDL for 16 hours and cholesterol efflux to media were determined (Figure 13 a). Female serum and HDL demonstrated significantly greater cholesterol efflux potential compared to those of males. BLT1, ICI, or BLT1 and ICI pretreatments were performed on macrophages and then incubations with female and male HDL were conducted. In all treatment combinations, female HDL mediated macrophage cholesterol efflux was reduced significantly whereas no changes were observed in the efflux to male HDL.
Figure 13: Macrophage cholesterol efflux potential to premenopausal female and male serum. a.) THP-1 human macrophages were loaded with acLDL and incubated with 0.5% serum, total HDL, or apoAI for 16 hours and [3H]cholesterol efflux percentage was determined as described in Materials and Methods. b.) Macrophages were pretreated with BLT1, ICI, or both and premenopausal female or male HDL was incubated with the cells. No acceptor = no HDL in media. Data is representative of three independent experiments and mean ± S.E. are shown. * P ≤ 0.05. Figure reprinted with permission.
6. Discussion

Cholesterol efflux potential and HDL-associated E\textsubscript{2} fatty acyl esters and the contribution of ABCA1 and SR-BI to these processes, respectively, were specifically addressed in this thesis. Additionally, LCAT mass and activity were evaluated in mice with a targeted SR-BI gene disruption in order to get insight to their possible co-regulation. The major outcomes listed in a connected order were first, that upon SR-BI deletion, LCAT becomes inactivated in plasma, but does not undergo catabolism. Second, SR-BI and LDL receptors contributed significantly to hepatic uptake of HDL-associated E\textsubscript{2} esters and these cells hydrolyzed the internalized E\textsubscript{2} ester into its free, unesterified form. Third, HDL-associated E\textsubscript{2} esters triggered enhanced cholesterol efflux from cholesterol loaded macrophage foam cells compared to non-E\textsubscript{2} containing HDL, and SR-BI and estrogen receptors were involved in this process. In addition, intracellular hydrolysis of HDL-associated E\textsubscript{2} fatty acyl esters into free E\textsubscript{2} occurred. These results and a summary of their physiological relevance in HDL-associated E\textsubscript{2} ester metabolism and RCT are depicted in Figure 14. Fourth, serum and HDL from premenopausal women showed enhanced macrophage cholesterol efflux potential compared to males and SR-BI and ERs were essential for this effect. Fifth, dietary soy isoflavone supplementation did not enhance macrophage cholesterol efflux potential compared to placebo in equol or equol non-producing postmenopausal women. This treatment did evoke a significant increase in preβ-HDL levels. The intention of this thesis was to get a better understanding about the metabolism and possible atheroprotective nature of HDL-associated E\textsubscript{2} fatty acyl esters and phytoestrogens.

Relationship among SR-BI, LCAT, and HDL-associated E\textsubscript{2}-FAE

Since defects in RCT and HDL metabolism could lead to the progression of atherosclerosis (Hovingh et al., 2005, Hoeg et al., 1996, Oram & Vaughan, 2006, Van Eck et al., 2005, Rigotti et al., 1997, Huby et al., 2006), the consequences of SR-BI gene deletion in this mechanism were first investigated (Study I). Previous studies in mouse models have shown that SR-BI gene deletions result in marked increases in plasma cholesterol concentrations, the generation of an abnormally large HDL fraction (HDL1),
and enhanced atherosclerosis (Huby et al., 2006, Rigotti et al., 1997). In Study I, human apoA-I transgenic mice that expressed different gene levels of SR-BI (hA-I<sup>Tg</sup> SR-BI) were used. This study was separate from related studies because this is the first to use human apoA-I transgenic mouse which has a heterogeneous HDL profile compared to wild type mice with a homogeneous profile (Rubin et al., 1991, Chajek-Shaul et al., 1991). Plasma lipids were characterized and plasma LCAT function was assessed. In hA-I<sup>Tg</sup> SR-BI<sup>−/−</sup> mice, plasma cholesterol content was markedly elevated, HDL1 comprised the majority of the HDL subfractions (data not shown), plasma LCAT activity was reduced by 75%, and the esterified to total cholesterol ratio, an indicator of LCAT function, was significantly lower compared to hA-I<sup>Tg</sup> SR-BI<sup>+/+</sup> mice (Table 1). Since LCAT mass is correlated to activity (Stokke & Norum, 1971, Albers et al., 1981), this observation suggested that LCAT was hypercatabolized from plasma. Measurements of LCAT mRNA abundance, in liver, and in plasma showed that this theory was incorrect. Gene and protein levels did not differ between the hA-I<sup>Tg</sup> SR-BI<sup>−/−</sup> and hA-I<sup>Tg</sup> SR-BI<sup>+/+</sup> (Figure 6 a and b). Another factor that dictates LCAT activity is HDL phospholipid surface composition (Bolin & Jonas, 1994, Bolin & Jonas, 1996). Phosphatidylcholine is the best substrate for LCAT for its esterification reaction while sphingomyelin is the poorest. The HDL1 subclass resembles LDL both in size and phospholipid composition (Lusk et al., 1979, Mahley, 1982) and LDL is a poor substrate for the LCAT reaction because of the presence of sphingomyelin (Kosek et al., 1999). HDL phospholipid distribution was analyzed and it was found that hA-I<sup>Tg</sup> SR-BI<sup>−/−</sup> mice had a 3-fold greater ratio of sphingomyelin to phosphatidylcholine compared to HDL from hA-I<sup>Tg</sup> SR-BI<sup>+/+</sup> mice (Table 1). This suggests that the phospholipid surface, therefore, of the HDL1 generated in hA-I<sup>Tg</sup> SR-BI<sup>−</sup>, is a poor substrate for LCAT and this provides a mechanistic explanation for why LCAT may remain inactive in plasma of hA-I<sup>Tg</sup> SR-BI<sup>−/−</sup> mice. Study I provides novel evidence that SR-BI is critical for optimal LCAT function and it may have implications on E<sub>2</sub> esterification in HDL. LCAT must be active to esterify E<sub>2</sub> in plasma (Höckerstedt et al., 2002) and modifications in SR-BI expression could influence the activity of this enzyme, and thereby the degree of cholesterol and E<sub>2</sub> esterification.

Hepatic SR-BI participates in the selective uptake of HDL-associated cholesterol esters (Rigotti et al., 2003) and the present data here demonstrates that hepatic cells also internalize HDL-associated E<sub>2</sub> esters (Study III). This study provides novel evidence for
the hepatic uptake of HDL-associated E2 esters by hepatocytes and also of the specific SR-BI and LDL receptor related mechanisms involved. SR-BI inhibition by BLT-1 reduced HDL-associated E2 ester uptake by more than 15% at all timepoints measured, with the greatest effect (32%) at 4 hours. Further, the combined blocking of the binding sites for the LDL receptor with excess amounts of LDL and chemical inhibition of SR-BI caused an even greater reduction in HDL-associated E2 ester uptake at all timepoints than SR-BI inhibition alone or LDL receptor blocking alone. This suggests that the LDL receptor and SR-BI are major contributors to hepatic HDL-associated E2 ester uptake. Mechanistically, hepatic SR-BI and the LDL receptor can bind and internalize apoE containing lipoprotein particles (Windler et al., 1980, Arai et al., 1999). ApoE can associate with HDL as well as LDL (Mackie et al., 1981). In a study by Arai et al., SR-BI uptake of total HDL was less efficient in apoE deficient mice which infers that apoE in HDL is a positive regulator of SR-BI-HDL uptake (Arai et al., 1999). In study III, E2 distribution analysis of the HDL showed that more than 80% of the total E2 radiolabel, existing as esters, associated with apoE containing HDL. ApoE might direct these HDL containing E2 fatty acyl ester particles to SR-BI and the LDL receptor in hepatic cells. This also suggests, although unproven, that LCAT-mediated E2 esterification may have selectivity for apoE containing HDL particles. This latter idea is supported by the fact that LCAT can esterify cholesterol in apoE containing synthetic HDL particles and apoE then functions as a co-factor for LCAT (Marcel et al., 1980, Matsuura et al., 2006). These findings are unique because they hint at an apoE, LDL receptor, and SR-BI-related mechanism for HDL-associated E2 ester hepatic cellular uptake.

SR-BI interacts with HDL particles containing E2 (Brodeur et al., 2008, Gong et al., 2003), but studies addressing specific HDL-associated E2 ester-SR-BI interactions are lacking. Since in vivo and in vitro investigations show that E2 associates with HDL mostly in its esterified state (Vihma et al., 2003, Helisten et al., 2001), study III provides a more physiologic representation of the relationship between SR-BI and HDL-associated E2 esters. A major finding in study III was the hepatic HDL-associated E2 ester delivery and subsequent intracellular hydrolysis. This hydrolysis yielded almost 50% intracellular unesterified E2 by 24 hours of HDL-associated E2 ester and hepatic cell incubations. This has potentially significant biological implications because the liver is a major site for apoA-I biosynthesis and E2 can modulate its gene expression (Lamon-Fava & Micherone,
This raises the possibility that internalized HDL-associated E₂ esters could affect circulating HDL levels, which would support a probable gender specific mechanism in the difference of HDL levels that exist between premenopausal women and men.

**Direct effects of HDL-associated E₂-FAE on RCT-related mechanisms**

Lipid-laden macrophage foam cells are thought to be targets for atherosclerosis prevention because reductions in the intracellular cholesterol ester content could decrease the detrimental factors that characterize this disease (Glass & Witztum, 2001, Li & Glass, 2002, Vainio & Ikonen, 2003). There are previous studies showing that HDL itself stimulates macrophage cholesterol efflux (Barter & Rye, 2007) and others show that E₂ treatment protects against atherosclerosis in animal models and triggers cholesterol efflux from cholesterol ester containing acLDL loaded macrophages (St. Clair, 1997, Napolitano et al., 2001). Based on these facts and the observations in study III demonstrating that HDL containing E₂ fatty acyl esters interact with cellular lipoprotein receptors, the research interest was shifted to investigating similar interactions with macrophage foam cells: the subject for study IV. This study addressed the cholesterol efflux potential from cholesterol ester containing acLDL loaded human macrophages to HDL containing E₂ fatty acyl esters and also probed the possible differences in this between premenopausal female and male HDL. Creation of HDL containing E₂ fatty acyl esters by loading of human male HDL, which naturally has low levels of E₂ (Gong et al., 2003), with E₂ oleate, was achieved with the intention to specifically demonstrate potential importance of the E₂ ester in HDL in mediating macrophage cholesterol efflux. In macrophages, male HDL-associated E₂ oleate stimulated almost 25% greater cholesterol efflux than male HDL lacking this. This finding led to the systematic exploration of cellular HDL receptors that may participate in this mechanism. SR-BI was considered as an ideal candidate based on previous studies addressing its role in HDL-associated E₂ mediated effects in endothelial cells and in uptake of HDL-associated E₂ in bone cells (Gong et al., 2003, Brodeur et al., 2008). Gong et al. showed that SR-BI inhibition with specific blocking antibodies eliminated HDL-associated E₂ interactions with endothelial cells (Gong et al., 2003). Consideration of ERs in cholesterol efflux mechanism was also based on Gong et al.’s investigation (Gong et al.,
In that study, HDL from female mice which had 0.015 pg E_2 mg^{-1} HDL protein or male mice with no detectable E_2 before E_2 loading in HDL and 0.020 pg E_2 mg^{-1} after HDL- E_2 loading, respectively were used. When endothelial cells were treated with an ER antagonist, ICI 182780, eNOS activity, in vitro, stimulated by either female HDL or male HDL-associated E_2 was drastically reduced (Gong et al., 2003). This evidence indirectly suggests that cellular ERs may be needed for SR-BI mediated HDL-associated E_2 cellular effects. Based on this study (Gong et al., 2003), inhibition of SR-BI, antagonism of ERs, or these combined in macrophage cells was performed to test the contribution of each in macrophage cholesterol efflux capacity to HDL-associated E_2 ester particles (study IV). SR-BI or ER inhibition alone or combined significantly reduced this capacity to similar levels as male HDL. These data link, mechanistically, SR-BI and ERs to HDL-associated E_2 ester mediated macrophage cholesterol efflux with a particular emphasis on the E_2 ester component.

**HDL acts as a major transporter of E_2 to cellular receptors**

The overall significance of HDL as a transporter of E_2 esters to macrophage cells was determined (study IV). BSA was loaded with E_2 oleate, in a similar manner as HDL, and incubated with acLDL loaded macrophages and cholesterol efflux was assessed. BSA-associated E_2 ester did not achieve as great cholesterol efflux as HDL-associated E_2 ester or male HDL without E_2 ester. Interestingly, however, BSA-associated E_2 ester did show greater macrophage cholesterol efflux potential compared to BSA lacking the ester. This suggests that first, E_2 ester uses an active, receptor specific cellular mechanism, and second, that HDL assists majorily in this mechanism. This is in line with Gong et al.’s investigation showing that HDL, but not BSA, is critical for E_2 effects on eNOS (Gong et al., 2003).

**ER’s contribute to HDL-derived E_2 cellular uptake**

In study III, cellular uptake of HDL-associated E_2 ester and SR-BI’s role in this was characterized. In addition, since ERs contribute to macrophage cholesterol efflux potential to HDL-associated E_2 ester particles, these were tested for involvement in HDL-associated
E₂ ester uptake. Macrophage cells internalized and hydrolyzed HDL-associated E₂ esters, after 16 hours, changing ratio of 91 to 9 E₂ ester:unesterified E₂ particle, prior to incubation, to approximately 70 to 30, respectively. In the absence or presence of inhibitors, this ratio was valid. The greatest reduction in total E₂ uptake was with BLT1 treatment or a combined BLT1 and ICI treatment. ICI treatment reduced the cellular uptake, but not as markedly as with BLT1. This is intriguing because although SR-BI contributes majorly to this uptake, the question arises: with a functional SR-BI, why does ER inhibition reduce the uptake of HDL-associated E₂ esters? A possible explanation could be that macrophage ERs, on the plasma membrane, may interact with SR-BI forming a functional signaling complex, similar to endothelial cells, to coordinate this uptake and if this partnership is broken, then the uptake is less efficient (Mineo & Shaul, 2006, Matveev et al., 1999). Further studies are warranted to confirm this interesting, novel mechanism. Nevertheless, in study IV, macrophage cells internalized HDL-associated E₂ esters by SR-BI and also hydrolyzed these esters into unesterified E₂. As speculated upon in study III, the biologically active, unesterified E₂ and not the inert E₂ ester (Zielinski et al., 1991, Katz et al., 1991, Banerjee et al., 1991), could have significant downstream effects on intracellular machinery, such as mobilizing macrophage cholesterol ester depositions for extracellular release (Tomita et al., 1996) or increasing cholesterol efflux transporters, e.g. ABCA1 (Srivastava, 2002)

**Human gender differences exist in foam cell cholesterol efflux to HDL**

Interest in gender specific differences in cardiovascular health exists (Jousilahti et al., 1999, Mendelsohn & Karas, 2005, Kauma et al., 1996). Establishing the basis for these has gained momentum and has potential for future therapies (Mendelsohn & Karas, 2005). Accordingly, whether the differences exist in RCT mechanisms remains poorly understood and demands clarification. To unravel this enigma, a study by Catalano et al. demonstrated that a clear gender difference exists between premenopausal women and age-matched men between HDL cholesterol and mass (defined as the total chemical mass of lipids and proteins) and in the cholesterol efflux potential mediated by specific HDL receptors (Catalano et al., 2008). Premenopausal women had significantly elevated HDL cholesterol and mass values, significantly increased apoA-I percentages, and significantly
Figure 14: Schematic depicting the interactions between HDL-associated E₂ esters and RCT plasma and cellular factors. In the plasma compartment, LCAT esterified free E₂ into E₂ esters along with cholesterol partitions it into the HDL, forming HDL-associated E₂ fatty acyl ester particles. Cholesterol efflux from macrophage cells, located in the arterial intima layer, and LCAT work to generate larger HDL particles (HDL2) and then CETP can transfer a portion of these E₂ esters to LDL. The remaining HDL-associated E₂ fatty acyl ester particle can be either destined for the liver or remain in circulation to facilitate macrophage cholesterol efflux. SR-BI, both at hepatic and macrophage locations, can internalize HDL-associated E₂ fatty acyl esters and these cells can hydrolyze the E₂ ester into its unesterified state. This may trigger internal cellular machinery that could upregulate genes important in the HDL biosynthetic pathway or cholesterol efflux. LDL receptors and perhaps LRP may facilitate apoE-enriched HDL-associated E₂ fatty acyl ester uptake and ERs in macrophage cells could also participate in cellular uptake. Illustration by RM Badeau 2009.
higher HDL-mediated hepatic SR-BI cholesterol efflux potential compared to men (Catalano et al., 2008). To extend this further, a major aim of this thesis was to assess specific macrophage cholesterol efflux potential to human premenopausal female or human male serum or isolated HDL from the respective serum (study IV). Female serum and HDL had significantly elevated levels of efflux compared to the male counterparts. Efflux differences existed between HDL$_2$ but not between HDL$_3$ particles (unpublished data). In addition, SR-BI and ER inhibition had a negative effect only on female HDL stimulated cholesterol efflux. Upon receptor inhibition, macrophage cholesterol efflux to female HDL was approximately equal to values obtained to male HDL. These observations are in line with the data obtained using male HDL-associated E$_2$ oleate particles as efflux acceptors. Taken together, evidence from study IV supports the E$_2$ ester component of HDL, to be potent atheroprotective factor, found exclusively in women.

A SR-BI-related mechanism was a consistent theme in three parts of this thesis (study I, III, IV). Since disruptions in this receptor caused marked reductions in HDL-associated E$_2$ fatty acyl ester uptake and internalization in both hepatic and macrophage cells and indirectly, a non-functional LCAT enzyme—which is required for E$_2$ esterification in HDL, it can be stated that this receptor is a critical determinant in any HDL-associated E$_2$ fatty acyl ester effect in RCT. Based on this thesis, it is most probable that SR-BI is an important route of estrogen delivery to cells and HDL can function as an E$_2$ transporter and a principle mediator of all this. Involvement of internalized HDL-associated E$_2$ esters and the relation to SR-BI in lipid efflux needs to be clarified. Studies that have assessed the contribution of SR-BI and ABCA1 in cholesterol efflux have shown that ABCA1 is the principle cholesterol efflux protein and not SR-BI (Wang et al., 2007). Study IV needs to be continued to determine if ABCA1, ABCG1, or SR-BI is responsible for the enhanced macrophage cholesterol efflux demonstrated in this study.

SR-BI is expressed in humans (Velasco et al., 2006, West et al., 2009, Jiang et al., 2008, Lopez & McLean, 2006). In women, there is evidence that the genotype of this gene and cellular expression could be critical determinants of HDL-cholesterol levels (Richard et al., 2005, West et al., 2009, Lopez & McLean, 2006). In addition, E$_2$ is a strong modulator of SR-BI cellular expression (Lopez et al., 2002, Velasco et al., 2006, Lopez & McLean, 2006). Taken together, HDL-associated E$_2$ fatty acyl ester hepatic and macrophage delivery via SR-BI could potentially increase the efficiency and potency of
RCT because if SR-BI can internalize E\textsubscript{2}, as shown in this thesis, then further upregulation of SR-BI could be achieved by the HDL-derived E\textsubscript{2} fatty acyl esters. This would increase the selective HDL-cholesterol ester removal, at hepatic cellular sites, and also promote the increase in macrophage internalization of E\textsubscript{2}, by SR-BI, and potentially allow E\textsubscript{2} to affect other cellular cholesterol efflux genes, such as ABCA1 (Srivastava, 2002). This needs to be tested, but the foundation for this mechanistical approach is in place by results uncovered in this thesis. If true, this could give solid evidence for the protection of premenopausal women against atherosclerosis that is afforded by E\textsubscript{2}.

**Phytoestrogen’s and their effects on RCT in postmenopausal women**

The RCT mechanisms affected by phytoestrogens are unclear. Isoflavone supplementation in postmenopausal women had no effect on SR-BI-dependent cholesterol efflux capacity to serum (Törmälä et al., 2006) compared to placebo. In addition, separation of this study group into equol and equol non-producers did not show any difference in cholesterol efflux to the respective serum acceptor. In parallel, Study II, a branch study from Nikander et al. (Nikander et al., 2004), addressed macrophage ABCA1-specific cholesterol efflux to serum, from these same postmenopausal women, and also between equol and equol non-producers, undergoing isoflavone treatment or placebo. No significant differences were detected in ABCA1-mediated cholesterol efflux in these groups. Isoflavone-rich serum from equol-producing postmenopausal women demonstrated a modest increase in ABCA1-dependent cholesterol efflux compared to equol non-producers upon isoflavone treatment, but the overall impact of this remains to be determined in future studies.

Plasma preβ-HDL levels were significantly elevated in postmenopausal women treated with isoflavones compared to placebo. This is a novel and interesting finding because this may relate to genetic analysis studies assessing genistein’s effect on apoA-I synthesis, the major building block of HDL, which demonstrated that genistein, at concentrations between 1 to 10 µmol\textsuperscript{-1} increases apoA-I biosynthesis rates in hepatic cells (Lamon-Fava & Micherone, 2004, Lamon-Fava, 2000). In Study II, the group of postmenopausal women had a mean plasma genistein concentration of about 440 nmol\textsuperscript{-1} which confounds the interpretation of this finding because it is below the effective
concentration range of genistein for apoA-I secretion found by Lamon-Fava et al. (Lamon-Fava, 2000). Since the intestine is a relatively important site of apoA-I biosynthesis, it would be interesting to test the effect of genistein on apoA-I synthesis rates there.

Consideration of the confounding factors in the clinical trial methodology employed is needed before a solid conclusion about the relationship between isoflavones and RCT can be made from Study II and related studies (Törmälä et al., 2006, Nikander et al., 2004). First, the isoflavone supplement contained 68% glycitein, 35% daidzein, and 6% genistein and the postmenopausal women received 114 mg of isoflavone daily. Genistein is the most potent isoflavone for ERβ (Kuiper et al., 1998) and also is able to promote equivalent amounts of liver apoA-I secretion into media to E2 and compared to daidzein, E2 and genistein caused 62% greater apoAI secretion (Lamon-Fava, 2000). In related studies (Nikander et al., 2004, Törmälä et al., 2006) and study II, this would mean that only 6.84 mg out of 114 mg daily total of isoflavone, consumed by the postmenopausal women, was genistein. Since consumption of 50 mg per day of genistein could yield between 1 to 4 µmol l\(^{-1}\) in plasma (Bhathena & Velasquez, 2002), this could influence apoAI biosynthesis and HDL levels. Therefore, a supplement containing over 50% genistein could, in principle, be more effective for effects on plasma lipids and RCT parameters.

Equol’s, a metabolite of daidzein, specific effects on macrophage ABCA1 cholesterol efflux to serum were not pronounced. The impact this isoflavone has on lipid metabolism is unclear. Neither Nikander et al., Törmälä et al., nor study II profiled the serum lipids or lipoproteins in equol and equol non-producers (Törmälä et al., 2006, Nikander et al., 2004). More in vitro and clinical investigations into equol-specific effects on RCT are warranted.

Presently, there exists a substantial gap in knowledge regarding the biochemical and lipid metabolic processes exerted by isoflavones and the interpretive data emanating from many clinical studies investigating CVD and soy- or isoflavones-mediated effects is conflicting, therefore, the nature of the role of isoflavones in atherosclerosis prevention cannot be stated beyond a doubt. Continued interest and more diligent clinical studies are essential to better understand the relationship between isoflavones, RCT, and atherosclerosis.
Conclusion

Relationships between cellular lipoprotein receptors and RCT interactions with HDL-associated E₂ fatty acyl esters and isoflavones were investigated in this thesis. SR-BI is a major receptor for HDL-associated E₂ ester uptake in hepatic and macrophage cells and if this receptor is disturbed, LCAT-mediated esterification of cholesterol, and indirectly, E₂, is dysfunctional. Hepatic LDL receptors also internalize HDL-associated E₂ fatty acyl esters. Cholesterol ester loaded macrophages have increased cholesterol efflux potential via stimulation by HDL-associated E₂ esters. HDL derived from premenopausal women compared to HDL lacking E₂ or male HDL, facilitates improved cholesterol removal from macrophages, respectively. Macrophage ERs also contribute to the internalization and cholesterol efflux responses of HDL-associated E₂ esters. Soy isoflavone treatment to postmenopausal women did not enhance ABCA1 mediated cholesterol efflux to serum compared to placebo. Preβ-HDL levels were significantly elevated upon isoflavone treatment, which may be indicative of modulation of RCT processes. These data present novel evidence for a protective function of E₂ and to a lesser extent, phytoestrogens, in atherosclerosis prevention afforded to women.
7. Summary and conclusions

The novel results of Studies I to IV can be summarized as follows:

I. LCAT esterification function of cholesterol is significantly impaired in SR-BI deficient mice. The altered HDL phospholipid compositions between the $hA-I^Tg$ SR-BI$^{-/-}$ and the $hA-I^Tg$ SR-BI$^{+/+}$ mice may be a strong mediator of this effect. Hepatic mRNA production or plasma clearance of LCAT mass was not observed between these strains of mice. This crosstalk between LCAT and SR-BI is physiologically important in terms of HDL containing E$_2$.

II. Preβ-HDL levels, in plasma, are significantly elevated after three-months of phytoestrogen treatment in postmenopausal women compared to baseline and placebo. This treatment does not affect macrophage ABCA1-specific cholesterol efflux potential to plasma and subclassification of these subjects based on their ability to produce equol further did not show any differences in this efflux capacity.

III. Hepatic SR-BI and LDL-receptors were critical for the cellular uptake of HDL-associated E$_2$ fatty acyl esters. Intracellular hydrolysis of E$_2$ fatty acyl esters into unesterified E$_2$ occurred over time.

IV. HDL containing E$_2$ fatty acyl esters and premenopausal female HDL particles stimulated maximum macrophage cholesterol efflux potential compared to HDL lacking E$_2$ esters or male HDL, respectively. SR-BI and ERs contributed significantly to this process and also to the cellular internalization of HDL-associated E$_2$ esters.

In conclusion, HDL-associated E$_2$ fatty acyl esters are key regulators of RCT-related mechanisms. This effect is principally mediated through HDL transport to specific cellular HDL receptors and the following uptake of E$_2$ esters at these sites. SR-BI is a major facilitator for these processes and disruption of SR-BI has consequences on LCAT esterification abilities, has implications on the initial stages of HDL and E$_2$ interactions, and also on HDL-associated E$_2$ uptake by macrophage and hepatic cells. Estrogenic compounds, such as soy phytoestrogens, did not have direct effects on RCT but did induce HDL precursor preβ-HDL levels and thereby HDL biosynthetic pathways. Taken together, these findings suggest direct relevance to cardiovascular protection mechanisms in women.
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9. References


