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LOW DENSITY LIPOPROTEIN PARTICLES AS CARRIERS FOR BIOACTIVE HORMONAL SUBSTANCES

Qinghe Meng

Helsinki 1999

Department of Medicine
Division of Cardiology
University of Helsinki, Finland

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ACADEMIC DISSERTATION

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. Meng QH, Pajukanta P, Ilmonen M, Schuster H, Schewe CK, Andersson LC, Tikkanen MJ. Analysis of novel apolipoprotein B mutations using a modified U937 cell line LDL binding assay. Clin Chim Acta 1996; 256:27-36.

II. Meng QH, Wähälä K, Adlercreutz H, Tikkanen MJ. Antiproliferative efficacy of lipophilic soy isoflavone phytoestrogens delivered by low density lipoprotein particles into cultured U937 cells. Life Sci 1999 (in press).

III. Meng QH, Lewis P, Wähälä K, Adlercreutz H, Tikkanen MJ. Incorporation of esterified soybean isoflavones with antioxidant activity into low density lipoprotein. Biochim Biophys Acta 1999; 1438(3):369-376.

IV. Meng QH, Höckerstedt A, Heinonen S, Wähälä K, Adlercreutz H, Tikkanen MJ. Antioxidant protection of lipoproteins containing estrogens: in vitro evidence for low- and high-density lipoproteins as estrogen carriers. Biochim Biophys Acta 1999; 1439(3):331-340.

ABBREVIATIONS

Apo	apolipoprotein
CAD	coronary artery disease
CD	conjugated dienes
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
CM	chylomicron
Da	dalton
DNA	deoxyribonucleic acid
DTNB	5,5´-dithio-*bis*(2-nitrobenzoic acid)
E_2	17β-estradiol
EC	endothelial cell
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FC	free (unesterified) cholesterol
FDB	familiar defective apolipoprotein B-100
GC-MS	gas chromatography-mass spectrometry
HDL	high density lipoprotein
HL	hepatic lipase
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HRT	hormone replacement therapy
IDL	intermediate density lipoprotein
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein
LPL	lipoprotein lipase
NO	nitric oxide
Ox-LDL	oxidized low density lipoprotein
PBS	phosphate buffered saline
PL	phospholipids
PUFA	polyunsaturated fatty acids
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S_f	Svedberg flotation unit
SMC	smooth muscle cell
SR	scavenger receptor
TBARS	thiobarbituric acid-reactive substances
TG	triglyceride
TRAP	total peroxyl radical trapping potential
VLDL	very low density lipoprotein

1. INTRODUCTION

Atherosclerosis-associated cardiovascular disease is the leading cause of death in Western societies. An elevated level of plasma low density lipoprotein (LDL) cholesterol is an independent risk factor for atherosclerosis and its clinical sequel, coronary heart disease (CHD). Epidemiological and clinical studies have shown that cholesterol-lowering therapy can reverse progression of atherosclerosis and reduce CHD events (Lipid Research Clinics Program. JAMA 1984, Blankenhorn et al. 1993, Levine et al. 1995, Scandinavian Simvastatin Survival Study (4S) Group, 1994). However, at any given plasma concentration of LDL there is great diversity in the extent of atherosclerosis and in the expression of clinical disease. On the other hand, continuing to reduce LDL-cholesterol did not abolish atherosclerosis (Steinberg 1997a), suggesting that not only the quantity but also the quality of LDL may be associated with atherosclerosis. A great deal of evidence has indicated that oxidatively modified LDL plays a critical role in the initiation and progression of atherosclerosis (Regnström et al. 1992, Ross et al. 1993, Steinberg et al. 1989, Witztum et al. 1991, Ylä-Herttuala 1998, Holvoet and Collen 1998, Heinecke 1998). A decrease of oxidative stress and antioxidant protection of LDL might therefore be promising for the prevention of atherosclerosis. Studies have demonstrated that dietary intake of antioxidants such as α-tocopherol (vitamin E) and flavonoids can reduce the risk of CHD (Stephens et al. 1996, Hertog et al. 1993). However, more beneficial evidence is needed before recommending specific amounts of antioxidant consumption.

LDL is the major carrier of plasma cholesterol. It consists of an apolar core of neutral lipids surrounded by a polar shell. In theory, other lipophilic substances could also be incorporated into the LDL in addition to lipoprotein lipids. The lipophilic antioxidants contained in LDL may increase oxidation resistance of these particles (Esterbauer et al. 1995). For example, the increase of α-tocopherol content of LDL correlates with the increase of LDL oxidation resistance (Esterbauer et al. 1997). Due to the binding property of LDL to the LDL receptors, some bioactive substances contained in LDL particles might be delivered into cells via the LDL receptor pathway influencing cellular functions, for example cell proliferation (Krieger et al. 1978, Lundberg et al. 1987, Vitols et al. 1990).

Several lines of studies have shown that human endogenous estrogens and diet-derived soybean isoflavone phytoestrogens play an important role in the prevention of atherosclerosis and CHD (Stampfer et al. 1991, Bush et al. 1987, Adlercreutz and Mazur 1997, St Clair 1998, Tham et al. 1998). In addition to the cholesterol-lowering effect, other mechanisms such as antioxidation of lipoproteins and antiproliferation of cells involved in the formation of atherosclerosis have received great interest. Esterified forms of 17β-estradiol (E_2) become associated with human lipoproteins in vitro (Tang et al. 1997, Larner et al. 1987, Shwaery et al. 1997, Leszczynski and Schafer 1990) and with rat lipoproteins in vivo (Larner et al 1987), reducing their susceptibility to oxidation (Shwaery et al. 1997, 1998). Furthermore, recent studies have demonstrated that LDL isolated from estrogen-treated postmenopausal women is associated with increased ex vivo Cu^{2+}-mediated oxidation resistance (Wakatsuki et al. 1998, Sack et al. 1994, Wilcox et al. 1997). Tikkanen et al. (1998) have also demonstrated that soy phytoestrogen intake could increase LDL oxidation resistance ex vivo. However, the underlying mechanisms of these findings have not been fully understood. In order to clarify these, we set up a model system by which LDL can act as a transport vehicle to carry some bioactive substances and investigated the following: (1) Assuming these substances could be incorporated into LDL, could these substances be delivered by LDL into U937 cells via the LDL receptor pathway and influence cell

proliferation? (2) Whether and to what extent lipophilic biosubstances such as estrogens and phytoestrogens could be incorporated into LDL and protect it against oxidation?

2. REVIEW OF THE LITERATURE

2.1. Plasma lipids and lipoproteins

Lipids play very important roles in maintaining the structure of cell membrane (cholesterol, phospholipids), cell growth (cholesterol), steroid hormone synthesis (cholesterol), and energy metabolism (triglycerides). Since lipids are highly hydrophobic, they have to be packed into lipoproteins as water-soluble particles in blood circulation (Gotto et al. 1986). A lipoprotein is a particle consisting of a core of hydrophobic lipids, i.e., triglycerides (TG), cholesteryl esters (CE), surrounded by a polar layer of phospholipids (PL), unesterified cholesterol (FC), and apolipoprotein(s) (Ginsberg 1990). Plasma lipoproteins are usually classified into five major subfractions based on their density (d), particle size, flotation rate (Sf), and electrophoretic mobility in agarose gel. Routinely, the lipoproteins are separated by sequential ultracentrifugation (Havel et al. 1955).

Table 1. Properties and apolipoprotein composition of the major human plasma lipoproteins

Classes*	Density range (g/ml)	Particle size‡ (diameter, nm)	Flotation rate (Sf)	Apolipoproteins
CM	<0.95	75-1200 (0)	> 400	B-48, A-I, A-II, C†, E
VLDL	0.95-1.006	30-80 (preβ)	20-400	B-100, C†, E
IDL	1.006-1.019	25-35 (β)	12-20	B-100, C†, E
LDL	1.019-1.063	18-25 (β)	0-12	B-100
HDL	1.063-1.210	5-12 (α)		A-I, A-II, C†, E

*CM, chylomicrons; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; HDL, high density lipoproteins.
C† There are three apoC subclasses: apoC-I, apoC-II, apoC-III.
‡ Electrophoretic mobilities in agrose are indicated in parentheses.
Source: Data from Zubay et al (1995), Schultz and Liebman (1997).

CMs are derived from dietary lipids (exogenous pathway) and assembled in the intestinal epithelial cells. TGs are the major constituents of the CM particles. The TGs in CM are hydrolyzed in the peripheral tissues by lipoprotein lipase (LPL) to form the CM remnants which are taken up by the liver in a process that probably involves apolipoprotein E (apoE) on the surface of the remnants and a hepatic receptor called LDL receptor-related protein (LRP) (Beisiegel 1995). VLDL particles are synthesized in the liver (endogenous pathway). They are the main liver-derived TG-rich lipoproteins and in circulation, their TGs are hydrolyzed by LPL and the VLDLs are then degraded into CE-enriched particles called IDL (Gotto et al. 1986). About half of the IDL particles are taken up by the liver via LDL receptor and remnant receptor (van Berkel et al. 1995), whereas the other half are converted into LDL by hepatic lipase (HL) (Taskinen and Kuusi 1987). LDLs are the major carriers of cholesterol in plasma. LDL metabolism is discussed in more detail in Section 2.2.2. Lipoprotein (a) [Lp(a)], which consists of an LDL particle covalently attached to apolipoprotein (a) [apo(a)], is a distinct class of CE-rich plasma lipoprotein. It can bind weakly to LDL receptors and play a role in the genesis of atherosclerosis (Jauhiainen et al. 1991).

HDLs consist of apoAI and apoAII as the main apolipoprotein constituents and carry about 20% cholesterol, most of which are CE (Ginsberg 1990). HDLs are synthesized in the liver and intestine (Franceschini et al. 1991). Also HDLs can be generated following the lipolysis of TG-rich lipoproteins whereafter plasma phospholipid transfer protein (PLTP) facilitates the transfer of phospholipids and some cholesterol into HDL pool (Eisenberg 1984, Jiang et al. 1999). In addition, the lecithin-cholesterol acyltransferase (LCAT) has a crucial role in the maturation of HDL particles. LCAT can catalyze the formation of CE which are then incorporated into the core of discoidal nascent HDL (Franceschini et al. 1991). HDLs (especially preβ-mobile HDL) play a major role in the transport of cholesterol from peripheral tissues to the liver, a process known as reverse cholesterol transport (Tall 1990). HDL CE are transfered by cholesteryl ester transfer protein (CETP) to apoB-containing particles which are finally removed from the circulation by the liver (Tall 1993). In addition, HDLs can be taken up by class B scavenger receptor (SR-BI)-mediated process in certain cells where this receptor mediates selective CE uptake leaving the HDL particles largely intact (Acton et al. 1996, Krieger 1998), or directly removed by the liver (Tall 1992).

2.2. Low density lipoproteins

2.2.1. LDL and atherogenesis

LDLs transport about 75% of the total cholesterol in blood circulation. Evidence exists that LDL cholesterol is a critical atherogenic factor (Grundy 1995,1997, Frishman 1998). A large number of epidemiologic studies have demonstrated a strong positive correlation between elevated LDL cholesterol levels and the development of coronary artery disease (CAD) (Kannell et al 1979, Krauss 1987, Genest and Cohn 1995, Frishman 1998). Genetic studies have also documented that inheritable hypercholesterolemias (familial hypercholesterolemia, familial defective apoB-100), mainly with elevated levels of LDL cholesterol, are the primary cause of premature CAD (Goldstein et al. 1973, Tybjaerg-Hansen et al. 1992). In addition, apoB and LDL particles have been identified in atherosclerotic plaques (Hoff et al. 1979a, Hoff et al. 1979b) and in vitro studies have shown that elevated LDL levels damage endothelial cell (EC) layer and penetrate into the arterial intima. The accumulation of LDL in the arterial wall initiates monocyte and smooth muscle cell migration and transforms macrophages and smooth muscle cells into cholesterol-loaded foam cells, which are the major cell components found in the plaque (Goldstein et al 1979, Brown and Goldstein 1983). Furthermore, pathological studies have demonstrated that the lowering of LDL-cholesterol is associated with reduced severity of atherosclerotic lesion and improvement of cardiac functional parameters (Zambon and Hokanson 1998). For example, reduction in cholesterol levels may reduce the susceptibility of LDL to oxidation which is a causal factor for the initiation and progression of atherosclerosis. Protection of LDL from oxidation could increase nitric oxide bioavailability and improve endothelium-dependent vasomotor, anti-inflammatory, and anticoagulant properties of the endothelium (Guetta and Cannon 1996). Finally, clinical studies have shown that lowering of LDL cholesterol has been associated with the reduction of CAD morbidity and mortality (Gotto 1995). The Scandinavian Simvastatin Survival Study (4S) showed that the lipid-lowering agent simvastatin significantly reduced the risk of coronary death and major coronary events in 4444 patients with coronary disease over the median follow-up period of 5.4 years (Scandinavian Simvastatin Survival Study Group 1994). These effects were presumed to be due to the beneficial reduction of serum lipids and lipoproteins, in which LDL cholesterol was reduced by 35%. The best evidence supporting lipid-lowering therapy for primary prevention comes from the West of Scotland Study (Shepherd et al. 1995). In this study, treatment with pravastatin

resulted in significant reduction in nonfatal myocardial infarctions and death due to CAD. Taken together, these studies strongly support the importance of LDL in atherogenesis.

2.2.2. LDL and receptor mediated metabolism

LDL is the most abundant cholesterol-carrying lipoprotein in plasma. CE, located in the hydrophobic core of LDL, is the main form of cholesterol carried in LDL. CE is supposed to be too hydrophobic to pass through cell membranes. The question is how can esterified cholesterol be delivered into cells for their use? The delivery problem is solved by the LDL receptors. The LDL receptors bind LDL and CE packed into LDL particles is delivered into the cell by receptor-mediated endocytosis. The receptor-mediated removal of LDL cholesterol occurs mostly via classical LDL receptors that have been observed in all mammalian cells tested except erythrocytes (Brown and Goldstein 1986) (**Fig. 1**). The liver plays a crucial role in receptor mediated uptake of LDL: about 75% of the LDL particles removed from the circulation are mediated by the liver. Of these, 75% of the clearance is LDL receptor-mediated, the remainder is by a nonspecific, receptor-independent low affinity process (Pittman et al. 1982, Billheimer et al. 1984). Also SR-BI mediates LDL binding but only CE is selectively delivered to the cell especially in non-placental steroidogenic tissues (van Berkel et al. 1995).

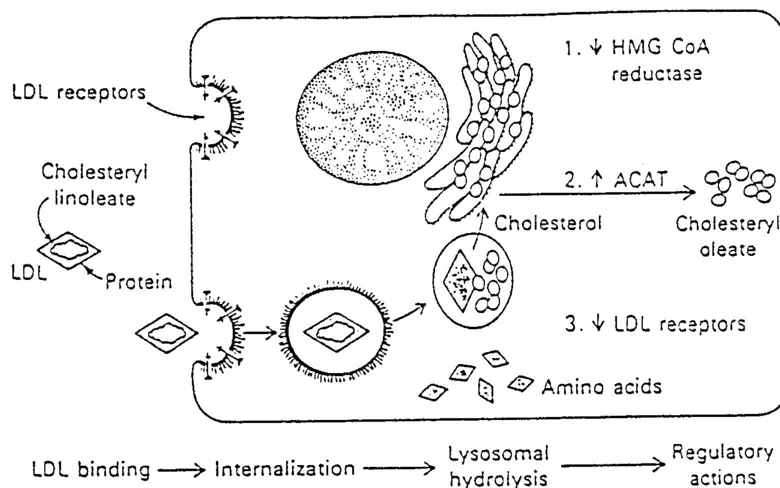

Figure 1. Steps in the LDL pathway in cultured human fibroblasts. HMG CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase; ACAT, acyl CoA:cholesterol acyltransferase (Brown and Goldstein, 1986).

The LDL receptor is a cell surface glycoprotein with a molecular weight of 164 kDa, with a coding gene on chromosome 19 (Francke et al. 1984). It is present on both hepatic and extrahepatic cells. The high binding interaction between LDL apoB and the LDL receptor is responsible for the receptor-mediated uptake and clearance of LDL from the circulation. The ApoE on apoE-containing lipoproteins (VLDL, IDL) is also capable of interacting with the LDL receptors and regulating the metabolism of these lipoproteins (Mahley 1990). Following the binding of LDL to its receptors, the lipoprotein is internalized and delivered into lysosomes where its CE is hydrolyzed. The liberated cholesterol is then used by the cell for the synthesis of plasma membranes, bile acids, and steroid hormones, or stored in the ester form. The increased intracellular cholesterol level will, in return, down regulate the LDL receptor activity, i.e., receptor synthesis. Subsequently, the number of the LDL receptors synthesized decreases when

the cellular cholesterol content increases, and vice versa (Brown and Goldstein 1986). Therefore, cellular cholesterol content is the major LDL receptor regulator. ApoB-100, one of the largest monomeric proteins known, is the major protein component of LDL and acts as a ligand for the LDL receptor.

ApoB-100 is a large (513 kDa), single chain glycoprotein composed of 4536 amino acid residues with a coding gene residing on the short arm of chromosome 2 (Knott et al. 1986, Yang et al. 1986). There is only one apoB-100 molecule in each LDL particle (Tikkanen and Schonfeld 1985, Cladaras et al 1986). ApoB-100 also is not transfered between lipoprotein particles during the metabolic conversion of VLDL into LDL. It is presumed that the apoB-100 binding site resides in the carboxyterminal portion of the molecule. However, the region assumed to be involved in LDL binding is not yet clear. So far three apoB mutations, called familial defective apolipoprotein B-100 (FDB) (Arg3500→Gln, Arg3500→Trp, Arg3531→Cys) have been reported to be related to hypercholesterolemia (Soria et al. 1989, Gaffney et al. 1995, Pullinger et al. 1995). However, none of these mutations have been found in Finland (Hämäläinen et al. 1990).

2.2.3. LDL particle structure

In blood circulation, TG and CE are packed into LDL particles forming a hydrophobic core surrounded by a surface monolayer of polar PL together with unesterified cholesterol (FC) and apoB. LDL normally also contains lipophilic antioxidants, mainly Vitamin E and β-carotene. LDL is a large spherical particle, molecular weight of about 3 x 10^6 Da, with a diameter of 22-28 nm and density between 1.019-1.063 g/ml. The core is composed of some 1,600 molecules of CE (long chain fatty acid) and 170 molecules of TG. The CE is the main lipid of the lipoprotein core with the most fatty acyl chain in these esters being linoleate (Krieger et al. 1978). This core is shielded by a layer of PL (700 molecules), FC (600 molecules), and 1 molecule of apoB-100 (Steinberg 1997b, Stryer 1988, Yang et al. 1989). In the percent mass composition, each LDL particle consists of 35-45% CE, 7-10% FC, 7-10% TG, 15-20% PL, and 20-25% protein (Deckelbaum et al. 1987, Schultz and Liebman 1997).

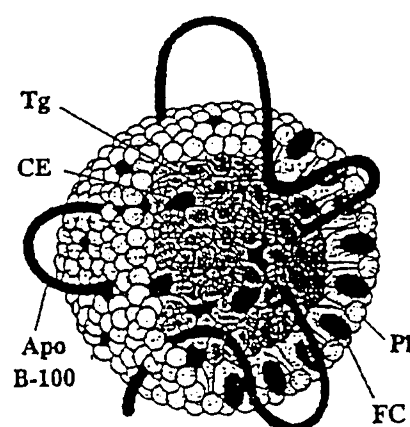

Figure 2. Schematic model of LDL particle.

Overall structure of the LDL particle is shown in **Fig. 2**. The PLs are arrayed so that their hydrophilic heads are on the outside, allowing the LDL to be dissolved in the blood or intercellular fluid. Embedded in this hydrophilic coat is one apoB-100 molecule. Effective and

efficient binding for apoB-100 to the LDL receptor is the prerequisite for cholesterol delivery into cells. In principle, LDL can also transport other lipophilic biosubstances into cells under the direction of apoB-100. On the other hand, apoB-100 gene mutation or conformational changes may affect LDL metabolism and plasma cholesterol levels (Innerarity et al. 1987, Aviram et al. 1988, Chen et al. 1994).

2.2.4. Oxidative modification of LDL

There is much evidence indicating that oxidized LDL (Ox-LDL) is present in atherosclerotic lesions in vivo (Ylä-Herttuala 1998). First of all, LDL isolated from atherosclerotic lesions is in part oxidatively modified (Ylä-Herttula et al. 1989). Second, immunological techniques have demonstrated that atherosclerotic lesions contain materials reactive with antibodies generated against Ox-LDL (Haberland et al. 1988, Palinski et al. 1989, Rosenfeld et al. 1990). Third, serum contains autoantibodies against Ox-LDL (Palinski et al. 1989, Salonen et al. 1992). Fourth, treatment with antioxidants can prevent the development or slow the progression of atherosclerosis (Carew et al. 1987, Kita et al. 1987, Steinberg 1997a).

In principle, any modified LDL in plasma could be rapidly removed by hepatic sinusoidal cells (Kupffer cells), which contain abundant scavenger receptors. Moreover, a variety of antioxidants remain in plasma. Therefore, it was presumed that Ox-LDL mainly occurred locally in the arterial wall after entrance of normal LDL, whereby it was sequestered from antioxidants in plasma (Steinberg et al. 1989, Witztum and Steinberg 1991). However, recent studies have suggested that very small amounts of Ox-LDL are also present in plasma (see review from Nielsen 1999). These changes could have occurred elsewhere, or during a previous transient passage through the artery wall. Such minimally modified LDL might then be "primed" for more rapid oxidative modification on a subsequent entry into the intima. Therefore, Ox-LDL in the arterial wall can be derived both from normal LDL oxidized locally in the arterial intima and from Ox-LDL in plasma (Nielsen 1999).

Lipid peroxidation presumably starts in the polyunsaturated fatty acids (PUFA) forming an ester bond with LDL-surface PLs, and then propagates to core lipids, resulting in oxidative modification not only of the PUFA, but also of the cholesterol moiety (mostly CE) and modification and degradation of apoB (Witztum 1994). Therefore, a wide variety of biologically active molecules can be formed, including oxidized sterols, oxidized fatty acids, and PL and protein derivatives generated by adduct formation with breakdown products of oxidized fatty acids. For example, malondialdehyde and 4-hydroxynonenal can subsequently react with lysine residues in apoB. Such adducts, and others, presumably create the epitopes on apoB that lead to recognition by scavenger receptors on macrophages.

In culture, all the vascular cells can initiate oxidation of LDL, but the relative contributions of ECs, monocytes and macrophages, or smooth muscle cells (SMC) to such modification in vivo are unknown (Heinecke 1998, Ylä-Herttuala 1998). In vitro, LDL can bind to copper which can promote rapid lipid peroxidation. However, it is not known whether sufficient free copper and iron, or complexes of these metals, exist in vivo to promote LDL peroxidation, although intact ceruloplasmin can act as a prooxidant. Therefore, several mechanisms are probably involved, and even the same cell type may use different pathways. For example, release of superoxide anion from ECs or SMCs might be responsible for initiation of oxidation in some settings, and thiols in others (Heinecke et al. 1986). In macrophages, enhanced 15-lipoxygenase activity could

generate increased cellular lipid hydroperoxides, which could be transferred to extracellular LDL, providing the "seed" that would lead to enhanced lipid peroxidation (Heinecke 1998).

The antioxidant defences that prevent oxidation of LDL need to be defined. The antioxidant content of the LDL particle is critical for its protection (Esterbauer et al. 1992) and, theoretically, if sufficient lipophilic antioxidants were present, the LDL particles would be protected from even profound oxidant challenge. In vivo, whether or not LDL becomes oxidized is a question of the balance between the extent of the prooxidant challenge and the capacity of the antioxidant defenses.

Although Ox-LDL is found in man, there are no conclusive intervention studies in man to support a quantitatively important role for this process. The ongoing antioxidant trials will no doubt add more beneficial evidence in the role of atherosclerosis prevention.

2.2.5. Determination of lipoprotein oxidation

LDL oxidation is a lipid peroxidation chain reaction driven by free radical intermediates, which is accompanied by characteristic changes of chemical, physiochemical, and biological properties. Therefore, a variety of methods may be used for determining the rate and extent of oxidation in vitro (Puhl et al. 1994) and in vivo (Jialal and Devaraj 1996). For in vivo LDL oxidation determination, the immunological method for autoantibody to Ox-LDL has been widely used (Jialal and Devaraj 1996), while a recent developed baseline diene conjugation measurement is a promising method for estimating LDL oxidation in vivo (Ahotupa et al. 1998). The in vitro estimation of LDL oxidation includes measurement of the increase of thiobarbituric acid-reactive substances (TBARS), total lipid hydroperoxides, defined lipid hydroperoxides, hydroxy- and hydroperoxy fatty acids, conjugated dienes (CD), oxysterols, lysophospholipids, aldehydes, fluorescent chromophores, measurement of disappearance of endogenous antioxidants and PUFA, oxygen uptake, and total peroxyl radical trapping potential (TRAP) (Puhl et al. 1994, Valkonen and Kuusi 1997). A convenient and very frequently used method for continuously monitoring the process of copper-induced LDL oxidation is to measure the conjugated diene formation at 234 nm as a time course in a UV spectrophotometer (Esterbauer et al. 1989).

2.2.6. Metabolism of oxidized LDL

Ox-LDL particles are taken up by so-called scavenger receptors (**Fig. 3**). In the liver, Kupffer cells are the main site for mediating the in vivo uptake of Ox-LDL from the circulation and might thus protect against circulating Ox-LDL (van Berkel et al. 1995). Increased LDL levels in plasma lead to an increase entry of LDL into the intima through the injured endothelium, resulting in accumulation of LDL in the intima. LDL in the artery wall can be oxidatively modified by all the major cells of the arterial wall, i.e. ECs, SMCs, monocytes/macrophages, and in cell-free system by transition metals, lipoxygenase, myeloperoxidase, and nitric oxide (NO). Ox-LDL is taken up by a family of scavenger-receptors (SR) on the surfaces of cells such as macrophages, platelets, and ECs. However, which part of the Ox-LDL particle is being recognized by scavenger receptors is not fully understood (Greaves et al. 1998). Certainly, both the lipids and the protein are oxidized under the oxidative condition. It was shown that the modified apolipoproteins extracted from Ox-LDL particles were efficiently internalized and degraded by macrophage scavenger receptors (Parthasarathy et al. 1987), while the oxidized lipids extracted from Ox-LDL were also recognized by scavengers (Terpsta et al. 1998). During

the oxidation of LDL, the PUFA are broken down to smaller fragments and become conjugated with the -amino groups of lysine residues. Therefore, Steinberg (1997b) proposed that the recognition of Ox-LDL by scavenger receptors appears to be due to the masking of lysine -amino groups and subsequent changes in protein charge and configuration. The SRs mediate the endocytosis of the Ox-LDL, where the process is not down regulated. Increasing uptake of Ox-LDL via scavenger receptors certainly promotes cholesterol ester accumulation and conversion to lipid-droplet filled foam cell formation which is the hallmark of fatty streaks and atherosclerotic plaques. The SR activity on the macrophages exhibits a remarkable broad binding specificity (Goldstein et al. 1979, Brown and Goldstein 1983); they not only recognize Ox-LDL but also other chemically modified proteins such as acetyl LDL (Ac-LDL), methylated LDL, suggesting they are multiligand receptors. So far, there are more than six classes of SRs (10 types) that have been shown to be responsible for endocytosis of Ox-LDL (Krieger 1997).

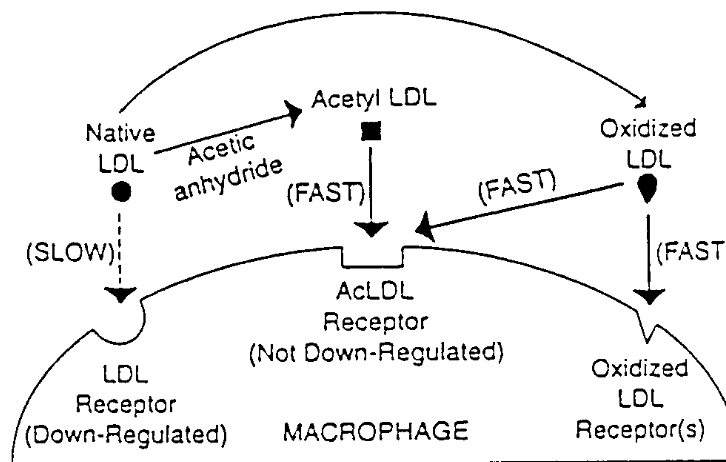

Figure 3. Scheme depicting the uptake of native and Ox-LDL by macrophage receptors (Steinberg 1997a).

2.2.7. Atherogeneity of oxidized LDL

In the early stages, uptake of Ox-LDL by macrophage may be protective. When too much Ox-LDL accumulates in macrophage, irreversible damage occurs, resulting in foam cell formation, cell death, and release of many modified molecules with diverse effects (Witztum et al. 1991, Witztum 1994). As a result of accumulation of lipid deposits in foam cells, the arterial wall evolves from the initial fatty streak to form the lipid-rich atheromatous core and an overlying dense fibrocellular layer, comprising primarily of SMCss.

Ox-LDL is the prerequisite for macrophage uptake and cellular cholesterol accumulation (Steinberg et al. 1989). Therefore, it has potential atherogenic properties in the initiation and development of the atherosclerotic lesion (Ross 1993, Steinberg 1997b). In addition to the foam cell formation, Ox-LDL can also play other roles. It promotes atherosclerosis by recruitment and retention of monocytes and T cells in the intima, migration of SMC from media into intima where these cells will become foam cell-like after having taken up Ox-LDL, by its cytotoxicity toward EC and by stimulating monocyte adhesion to the endothelium. In addition, the Ox-LDL or its products may induce cellular expression of potent chemotactic factors, such as monocyte chemotactic protein 1, and secretion of colony-stimulating factors, such as macrophage colony-stimulating factor, which can stimulate SMC proliferation and differentiation of monocytes into macrophages. Ox-LDL is also immunogenic with antibodies to epitopes of Ox-LDL found in

plasma and in lesions associated with immune complexes. In addition, CD4+ T cells have been isolated from human atherosclerotic plaques and up to 10% of these clones proliferate and release cytokines on exposure to Ox-LDL. This response is dependent on autologous antigen-presenting cells and restricted by HLA-DR. Thus there is both a humoral and a cell-mediated immune response, typical of an inflammatory lesion (Witztum 1994). Ox-LDL enhances platelet adhesion and aggregation, which may stimulate macrophage foam cell formation and SMC proliferation.

Ox-LDL may alter other vital properties of the arterial wall with clinical or fatal consequences such as impairing vasodilation (Holvoet and Collen 1998). Ox-LDL can induce vascular cell apoptosis which is involved in atherosclerosis formation (Nishio et al. 1996, Li et al. 1998). Ox-LDL, or its products, can profoundly impair the nitric-oxide-mediated vasorelaxation of coronary arteries in response to agents such as acetylcholine. Hypercholesterolaemia, by generation of more Ox-LDL in the intima or by creation of a prooxidant environment that stimulates EC to release more superoxide anion, may contribute importantly to vasospasm even in the absence of significant lesions. Certain products of Ox-LDL, such as oxysterols, are highly toxic to EC and could initiate breaks in endothelial integrity. Other products may stimulate tissue-factor release and initiate coagulation and thrombosis. Because the shoulder regions of even established lesions are rich in macrophage-filled foam cells containing Ox-LDL (it is at these sites that plaque rupture and thrombotic events occur), Ox-LDL probably participates in this late stage as well. Thus, Ox-LDL may contribute to atherogenesis and CHD by mechanisms other than macrophage foam-cell formation alone. The pathological process of Ox-LDL initiated atherosclerotic lesion formation is shown in **Fig. 4**.

Accordingly, the intervention and prevention of Ox-LDL should be the primary goal of therapy.

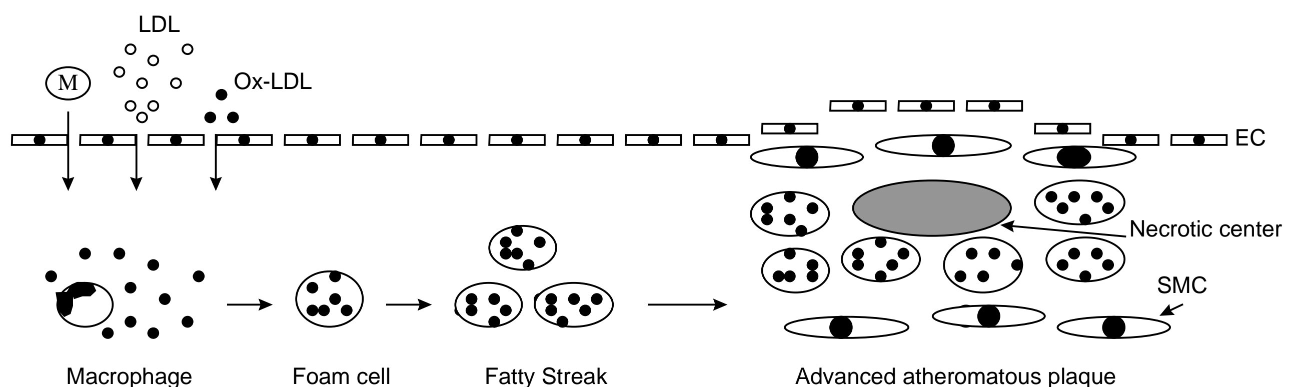

Figure 4. Pathway for Ox-LDL initiated atherosclerotic lesion formation
EC: Endothelial cells, M: monocytes, SMC: smooth muscle cells

2.2.8. LDL composition, oxidizability, and endogenous antioxidants

In LDL particle, cholesteryl linoleate represents quantitatively the single most important PUFA which is the substrate for peroxidation. If PUFA becomes oxidized to lipid hydroperoxides, their isolated carbon-carbon double bonds are converted to conjugated double bonds showing a strong UV-absorption at 234 nm, designated as conjugated diene (CD) formation (Esterbauer et al 1993). Therefore, its content in LDL may influence the determination of oxidation resistance measured by CD or TBARS formation (Ziouzenkova et al. 1996). Reaven et al. (1993) reported that LDL particles rich in PUFA are more readily oxidized than LDL particles enriched in

saturated fatty acids or monounsaturated fatty acids. In addition, elevated levels of performed lipid hydroperoxides and cholesterol in LDL were associated with increased oxidation susceptibility (Frei and Gaziano 1993). Studies have shown that monoenic fatty acids enriched LDL, for example oleic acid was remarkably resistant to oxidative modification as measured by decreased formation of CD and TBARS (Parthasarathy et al. 1990). The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase inhibitors reduced the susceptibility of LDL to oxidation by altering the LDL particle composition containing less lipid relative to protein (Lavy et al. 1991) or preserve endogenous antioxidants (Chen et al. 1997). In deed, most of these HMG-CoA reductase inhibitors themselves are antioxidants and could become bound to lipoprotein in the circulation to protect them against oxidation (Lennernas and Fager 1997, Girona et al. 1999). LDL particle size could also influence its oxidation susceptibility. For example, small, dense LDL particles display diminished resistance to oxidative stress in vitro (Tribble et al. 1992, de Graaf et al. 1991). Since LDL is the major extracellular transport vehicle for lipid-soluble antioxidants, it contains relatively large amounts of α-tocopherol, γ-tocopherol, β-carotene, and ubiquinol-10 and among them α-tocopherol is the most important antioxidant known with 6 molecules per LDL particle (Esterbauer et al. 1995). As the oxidation goes on, α-tocopherol is the first and β-carotene is the last endogenous antioxidant in LDL particles to be depleted (Esterbauer et al. 1992).

The endogenous antioxidants contained in LDL particles might influence oxidation resistance (Tribble et al. 1994). Dietary antioxidant supplementation could increase their content in LDL and therefore, increase oxidation resistance of lipoproteins (Nyyssonen et al. 1994, Jialal et al. 1995). The composition of native LDL and the antioxidants contained in LDL are list in **Tables 2,3**.

Table 2. Composition of human LDL

Components	nmol/mg LDL protein (mean)	mol/mol LDL (mean)
Protein		
ApoB-100	NA	1
Lipids		
PL	1300	700
TG	304	170
FC	1130	600
CE	2960	1600
Total cholesterol	4090	2200
Free fatty acids	48	26
Total fatty acids	4756	2616
Total PUFAS	2330	1280

Data derived from Esterbauer et al. (1995). NA, not applicable

Table 3. Antioxidants in native human LDL

Antioxidant	nmol/mg LDL protein (mean)	mol/mol LDL (mean)
α-tocopherol	11.58	6.37
γ-tocopherol	0.93	0.51
β-carotene	0.53	0.29
α-carotene	0.22	0.12
Lycopene	0.29	0.16
Cryptoxanthine	0.25	0.14
Cantaxanthine	0.04	0.02
Lutein + zeaxanthine	0.07	0.04
Ubiquinol-10	0.18	0.10

Data were adopted from Esterbauer et al. (1995)

2.2.9. Antioxidants in the pathogenesis of atherosclerosis

As discussed in section 2.2.7, Ox-LDL is involved in several steps of atherosclerosis. It is believed that antioxidants can interfere to different extents with these steps (Faggiotto et al. 1998). Kleinveld et al. (1993) reported that 18 weeks of pravastatin or simvastatin administered to 23 hypercholesterolemic patients (15 men, 8 women) decreased LDL cholesterol levels by 36% and significantly reduced the rate and extent of copper-catalyzed LDL oxidation. LDL particles after therapy were changed in composition to contain less lipid relative to protein, possibly rendering the particle less susceptible to oxidation (Lavy et al. 1991). HMG-CoA reductase inhibitors rather than reducing LDL levels and changing its particle composition, may also be of antioxidant importance. In this regard, Giroux et al. (1993) reported that simvastatin diminished superoxide anion formation and LDL oxidation by human macrophages in tissue culture. Fruebis et al. (1994) have found that atherosclerosis in WHHL rabbits is inhibited by probucol (a potent antioxidant) but not by its analogue with similar structure. Thus, for any given prooxidant stress, there may be a threshold of protection that must be achieved. Protection of LDL from oxidation could increase nitric oxide bioavailability and improve endothelium-dependent vasomotor, anti-inflammatory, and anticoagulant properties of the endothelium (Guetta and Cannon 1996). Recently, Yasunari et al. (1999) observed that antioxidants probucol and vitamin E prevent smooth muscle cell migration and proliferation via reducing intracellular oxidative stress. Since endogenous antioxidants (superoxide dismutase, H_2O_2-removing enzymes, and metal binding proteins) may become inadequate to prevent LDL oxidation, exogenous antioxidants (diet-derived or supplemented) would seem, therefore, important to maintain such effect in vivo. The direct provision of lipophilic antioxidants into the LDL particle should be the most effective strategy. The most abundant natural antioxidant in LDL is α -tocopherol (Esterbauer et al. 1992), and supplementation of the diet with vitamin E can increase the vitamin E content of LDL and lead to enhanced protection of such LDL from in vitro oxidation. The vitamin E content in LDL particles is positively correlated with oxidation resistance (Tesoriere et al. 1998). However, in man, supplementation at about 1.2 g per day saturates the LDL, and at this degree of enrichment (about a 2 1/2-fold increase) the lag time for CD formation, a sensitive index of susceptibility to lipid peroxidation, is only prolonged by 50% (Reaven and Witztum 1996). Tikkanen et al. (1998) have observed that soybean phytoestrogen

intake prolonged the lag time by 20 min. However, we do not know if this degree of lag time prolongation is sufficient to protect LDL under all conditions and its correlation to the prevention of CAD. For example, in hypercholesterolaemia, the residence of LDL in the artery is prolonged. If the pro-oxidant stress is continuous, more potent antioxidant activity would be required. Beta-carotene is the next most common antioxidant in LDL (Esterbauer et al. 1992) and theoretically should provide enhanced antioxidant protection. However, data have not supported this effect even when the β-carotene content was increased more than 20-fold by dietary enrichment (Heinecke 1998, Anderson et al. 1998). By contrast, the administration of probucol to volunteers, such that the probucol content of the LDL is 2-4 μg/mg of LDL protein, can lead to near-total protection against oxidative stress for as long as 16 hours. Vitamin C, a water-soluble antioxidant, also provides significant protection for LDL in vitro, presumably by maintaining or regenerating vitamin E in the LDL particle in its reduced antioxidant state.

Another strategy to protect LDL against oxidation is to reduce its content of PUFA by dietary substitution with oleic acid (Reaven et al. 1993). The diet-enriched flavonoids and isoflavonoids, may have great nutritional benefits against atherosclerosis as they appear to constitute a major source of dietary antioxidants (Hertog et al. 1993).

A reduction in prooxidant activity can also be achieved by enhancing the antioxidant content of cells, for example, by enriching them with ascorbate, or with vitamin E or β-carotene. Navab et al (1991) have developed a co-culture of ECs and SMCs that can oxidatively modify LDL. Enrichment of this culture with vitamin E, or β-carotene, decreases the ability of the cells to modify LDL.

In theory, antioxidant protection could be achieved with changes in lifestyle, diet and even with pharmacological approach. Despite the impressive ability of lipid-soluble antioxidants to block atherosclerosis in hypercholesterolemic animals, some studies are controversial because these antioxidants may have other antiatherogenic effects such as a hypolipidemic effect which may confound the conclusion. However, randomized clinical trials will eventually resolve the question as to whether these antioxidants deserve greater importance in inhibition of atherosclerosis.

2.3. Dietary isoflavone phytoestrogens

2.3.1. Isoflavone phytoestrogens in man

Phytoestrogens are defined as plant substances which are structurally or functionally similar to 17 β-estradiol (E_2) and consist of two major classes: isoflavones and lignans. Isoflavones make up the most common form of phytoestrogens. Isoflavones are found in a variety of plants, including fruits and vegetables, but they are predominantly found in leguminous plants and are especially abundant in soybean and soybean products (Adlercreutz and Mazur 1997). The single largest source of phytoestrogens in human diet is from soy or soy products. In plants, isoflavones are inactive in the form as glycosides, but when sugar residue is removed (deconjugation), these compounds become activated (Setchell 1998). The two major isoflavones, genistein and daidzein, are also metabolized (demethylation) from the precursors, biochanin A and formononetin, respectively (Tham et al. 1998). These plant compounds undergo fermentation by intestinal microflora, with both metabolites and the unfermented parent compounds (aglycone) being liable to absorption. After absorption, isoflavones undergo entero-hepatic circulation,

present either as glycoside conjugated or unconjugated (aglycone) forms in the blood. In the colonic microflora, daidzein may be further metabolized to equol or O-demethylangolensin (O-Dma) and genistein may be metabolized to p-ethyl phenol. Colonic microflora plays an important role in determining the metabolism and bioavailability of isoflavones (Murkies et al. 1998) and there is considerable variation among individuals with respect to isoflavone metabolism. Both absorption and intestinal degradation are important factors in determining bioavailability. Daidzein, genistein, equol, and O-Dma are the major phytoestrogens detected in human blood and urine.

2.3.2. Structural characteristics and binding properties of phytoestrogens

Isoflavones have three phenolic rings referred as A, B, C (or pyrane), with different hydroxyl group substitution giving genistein and daidzein (**Fig. 5**).

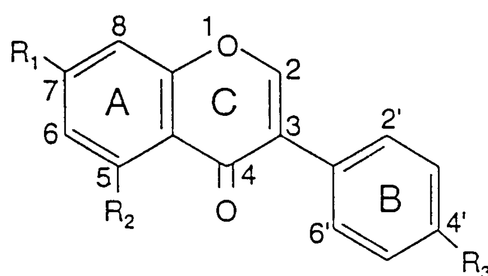

Figure 5. The generic structure of isoflavones

	R_1	R_2	R_3
Genistein	OH	OH	OH
Daidzein	OH	H	OH

They have a common diphenolic structure that resembles the structure of E_2. The structure of isoflavones is also similar to the antiestrogen tamoxifen, which is used for the treatment of estrogen associated cancers. Thus they can bind to the estrogen receptors, with binding affinity 10^{-2}-10^{-4} of E_2 (Messina et al. 1994, Martin et al. 1978). Depending on the concentrations and tissues, these compounds act as estrogen agonists and antagonists (Setchell 1985, Miksicek 1993). The recent discovery of a second estrogen receptor subtype, estrogen receptor-beta, may significantly advance our understanding of the specific effects of phytoestrogens and explain their potential benefits to humans (Kuiper et al. 1996). The binding affinity of some phytoestrogens to the estrogen receptor-beta is greater than estrogen receptor-alpha and, therefore, greater effects of phytoestrogens may be observed in estrogen receptor-beta expressing tissues (Cassidy 1999). For example, the binding affinity of genistein to the estrogen receptor-beta is 20-fold higher than to the estrogen receptor alpha (Kuiper et al. 1998), which is almost as potent as E_2. These findings provide convincing evidence that their antiestrogenic effect may also be mediated via this receptor and may play a crucial role in antiproliferation and antiangiogenesis. Relative potencies of the isoflavones compared with E_2 (value 100) which were determined by cell assays are as follows: genistein (0.084) and daidzein (0.013) (Knight et al. 1996). Although genistein has only about $1/1000^{th}$ of the estrogenic activity of E_2, its circulating concentration in individuals consuming a moderate amount of soyfoods is nearly 1000-fold higher than peak levels of endogenous E_2 in premenopausal women (Adlercreutz et al. 1995). Therefore, soy-derived genistein and daidzein could potentially play an important role in reducing the incidence of estrogen associated diseases. The presence and position of the

hydroxyl groups on the phytoestrogens and estrogens are considered to be one of the prerequisites for estrogen receptor binding affinity and activity (Martuci et al. 1993). Also the presence and position of hydroxyl groups make these compounds possess a potential antioxidant activity. As estrogen antagonists, the antiestrogenic effect of isoflavones have some advantages in their application compared to estrogens, for example, in relation to some hormone associated disease. Some other effects such as antioxidant effect may not be related to their estrogenic effect. Isoflavone phytoestrogens are known to bind tightly to soy protein (Pratt and Birac 1979) and sex hormone-binding globulin (Adlercreutz and Mazur 1997).

2.3.3. Phytoestrogens and cardiovascular disease

Several lines of evidence suggest the inverse correlation between phytoestrogens and cardiovascular disease (Tham et al. 1998). The lower rates of cardiovascular disease in Asian population compared to the West might be related to the high intake of soybean phytoestrogens (Adlercreutz 1990, Adlercreutz and Mazur 1997). CHD can also be described as a hormone associated disease. Postmenopausal women have lower CHD rates compared with age-matched man and hormone replacement therapy (HRT) can reduce the incidence of CHD. Studies in animal models have provided evidence for a role of soy in the prevention of atherosclerosis development and subsequent CHD. In one study (Huff et al. 1982), rabbits fed with a diet containing soy protein had a significantly reduced amount of atherosclerosis in both the aortic arch and thoracic aorta compared with control group fed with casein. To directly address the effect of soy phytoestrogens on atherosclerosis, Anthony et al. (1997) compared the effect of soy protein with and without isoflavones on atherosclerosis in cholestrol-fed male cynomolgus monkeys. The group receiving soy protein containing isoflavones had the least atherosclerosis compared with other groups, suggesting that the isoflavones play a key role in CHD. Another study has investigated the effects of soy protein in male cynomologus macaques, showing that the monkeys fed soy protein with isoflavone intact had negligible coronary artery atherosclerosis compared with the others without isoflavone intake in whom atheromatous plaques were detected. Furthermore, the soy isoflavone diet enhanced dilator responses of atherosclerotic coronary arteries to acetylcholine in female rhesus monkeys (Honore et al. 1997). Similar results were obtained by Kirk and coworkers (1998), showing that dietary isoflavones reduced atherosclerotic lesion in C57BL/6 mice. The potential protective mechanisms for phytoestrogens are assumed to include cholesterol lowering, inhibition of lipoprotein oxidation, and inhibition of cell proliferation and angiogenesis. In addition, other mechanisms such as inhibition of platelet aggregation and vascular tone may be involved. In the following discussions, we focus on the effects of isoflavones on lipids, antioxidation, and cell proliferation.

2.3.4. Phytoestrogens and lipid-lowering effect

There is a large body of evidence showing that substitution of soy protein in humans can lower VLDL, LDL, and total cholesterol (Anderson et al. 1995, Sirtori et al. 1995). Researchers postulated that soy phytoestrogens might be responsible for this effect (Anderson et al. 1995, Setchell et al. 1985). However, this conclusion remains open. In one study, no significant cholesterol reduction was reported when 20 healthy men were fed supplements of 60 g/day of soy protein for 4 weeks despite high plasma levels of genistein and daidzein (Gooderham et al. 1996). In contrast, another study reported a significant reduction in total cholesterol in women with elevated levels of cholesterol when they consumed soy isoflavones but not in normolipidemic women (Cassidy et al. 1995), while Wong and coworkers (1998) reported a cholesterol-lowering effect of soy protein in both normocholesterolemic and

hypercholesterolemic men. Recently, Baum et al. (1998) have reported that isoflavones can reduce LDL cholesterol and increase HDL cholesterol in postmenopausal women with hypercholesterolemia. It seems plausible that isoflavone phytoestrogens are responsible for cholesterol lowering effects mostly in patients with pre-existing hypercholesterolemia. Several animal studies also support the findings that isoflavones lower VLDL, LDL, and total cholesterol with or without increasing HDL cholesterol (Anthony et al. 1996, Kirk et al. 1998).

The mechanisms for the cholesterol lowering effect of soybean have not yet been fully clarified. With regard to the metabolism of cholesterol, soy protein consumption can increase activities of both cholesterol 7α-hydroxylase and hepatic HMG CoA reductase (Potter 1998), so that the removal of LDL and bile acid synthesis and excretion are increased. It was also proposed that the cholesterol-lowering effect of soybean is in some way because of the up-regulation of LDL receptors (Sirtori et al. 1995, Carroll and Kurowska 1995). For example, Lovati et al. (1987) reported a sevenfold increase in monocyte LDL receptor activity in hypercholesterolemic patients with soy protein intake. Recently, Baum et al. (1998) have demonstrated that LDL receptor mRNA levels in mononuclear cells were elevated by 75% in individuals with soy isoflavone consumption. Increased LDL receptor activity has also been reported in animal study after soy protein supplement (Sirtori et al 1984). Interestingly, some studies have shown that low concentrations of genistein could up-regulate LDL receptor activity and increase LDL receptor gene expression (Liu et al. 1993, Kanuck et al. 1995). All these findings suggest that soy isoflavones might be responsible for their lipid-lowering effect by increasing LDL receptor expression.

2.3.5. Phytoestrogens and lipid oxidation

Several lines of studies have shown that isoflavone phytoestrogens are potent antioxidants in vitro (Pratt et al. 1979, Wei et al. 1995, Record et al. 1995, Cao et al. 1997) and can protect lipoprotein against oxidation (Hodgson et al. 1996, Ruiz-Larrea et al. 1997). In an in vivo study, Vinson et al. (1995) detected that genistein could become bound to LDL and prevent it from oxidation following plasma incubation with higher amounts of genistein. Kapiotis et al. (1997) have demonstrated that dietary-derived genistein can inhibit cell-free and cell-mediated LDL oxidation as well as protect endothelial cells from damage by Ox-LDL. Interestingly, Tikkanen et al. (1998) and others (Kanazawa et al. 1995) have reported that soy isoflavone intake inhibited oxidation of LDL isolated from individuals ex vivo, suggesting that isoflavone phytoestrogens are particularly important in the prevention of LDL from oxidation in vivo. These findings provide a further beneficial effect on cardiovascular disease from food-derived lipophilic antioxidant administration. In addition, genistein can increase the activities of several antioxidant enzymes (Cai and Wei 1996). The antioxidant activity of isoflavones is structure-related (Cao et al. 1997, Rice-Evans et al. 1996, Cook and Samman 1996, Ruiz-Larrea et al. 1997) and the hydroxyl groups are considered to be of prime importance for the free radical-scavenging properties. In addition to the number and position of hydroxyl substitutions in its backbone structure, other factors such as hydrophilicity and the interaction with lipoprotein may also affect the antioxidant efficacy (Chen et al. 1996). In summary, the antioxidant activity of isoflavones (**Fig.5**) is dependent on: 1) The number of hydroxyl groups. 2) The additional presence of a C-3 or C-5 hydroxyl group. 3) the presence of a hydroxyl group at 4´ position of the B ring (Ruiz-Larrea et al. 1997), some have proposed that 5 position is also important (Wei et al. 1995). 4) A double bond between carbons two and three (C2-C3) of the C ring. 5). The carbonyl group at C-4 of the C ring is necessary for antioxidant activity (Cook and Samman 1996). The exact mechanism by which isoflavones inhibit LDL oxidation are still not clear. It

has been proposed that isoflavones break the chain radical reaction by donating hydrogen atoms to the peroxy radical. Isoflavones may maintain α-tocopherol content in LDL and regenerate α-tocopherol by donating a hydrogen atom to the α-tocopheroxyl radical (Frankel et al 1993). Isoflavone phytoestrogens known to bind to soy protein could become attached to binding sites on apo B normally occupied by Cu^{2+} for oxidation initiation to inhibit the oxidation initiation and development (Tikkanen et al. 1998).

2.3.6. Effects of phytoestrogens on cell proliferation and angiogenesis

Beyond the role of cholesterol reduction and antioxidation, studies have demonstrated that isoflavone genistein and daidzein can inhibit all kinds of vascular cell proliferation associated with atherosclerotic lesion formation (Raines and Ross 1995, Anthony et al. 1998). Genistein has been shown to cause both proliferative (estrogenic) and antiproliferative (antiestrogenic) effects in human cell lines (Wang et al. 1996). These biphasic effects are concentration dependent, with stimulation of cell growth occurring at low concentrations of genistein (10^{-5}-10^{-8} M) and inhibition at higher concentrations (10^{-4}-10^{-5} M), suggesting different mechanisms being involved (Wang et al. 1996). Fotsis et al.(1993, 1995) have demonstrated that genistein can inhibit EC proliferation and angiogenesis in a concentration dependent manner. The inhibitory effect of genistein on cell growth targets only proliferating cells, leaving quiescent, nondividing cells unaffected (Fotsis et al. 1993). Phytoestrogens profoundly inhibit chemotaxis and multiple steps of cell cycle of vascular smooth muscle cells (Shimokado et al. 1994; 1995). However, high concentrations of genistein are needed in order to inhibit SMC proliferation (Fujio et al. 1993). The inhibitory effects of isoflavones are likely mediated by inhibition of tyrosine kinases (Akiyama et al. 1987, Fotsis et al. 1993). Since atherosclerosis is an inflammatory process, the presence of monocytes or macrophages and lymphocytes composes an important part of atherosclerotic lesion. Their functions such as chemotaxis, cell adhesion, cell immigration, and immune responses, which are involved in the atherosclerotic lesion formation, can be inhibited by isoflavones (Raines and Ross 1995). Genistein can also inhibit thrombin formation and platelet activation as well as growth factor activity, which may contribute its protection against thrombosis (Wilcox and Blumenthal 1995). All these properties are probably responsible for the beneficial effects of isoflavone-containing diets on the prevention of atherosclerosis.

2.4. Human endogenous estrogens

2.4.1. Protective effects of estrogens on cardiovascular disease

Cardiovascular disease is the leading cause of mortality in women. Epidemiological, experimental, and clinical studies have shown that CAD is lower in premenopausal women than that in men (Barrett-Connor and Bush 1991) and treatment of postmenopausal women with estrogen replacement reduces the incidence of coronary events, even in those with established CAD (Stampfer et al. 1991, Ettinger et al. 1996). Evidence from more than 30 case-control and cohort studies shows that postmenopausal estrogen replacement therapy reduces the risk of CAD by almost 50% (Sullivan et al. 1990, Miller 1994). Grodstein et al. (1997) reported that postmenopausal hormone users with coronary risk factors had the largest reduction in mortality (relative risk 0.51). On the basis of these studies, it is clear that estrogens have a potent role in the prevention and treatment of cadiovascular disease. However, the mechanisms of this effect are not fully understood. It has been suggested that the beneficial effects of estrogens on

cardiovascular disease may involve the improvement of lipids and lipoproteins, antioxidative protection, inhibition of cell proliferation, altering vascular response to vasoactive agents, and increasing insulin sensitivity. Estrogens are now known to have potent anti-atherogenic properties through lipid and non-lipid mechanisms; both will be highlighted in the following reviews. Non-lipid mechanisms of estrogen action include decreasing insulin resistance, serum fibrinogen, factor VII and plasminogen activator inhibitor-1 (PAI-1). Moreover, estrogens maintain EC integrity, decrease expression of adhesion molecules, lower systemic blood pressure, promote vasodilatation, decrease platelet aggregability, inhibit vascular smooth muscle cell proliferation, possess potent antioxidant and calcium antagonist activities.

2.4.2. Effects of estrogens on lipid metabolism

Before menopause, plasma LDL cholesterol levels are lower and HDL cholesterol levels are higher in women compared with men of the same age. After menopause, LDL cholesterol levels rise, exceeding those of age-matched men, with a shift to smaller, more dense, and potentially more atherogenic particle sizes, and HDL cholesterol levels decline (Stevenson et al. 1993, Campos et al. 1988, Brown et al. 1993). Estrogens have the beneficial effects on the lipoprotein profile by increasing HDL cholesterol, decreasing LDL cholesterol and lipoprotein (a) [Lp(a)] (Tikkanen 1990, Nabulsi et al. 1993, Guetta et al. 1996, Skafar et al. 1997, Tuck et al. 1997). Silliman et al. (1993) reported that HDL-cholesterol and apoA-I levels were 20% higher in late pregnancy when the mean serum E_2 level was about 20,000 pg/ml, compared with 59 pg/ml postpartum, suggesting the blood E_2 level is the critical determinant of HDL levels. Orally administered estrogen reduces LDL cholesterol levels and increases HDL cholesterol levels in postmenopausal women with normal or elevated baseline lipid levels (Tikkanen et al. 1978, Walsh et al. 1991, The Writing Group for the PEPI Trial 1995). Brussaard et al. (1997) reported that E_2 reduced LDL cholesterol (-16%), apoB (-11%), and increased HDL cholesterol (20%) and apo A-I (14%) in postmenopausal women with type II diabetes mellitus. However, transdermally administered E_2 has no effect on lipoprotein levels, suggesting that the hepatic effects of estrogen absorbed through the gut are important for changes in lipoprotein levels (Walsh et al 1991).

TG levels have been known to increase in women taking estrogen who initially had normal TG levels, but the TG enriched VLDL composition may not be atherogenic (Sacks and Walsh 1994). Estrogen also significantly reduced the size of LDL particles. The LDL particle diameter correlated negatively with the plasma level of TG (Wakatsuki et al. 1997). Lp(a), a lipoprotein with structural features of LDL and plasminogen, believed to be potentially atherogenic and thrombogenic, has been shown to be an independent cardiovascular risk factor that links LDL cholesterol abnormalities with plasminogen activity (Frishman 1998). Taskinen et al. (1996) have demonstrated that HRT was highly effective in lowering elevated Lp(a) levels in postmenopausal women. Espeland et al (1998) also noted that estrogen therapy produced consistent and sustained reductions in plasma Lp(a) concentration.

Estrogens may affect lipoproteins with different mechanisms. VLDL levels increase because of enhanced production of apoB and TG (Walsh et al. 1991, Sacks and Walsh 1994). In contrast, the reduction in LDL cholesterol levels is probably a result of accelerated conversion of hepatic cholesterol to bile acids (Kushwaha and Born 1991) and increased expression of LDL receptors on cell surfaces (Windler et al. 1980) resulting in enhanced clearance of LDL from the plasma.

The increase in HDL levels is due to the increased production of apoA-I (Walsh et al. 1991) and decreased hepatic lipase activity which could increase HDL clearance (Tikkanen et al. 1982).

Nevertheless, the estrogen-induced lipoprotein changes are currently thought to be responsible for approximately 25% to 50% of estrogen's cardioprotective benefit. Accordingly, other cardiovascular properties of estrogens may also be important in accounting for the cardioprotective effect of the hormone.

2.4.3. Antioxidant effects of estrogens

Estrogens share structural similarity with lipophilic antioxidants such as probucol and vitamin E. Since estrogens have hydroxyphenol groups, the hydrogen atom of the hydroxy group and its single electron can easily be donated to lipid peroxyl free radicals, thus terminating a chain propagation of oxidation along the fatty acids of lipoprotein membrane PL (Niki 1987, Esterbauer et al. 1993). Estrogens have been shown to be powerful antioxidants, effectively preventing lipid peroxidation (Nakano et al. 1987, Mukai et al. 1990, Huber et al. 1990, Maziere et al. 1991, Rifici and Khachadurian 1992, Subbiah et al. 1993), and the antioxidant efficacy is structure-related, a free phenolic hydroxyl group being necessary for this potential (Nakano et al. 1987, Ruiz-Larrea et al. 1994, Miller et al. 1996, Mukai et al. 1990). A-ring metabolites (catecholestrogens) emerged as more potent inhibitors of LDL oxidation than the parent substance E_2, its D-ring metabolites, and vitamin E (Seeger et al. 1998). The antioxidant effect of estrogens were not negated by progestins (Mueck et al. 1998, Arteaga et al. 1998), providing further support for the combination of estrogen and progestin in hormone replacement therapy for postmenopausal women in addition to their beneficial effects on lipids.

Hoogerbrugge and his associates (1998) demonstrated that estrogen administration reduced Ox-LDL antibodies in vivo in postmenopausal women. Sack et al. (1994) have found that the acute administration of E_2 into the brachial arteries of postmenopausal women significantly delayed the onset and rate of copper-catalyzed oxidation of LDL. After E_2 administration via a transdermal preparation for 3 weeks, LDL was protected against oxidation by a degree similar to that noted in the short-term infusion study. One month after discontinuation of treatment, the LDL lag time had returned to baseline level, suggesting that prolonged HRT would be required to maintain the cardioprotective benefits. This finding was further confirmed by two recent studies showing that LDL isolated from estrogen-treated postmenopausal women is associated with reduced oxidation susceptibility (Wakatsuki et al. 1998, Wilcox et al. 1997). However, the content of estrogens in LDL particles were not reported after estrogen administration. Therefore, Shwaery et al. (1997,1998) have demonstrated that physiological concentrations of E_2 incubated in plasma could be converted to fatty acid ester derivatives that incorporate into LDL and, thus, reduce the LDL susceptibility to oxidation. In contrast, another group proposed that physiological concentrations of E_2 is unlikely to act as an antioxidant (Santanam et al. 1998). Nevertheless, it is postulated that the the antioxidant activity is considered to be one mechanism by which estrogen confers cardioprotection (Bush et al. 1987).

2.4.4. Estrogens and vascular cell proliferation

Smooth muscle cell proliferation is a major aspect in the development of atherosclerosis. Many studies have reported that estrogens decreased SMC proliferation (Rhee et al. 1977, Morey et al. 1997, Lou et al. 1997) and migration (Kolodgie et al. 1996). E_2 affects a number of vascular smooth muscle cell (VSMC) functions, including contractility and growth. Espinosa et al. (1996)

observed that E_2 induced a remarkable inhibition of SMC proliferation in female rats but not in male rats and this effect is mediated by E_2 receptors. Foegh et al. (1994) reported that E_2 inhibits neointimal formation (VSMC proliferation) after balloon injury of iliac arteries of rabbits. Myointimal proliferation after balloon injury of the carotid artery in rats (Chen et al. 1996) and the abdominal aorta of rabbits (Farhat 1996) is also attenuated by E_2. This effect may be mediated via estrogen receptors present in SMC. Interestingly, E_2 inhibits increases in vascular medial area and VSMC proliferation after vasculature injury in transgenic mice lacking estrogen receptor alpha (ER-α) as well as in wild-type mice, suggesting that a novel mechanism independent of the classical ER-α, such as ER-β, is involved (Iafrati et al. 1997).

E_2 may also affect EC regeneration and angiogenesis. It promotes neovascularization and migration as well as proliferation of EC in vitro and in vivo (Morales et al. 1995). Moreover, E_2 can stimulate growth factor expression such as fibroblast growth factor, vascular endothelial growth factor (VEGF), and tumour necrosis factor-α (TNF-α) and augment these growth factor-induced angiogenesis (Skafar et al. 1997).

2.4.5. Esterification of estrogens

Only recently has the existence of naturally occurring fatty acid esters of steroids been confirmed. Earlier, Schatz and Hochberg (1981) demonstrated that estrogens could also be esterified in many tissues. After incubation of radiolabled E_2 with several tissues of a rat, a nonpolar, saponifiable metabolite, the lipoidal derivative of E_2, was detected. The metabolite was further characterized to have properties that are consistent with E_2 esters at C-17: it was saponifiable, resistance to oxidation, able to be acetylated (indicating the presence of a free hydroxyl group), and less polar than E_2 17-acetate. Since the metabolite was protected from oxidation but had a free hydroxyl, the site of esterification had to be C-17 and not the phenolic C-3 hydroxyl group. In a further study, it was confirmed that esterification of E_2 occurs only at C-17 and most of them are heterogeneous, unsaturated fatty acids (Mellon-Nussbaum et al. 1982). Further studies from Hochberg´s laboratory have demonstrated that the endogenous estrogen esters were also present in human blood, ovarian follicular fluid, and breast cyst fluid (Hochberg 1998, Janocko and Hochberg 1983, Larner et al 1993). The ester composition is varied in different tissues. The percent composition of the individual esters from ovarian follicular fluid was: E_2 17-linoleate (43%), E_2 17-palmitate (20%), E_2 17-arachidonate (19%), E_2 17-oleate (14%), and E_2 17-stearate (4%) (Larner et al. 1993). Almost every family of steroid hormones is esterified in a variety of tissues to become nonpolar derivatives, circulating in human blood (Janocko and Hochberg 1983). Other estrogen conjugates, such as sulfates and glucuronides are hormonally much less active or totally inactive, fatty acid esterification appeares to be the only form of metabolism that does not deactivate the biological effects of E_2. In fact, the fatty acid esters of E_2 are the most potent naturally occurring estrogen known (Zielinski et al. 1991, Larner et al. 1993). The fatty acid esters of E_2 are long-acting estrogens (Larner et al. 1985, Vazquez-Alcantara et al. 1989). This long-lasting effect may be due to its slow metabolic rate in vivo. Studies have shown that the longer the chain length of the esters, the slower the metabolism (Larner and Hochberg 1985). The fatty acid esters are cleared from blood at a slower rate than free E_2. At the dimensions of the naturally occuring esters of lipoidal derivative of E_2, such as C_{18}, metabolism was extremely slow. It is evident that fatty acid esterification of the D-ring estrogens dramatically protects the steroid nucleus from metabolism

and prolongs their biological effect. It is this protection from metabolic deactivation that causes a long-lived hormonal stimulation. Its striking potency indicates that it could be a physiologically important form of hormone. Paris et al. (1994) investigated the lipoidal derivative formation of E_2 in vivo after the administration of E_2. Shortly after the last injection, the ^{3}H-labeled matabolites extracted from peripheral fat were characterized. Approximately 25% of the radioactivity in the fat were present as nonpolar lipophilic derivatives, suggesting that E_2 could be esterified in vivo. Studies have suggested that the estrogen is esterified by the same enzyme that esterifies cholesterol in blood, mostly by LCAT (Jones and James 1985, Leszczynski et al. 1989, Pahuja et al. 1995). Although steroid esters are potent hormones, they do not act directly at the level of the receptor. They require hydrolysis by esterase to the parent steroid for their hormonal action (Larner and Hochberg 1985). These naturally occuring esters have the potential of being extremely useful pharmacological agents for long-lived estrogenic stimulation.

2.4.6. Binding of estrogen esters to lipoproteins

As known, the parent steroid, E_2, is bound to sex hormone-binding globulin (SHBG) in human plasma (Rosenbaum et al. 1966). The question was raised how E_2 esters are transported in blood. Larner et al. (1987) found that E_2 17-fatty acid esters are bound almost entirely to lipoproteins in serum and speculated that they may be transported into target cells via the lipoprotein receptors. The preferential binding to lipoproteins may exert an important biological function. Roy and Belanger (1989a) found that dehydroepiandrosterone was converted to lipoidal derivatives in human serum and these derivatives were present in the lipoprotein fractions, predominantly HDL and LDL. In another study, Leszczynski and Schafer (1990) found that several of the steroids including E_2 appeared to bind to HDL in a nonequilibrium manner, indicating metabolic conversion of the steroid hormones. It was presumed that LCAT might be responsible for the lipophilic conversion activity (Leszczynski et al. 1989). These authors suggested that incorporation of E_2 was dependent on its esterification by LCAT which is associated with HDL. In contrast to the continuous LCAT-induced accumulation of lipophilic E_2 in HDL, the E_2 uptake into LDL was limited reaching an equilibrium after 10 hours (Leszczynski and Schafer 1990). With radiolabeled E_2 incubation in plasma, Tang et al. (1997) have shown that hydrophobic derivatives of E_2 remained associated with HDL, but its association with VLDL and LDL increased significantly as compared with free E_2, indicating that the hydrophobicity of estrogen might enhance its association with LDL. Recently, Shwaery et al. (1997, 1998) made an interesting finding of the relationship between E_2 ester formation and antioxidant protection. They showed that incubation of physiological concentrations of E_2 (approximately 1 nmol/L) in plasma could lead to the formation of E_2 fatty acid esters. The esterification can be abolished by a sulphydryl group reagent, 5,5´-dithio-*bis*(2-nitrobenzoic acid) (DTNB). This might suggest that LCAT is the esterifying enzyme because it is also inhibited by DTNB (Jauhiainen et al. 1989). These esters can bind to LDL and prevent it from oxidation under physiological concentrations.

The estrogen esters bound to LDL are taken up by cells via LDL receptor-mediated pathway (Hochberg 1998). Although their physiological role is not understood, esterification of estrogens, binding to the lipoproteins and their transfer to cells may produce some biological effects that aid in the prevention of CAD.

3. AIMS OF THE STUDY

The main aim of the present study was to investigate the possible roles of lipophilic isoflavone phytoestrogens and estrogens associated with LDL as follows:

1. To set up a human myelomonocytic cell culture model (U937 cell line) exploring the role of LDL as a transport vehicle for delivery of lipophilic bioactive substances into cells via the LDL-receptor mediated endocytotic pathway.

2. Using the cell culture model, to analyze the putative antiproliferative efficacy of isoflavones and their esters delivered by LDL into cultured U937 cells.

3. To investigate the incorporation of isoflavones and E_2 as well as their fatty acid esters into LDL and analyze the antioxidant capacity of LDL containing such substances.

4. MATERIALS AND METHODS

4.1. Blood and plasma samples

Blood samples from healthy volunteers (age 28-37 for women, 25-55 for men) were drawn into vacuum tubes containing K_3EDTA (4.7 mmol/L) after overnight fasting. These participants were normolipidemic, consumed their habitual diets, and did not take any antioxidants or medication during that time. Plasma was prepared by centrifugation (1,000g) at 10°C for 10 min.

4.2. Synthesis of isoflavones and 17β-estradiol esters

Daidzein (4´,7-dihydroxyisoflavone) and genistein (4´,5,7-trihydroxyisoflavone) were synthesized by a one pot procedure (Wähälä and Hase 1991) from the appropriately substituted phenol and arylacetic acid, involving the initial condensation of these substances in boron trifluoride etherate, followed by an in situ cyclization of the resulting deoxybenzoin intermediates to the corresponding isoflavones. These were then esterified using potassium t-butoxide and oleyl or stearoyl chloride to give daidzein 4´-oleate, daidzein 7-oleate, daidzein 4´,7-dioleate, genistein 4´-stearate, genistein 7-stearate, genistein 4´,7-distearate, genistein 4´-oleate, genistein 7-oleate, genistein 4´,7-dioleate (Study III, Fig. 1). The purity of the synthesized isoflavones was >95% after flash-chromatography (silica gel) (Still et al. 1978). The details of synthesis and structural characterization of the isoflavone fatty acid esters were described in a separate paper (Lewis 1998).

E_2 17-oleate was synthesized as follows: E_2 was esterified in dry pyridine with oleic acid chloride in the presence of dimethylaminopyridine under argon atmosphere at 50°C (Höfle et al. 1978). After the reaction the mixture was poured into 10% HCl-solution, extracted with diethyl ether and washed with 10% sodium bicarbonate solution and water. The evaporation of the organic solvent furnished the solid crude product mixture of E_2 esters (the yield 90%). The purification of E_2 17-oleate from the synthetic mixture also containing trace amounts of E_2 3-oleate and E_2 3,17-dioleate was carried out by flash-chromatography (silica gel) as isoflavone esters, eluting with hexane:ethyl acetate 3:1, E_2 17-oleate, R_f=0.39. Recrystallization from chloroform gave E_2 17-oleate in >95% purity according to GC, PMR, ^{13}C-NMR and MS [70 eV, M^+ 536 (18%), 255 (100)] data (Mellon-Nussbaum et al.1982).

4.3. Methods

4.3.1. Lipoprotein preparation

After plasma preparation, the lipoprotein fractions, LDL and HDL were isolated by sequential ultracentrifugation (Havel et al. 1955) at density range 1.019 to 1.063 g/ml (38,000 rpm for 18 hours), and density range 1.063 to 1.21 g/ml (50,000 rpm for 24 hours), respectively, in a Beckman TL ultracentrifuge (Beckman, Palo Alto, USA). The LDL and HDL samples were prepared individually and applied for experiments immediately after isolation.

4.3.2. Cell culture

U937 cells, a human myelomonocytic cell line which originally derived from a histiocytic lymphoma, were established by Sundström and Nilsson (1976). These cells lack the 3-ketosteroid reductase activity in the cholesterol synthesis pathway, and they cannot synthesize cholesterol for their growth (Billheimer et al. 1987). Due to this defect, the presence of exogenous LDL cholesterol is critical for U937 cell growth (Esfahani et al. 1984).

The U937 cell line was obtained from Prof. K. Nilsson (Uppsala University, Sweden). The cells were grown in 25 cm^2 flasks in RPMI 1640 medium supplemented with glutamine, 10% heat-inactivated FBS and 10,000 U/ml penicillin (Gibco BRL, Glasgow, UK), and kept at 37°C in an atmosphere of 5% CO_2. Fresh medium was added three times a week and cells were kept at a density between 2-8 x 10^5 cells/ml. The viability of cells was assessed by trypan blue exclusion test and kept above 90%.

4.3.3. Cell proliferation assay

In order to achieve intracellular cholesterol depletion, U937 cells were washed three times with phosphate buffered saline (PBS), transferred to RPMI 1640 medium without FBS and cultivated for 24 hours. They were then resuspended in fresh RPMI medium at fixed density of 1.5 or 2 x 10^5 cells/ml. These cells were seeded out in 96-well tissue culture plates (Nunc, Roskilde, Denmark) with 100 μl cell suspension per well. After that, various amounts of LDL cholesterol (5 to 30 μg /ml) were added to each well. Each LDL sample was analyzed in quadruplicate or sixtuplicate. Incubation of cells was then carried out for 48 hours at 37°C, and 25 μl (0.5 μCi) ^{3}H-thymidine (Amersham international, Amersham, UK) were added to each well 6-8 hours before the end of the 48 hours incubation. After the incubation, the cells were precipitated in glass-fiber filters by means of an automatic cell harvester (SKATRON instruments, Norway). The DNA synthesis reflecting cell proliferation was evaluated by ^{3}H-thymidine incorporation. The amount of incorporated ^{3}H-thymidine was determined in a liquid scintillation counter (Wallac 1409, Turku, Finland).

4.3.4. Incubation and esterification of estrogens in plasma

Plasma was incubated with E_2 or E_2 17-stearate at final concentrations ranging from 10 to 5000 nmol/L (final ethanol concentration, 0.1% v/v) for 4 hours at 37°C in the dark under N_2 as described by Shwaery et al. (1997). To inhibit E_2 esterification, plasma was incubated with 100 μmol/L E_2 as described above in the presence of 1.4 mmol/L 5,5´-dithio-*bis*(2-nitrobenzoic acid) (DTNB) (ICN Biomedicals, Inc). LDL and HDL were subsequently isolated by sequential ultracentrifugation as described above and purified by gel filtration on a Sephadex G-25 as well as deionized by Chelex-100 resin (Bio-Rad Laboratories, USA) in order to remove unbound estrogens, EDTA, as well as contaminating metal ions before carrying out the oxidation experiments.

4.3.5. Incorporation of isoflavones and 17β-estradiol as well as their esters into lipoproteins in Celite dispersion

The incorporation of isoflavones or estrogens into LDL particles was investigated using a modified Celite 545 transfer system (Avigan 1959, Lundberg et al. 1982). This method allows transfer of relatively large amounts of isoflavone or estrogen derivatives from Celite particle surfaces to LDL without exposing LDL particles to organic solvents needed for solubilization of isoflavone or estrogen esters. Exposure of LDL to organic solvents may alter lipoprotein structure and denaturate apoB which results in impaired binding affinity or altered oxidation resistance of the particles. In this procedure, various amounts of free or esterified isoflavones and estrogens dissolved in methanol or chloroform were added to a test tube containing a Celite 545 dispersion (Fluka Chemie AG CH-9470 Buchs). After evaporating the organic solvent to dryness under $N_{2,}$ 1 mg of LDL or HDL protein in 0.1 M Tris-HCl (0.01% EDTA, 0.002% NaN_3 pH 7.8) was added to the Celite dispersion. The mixture was incubated at 37°C under N_2 in a shaking water bath for 22 hours. After incubation, the LDL or HDL was reisolated by centrifugation at 3000 x g for 30 min and filtered through a Millipore filter (0.22 μm). Before the cell assay or oxidation experiments, LDL was dialyzed thoroughly against PBS or purified by gel filtration on a Sephadex G-25 column (1.5 x 15 cm) in order to eliminate EDTA and any isoflavones or estrogens in the water phase. Control LDL and HDL samples in the absence of isoflavones or estrogens were processed in the same way.

4.3.6. Separation of ester fraction from free isoflavones or 17β-estradiol

To separate isoflavone and E_2 esters from unesterified ones, a column chromatographic method was developed using Sephadex LH-20 (Pharmacia Biotech.) (0.5 x 5 cm column in n-hexane/chloroform 1:1 v/v). The samples were applied to the column in 200 μl of n-hexane/chloroform (1:1 v/v) and the ester fraction was eluted with 6 ml of the same solvent. The unesterified isoflavone or E_2 was then eluted with 3 ml of methanol. The accuracy of the separation procedure was confirmed by chromatography of synthetic E_2 esters and unesterified E_2.

4.3.7. Quantification of phytoestrogens and estrogens in lipoproteins

The deuterated isoflavone or E_2 internal standard (for free fraction determination) was added to LDL or HDL samples (250 μg protein) which were then extracted twice with 2 volumes of diethyl ether/ethylacetate (1:1 v/v) and evaporated to dryness under N_2. After evaporation, 200 μl of n-hexane/chloroform (1:1 v/v) were added to dissolve the residue and the sample solution was applied to LH-20 chromatography to separate isoflavone or E_2 ester and free isoflavone or E_2 fractions as described above (4.3.6). Both fractions were evaporated to dryness. The free isoflavone or E_2 fraction was silylated, evaporated, and dissolved in n-hexane and subjected to gas chromatography-mass spectrometry (GC-MS) in the selected ion monitoring mode for quantitative determination of isoflavone or E_2 as described for other phenols (Adlercreutz et al. 1993). The isoflavone or E_2 ester fraction was saponified by incubating with 1 ml of 1 M KOH in MeOH for 2 hours at 60°C. After incubation, 1 ml of water was added, and the samples were neutralized by adding 250 μl of 4 M HCl, extracted twice with 3 ml of diethyl ether, and

deuterated isoflavone or E_2 internal standard was added. Following evaporation of the combined ether phase to dryness, the samples were further purified by Lipidex-5000 reversed phase chromatography (PACKARD Instrument Company, The Netherlands), evaporated to dryness, and silylated. After evaporation, the samples were dissolved in n-hexane and subjected to GC-MS for quantitation as free isoflavone or E_2 fraction.

4.3.8. Determination of antioxidant capacity of LDL carrying antioxidants

For the oxidation experiments, the protein concentration of LDL or HDL was adjusted to 100 μg/ml with PBS. Oxidation was initiated by adding freshly prepared $CuSO_4$ solution (3 or 10 μmol/L final concentration) at 21°C. The oxidation of lipoproteins was determined by monitoring, at 4 min intervals, the absorbance changes at 234 nm, which correspond to the conjugated diene formation (Esterbauer et al. 1989). The absorbance change was continuously recorded in a thermostat-controlled computerized UV-1202 spectrophotometer equipped with a 6-position automatic sample changer for 6.6 hours (Shimadzu, Japan). The oxidation parameters (lag time, slope) were then calculated.

4.3.9. Other analytical procedures

ApoB was measured by an immunochemical assay (Orion Diagnostica, Finland). Cholesterol, TG and PL were determined by enzymatic colorimetric methods (Boehringer Mannheim). Protein concentrations of lipoprotein fractions were determined by the method of Lowry et al (1951). To ensure that the apoB remained intact after the incorporation of estrogens or phytoestrogens into LDL, the LDL was submitted to 3% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

4.3.10. Statistical analyses

Statistical analyses were performed with SYSTAT (Version 6.0.1, Michigan, USA). Results from quadruplicate experiments or more are expressed as means ± SD. The significance of differences between means versus control was assessed with Student´s t test. If the data did not fit the constraints of this parametric test, data were analyzed with the non-parametric Wilcoxon test. Statistical significance was accepted if $P < 0.05$.

5. RESULTS

5.1. LDL receptor mediated delivery of LDL cholesterol into U937 cells

Since the U937 cells lack the 3-ketosteroid reductase activity needed for cholesterol synthesis, their growth is dependent on the exogenous cholesterol uptake. To determine to what extent the U937 cell proliferation is dependent on the presence of native LDL cholesterol in the culture medium, cells (2.0 x 10^5/ml) were seeded out in RPMI 1640 containing various amounts of LDL cholesterol. At cholesterol concentrations below 1 µg/ml , cell proliferation was similar to that observed with RPMI 1640 only (data not shown), while increasing concentrations of LDL cholesterol from 2 to 20 µg/ml caused a marked increase of cell growth (**Study I Fig.1**). Increments in LDL cholesterol above 20 µg/ml did not further increase U937 cell proliferation at this cell density.

In order to further clarify if the cholesterol delivery into cells is dependent on the efficient binding of LDL apoB to its receptors, we investigated the effects of methylated LDL and familial defective apolipoprotein B-100 (FDB) LDL (with known defective apoB binding affinity) on the cell proliferation. Methylated LDLs and LDLs from FDB patients were less effective in supporting cell proliferation compared with native LDL (**Study I**) .

This study demonstrated that LDL can transport cholesterol to cells for their growth via LDL receptor mediated pathway.

5.2. Esterification of 17β-estradiol in plasma

Small but significant amounts of E_2 in esterified form could be quantitated in LDL and HDL after isolation from plasma incubation with 1,000 nmol/L or more of free E_2 (**Table 3**).

Table 3. Content of free and esterified E_2 in LDL and HDL following plasma incubation

	free E_2	E_2 esters	free E_2	E_2 esters
E_2 (nmol/L)	pmol/mg LDL protein		pmol/mg HDL protein	
0	1.27 ± 2.17	0.72 ± 0.72	0.40 ± 0.65	0.26 ± 0.16
10	3.50 ± 2.75	0.50 ± 0.61	0.48 ± 0.59	0.23 ± 0.34

100	2.26 ± 2.99	0.83 ± 0.73	0.74 ± 1.33	0.70 ± 0.77
1000	1.13 ± 1.95	2.59 ± 1.78*	0.40 ± 0.62	2.81 ± 1.11*
5000	1.87 ± 2.90	14.69 ± 5.20 **	0.42 ± 0.64	12.98 ± 4.83**

LDL and HDL samples were isolated and gel-filtered following incubation of plasma with free E_2 for 4 hours at 37°C. The separation and quantification of free and esterified E_2 in LDL and HDL were carried out as described in the method section. Data are represented as the mean ± SD of five experiments with samples from different donors. *P< 0.05, **P< 0.01 vs. control (paired t test).

To demonstrate the possible role of LCAT in the esterification of E_2, we incubated plasma with 100 µmol/L of unesterified E_2 in the presence of 1.4 mmol/L of DTNB, a sulphydryl reagent also known to inhibit LCAT (Jauhiainen et al. 1989). The esterified E_2 detected in LDL and HDL were reduced by approximately 97% (**Study IV, Table 5**). Small proportions of esterified E_2 were also detected in HDL following incubation of isolated HDL (1 mg protein) with 3,000 nmol of unesterified E_2 in Celite dispersion (**Study IV, Table 7**).

5.3. Binding of isoflavone and 17 β-estradiol esters to lipoproteins

The amounts of free and esterified isoflavones incorporated into LDL following in vitro incubation in Celite dispersion are summarized in Table 4 (**Studies II, III**). In addition, some unpublished data is included in this table. The mean molar ratios of incorporated free genistein and daidzein per mole of LDL were 0.07 and 0.22, respectively. Among esterified isoflavones, genistein and daidzein stearates were least incorporated, with molar ratios ranging from 0.06 to 0.43. Incorporation of both genistein and daidzein oleates into LDL was more effective, with molar ratios ranging between 2.43 and 7.87. The incorporation of daidzein dilinoleate was the most effective, with the molar ratio reaching 17.

Table 4. Incorporation of unesterified and esterified isoflavones into LDL in Celite dispersion

Isoflavone	Isoflavone in LDL (nmol/mg protein)	Molecules/LDL
Genistein	0.13 ± 0.07	0.07 ± 0.03
Daidzein	0.43 ± 0.20	0.22 ± 0.10
Genistein 4´-stearate	0.64 ± 0.35	0.32 ± 0.18
Genistein 7-stearate	0.21 ± 0.09	0.11 ± 0.05
Genistein 4´,7-distearate	0.16 ± 0.09	0.07 ± 0.05
Genistein 4´-oleate	15.73 ± 7.09	7.87 ± 3.55
Genistein 7-oleate	8.07 ± 1.41	4.04 ± 0.71
Genistein 4´, 7-dioleate	9.75 ± 4.43	4.88 ± 2.21
Daidzein 4´-stearate	0.86 ± 0.36	0.43 ± 0.18
Daidzein 7-stearate	0.56 ± 0.24	0.28 ± 0.12
Daidzein 4´,7-distearate	0.12 ± 0.06	0.06 ± 0.03
Daidzein 4´-oleate	10.74 ± 5.61	5.37 ± 2.81

Daidzein 7-oleate	5.74 ± 2.54	2.87 ± 1.27
Daidzein 4´,7-dioleate	4.86 ± 1.85	2.43 ± 0.93
Daidzein 4´,7-dilinoleate	33.74 ± 2.86	16.87 ± 1.43

LDL (1mg protein) was incubated with 3,000 nmol of various isoflavones in Celite dispersion for 22 hours at 37°C. The isoflavones incorporated into the LDL particles were quantified by GC-MS using deuterated internal standards. Values are means ± SD of seven to eight independent experiments (from **Studies II,III** and other unpublished data). The isoflavone-ester described in this thesis is same as isoflavone-*O*-ester in **Study III**.

The incorporation of free or esterified E_2 into LDL and HDL particles was analyzed following incubation in plasma. Only trace amounts of free E_2 could be detected in gel-filtered LDL. However, small amounts of E_2 in esterified form could be quantitated in LDL following addition of 1,000 nmol/L or more of free E_2 to plasma (**Table 3**). Incubation of plasma with increasing amounts of E_2 17-stearate resulted in increasing proportions of E_2 in the ester fraction in LDL. With 100 nmol/L of E_2 17-stearate significant incorporation was achieved compared with control (**Study IV, Table 3**).

Only negligible amounts of free E_2 were detected in HDL following incubation with plasma, but small amounts of esterified E_2 could be demonstrated in this lipoprotein after addition of 1,000 nmol/L or more of free E_2. Incubation with E_2 17-stearate resulted in incorporation of E_2 ester into HDL comparable to that in LDL following incubation with E_2 17-stearate (**Study IV, Table 4**).

Substantial amounts of E_2 esters were detected in LDL following incubation with E_2 17-stearate and E_2 17-oleate in Celite dispersion, but not with free E_2 (**Study IV, Table 6**). The incorporation of E_2 17-stearate and E_2 17-oleate into LDL was proportional to the concentration added to the Celite dispersion, with values reaching 1.38 ± 0.26 molecules/LDL particle for 3,000 nmol of E_2 17-stearate and 1.93 ± 0.36 molecules/LDL particle for 3,000 nmol of E_2 17-oleate.

In similar studies with HDL, both $E_2$17-stearate and E_2 17-oleate were efficiently incorporated into this lipoprotein, the amounts reaching 4,125 pmol/mg HDL protein for 3,000 nmol of E_2 17-stearate added and 8,233 pmol/mg HDL protein for 3,000 nmol E_2 17-oleate added (**Study IV, Table 7**). The incorporation ratio of E_2 esters in HDL was higher than that in LDL following Celite incubation (**Study IV, Table 6**).

The incorporation of isoflavone or E_2 esters did not change the LDL lipid or apoprotein mass composition (cholesterol, phospholipids, triglyceride, and apoB) as compared with control LDL that was processed in the same way except in the absence of isoflavones or E_2. Incubation of LDL with isoflavones or estrogens did not result in detectable apoB-100 degradation or other changes as detected on 3% SDS-PAGE (data not shown).

5.4. Antiproliferative effects of lipophilic isoflavones delivered by LDL into U937 cells

The U937 cell culture method (**Study I**) was originally set up in order to investigate the effect of apoB mutations on the receptor binding of LDL. As this method also provides a possibility to investigate cellular effects of bioactive substances carried in LDL particles, we emphasized the system for studying effects of isoflavones.

The U937 cell proliferation was remarkably inhibited by free genistein and daidzein added directly to the cell culture medium in the presence of native LDL, although the lowest concentrations (0.1-0.5 µmol/L) of genistein induced a temporary increase in cell growth. The inhibitory effect was concentration dependent, with a 50% inhibitory concentration (IC_{50}) of 16.0 µmol/L for genistein and 39.6 µmol/L for daidzein (**Study II, Fig. 2**).
The LDLs incorporated with small amounts of unesterified genistein, daidzein or any of the genistein stearates did not exhibit inhibitory effects on U937 cell proliferation. However, the LDLs containing some isoflavone mono- and dioleates and dilinoleate significantly reduced the cell proliferation by 36% (genistein 7-oleate), 43% (genistein 4´,7-dioleate), 33% (daidzein 7-oleate), 30% (daidzein 4´,7-dioleate), and 93% (daidzein 4´,7-dilinoleate), compared with control LDL (**Study II, Fig. 3**). The corresponding concentrations of these isoflavone esters in cell culture medium calculated from their content in LDL were 0.36, 0.42, 0.24, 0.22, and 1.36 µmol/L, respectively. Neither of the 4´-oleates of genistein and daidzein contained in LDLs inhibited cell proliferation although relatively greater amounts of them were incorporated into LDL (**Study II, Table 1, Fig. 3**).

5.5. Antioxidant capacity of isoflavone-bound LDL

The Cu^{2+}-initiated oxidation of LDL was significantly inhibited in a concentration-dependent manner by adding free genistein or daidzein directly to the LDL oxidation mixture (**Study III, Table 2**). Incubation of LDL with unesterified isoflavones or genistein stearic acid esters did not result in prolongation of lag times compared with control LDL incubated in the absence of any isoflavone (**Study III, Table 3**). However, the LDLs containing 4´,7-dioleates of daidzein and genistein significantly prolonged lag times by 46% ($P<0.05$) and 202.1% ($P<0.01$), respectively (**Study III, Table 3, Figs. 2,3**). In addition, a small but significant increase in lag time (20.5%, $P<0.01$) was brought about by daidzein 7-oleate. A trend prolongation of lag time was induced by genistein 7-oleate but did not reach statistically significance. In contrast, neither of the 4´-oleates of daidzein and genistein incorporated into LDL could protect this lipoprotein against oxidation as judged by the lag time. There was no correlation between the concentrations of any of the isoflavones in LDL particles and lag times.

5.6. Antioxidant capacity of LDL carrying estrogens

The Cu^{2+}-induced LDL oxidation was significantly inhibited in a dose-dependent manner by adding free E_2 directly to the LDL oxidation mixture (**Study IV, Table 1**). The minimum effective concentration of E_2 for oxidation inhibition was 100 nmol/L, with 22% increase ($P=0.024$) in lag time compared with control.

LDL isolated after incubation of plasma with free E_2 or E_2 17-stearate (maximum concentration, 5000 nmol/L) did not prolong the lag time significantly compared with controls (**Study IV, Table 3**). However, significant lag time prolongations were observed for LDL (1 mg protein) incubated with 500 and 3000 nmol E_2 17-stearate or E_2 17-oleate but not with unesterified E_2 in

the Celite dispersion (**Study IV, Table 6, Figs. 1A,B**). There was a positive correlation between the lag time and E_2 -17-stearate or E_2 17-oleate concentration in LDL ($r=0.979$, $P< 0.001$, $n=8$).

Similarly, no oxidation resistance of HDL could be detected either after isolation of HDL from the same plasma incubation with estrogens as LDL (**Study IV, Figs. 2A,B**). However, prolongations of lag time were observed when greater amounts of E_2 17-stearate and E_2 17-oleate were incorporated into HDL using the Celite transfer system but not with unesterified E_2 (**Study IV, Figs. 2C,D**).

6. DISCUSSION

6.1. Evaluation of the method on LDL cholesterol delivery via LDL receptor pathway into U937 cells

U937 cells lack the 3-ketosteroid reductase activity in the cholesterol synthesis pathway, thus they cannot synthesize cholesterol for their growth (Billheimer et al.1987). Due to this defect, the presence of exogenous LDL cholesterol is critical for U937 cell growth (Esfahani et al. 1984). Alternatively, the cell growth rate can be influenced by the cholesterol uptake, which is mediated by the binding of LDL apoB to the LDL receptors (Brown et al. 1981). Accordingly, the growth rate of U937 cells can be used to determine the binding properties of LDL to its receptor and the delivery of exogenous cholesterol by LDL.

LDLs are the major cholesterol carrier in plasma, and CE is the major cholesterol component carried in LDL particles. The hydrophobic CE packed in LDL particles can be delivered into cells for their use via LDL-receptor mediated endocytotic pathway. Therefore, apoB mutations might impair the binding affinity to its receptors and the delivery of cholesterol into cells. The apoB Arg3500→Gln mutation was the first apoB gene mutation reported causing a hypercholesterolemic disorder defined as FDB as a result of reduced binding of LDL apoB to the LDL receptors. The U937 cell growth was therefore markedly decreased due to the defective binding of LDL apoB (FDB) to its receptors (Frostegård et al. 1990, Schewe et al. 1994, Van den Broek et al. 1994).

Our modified assay confirmed the previous findings that the apoB Arg3500→Gln mutation could reduce the binding affinity and cholesterol uptake, and therefore, the cell proliferation. Methylation of LDL impaired its ability to be taken up into U937 cells and failed to promote the cell growth. In addition, our findings have also demonstrated that the U937 cell proliferation is proportional to its cholesterol uptake before reaching saturation. These studies indicated that cholesterol (mostly CE) can be delivered by LDL into cells via LDL receptor-mediated pathway for their growth. The delivery is dependent on the effective and efficient binding of LDL apoB to the LDL receptors. This assay can be used for determining the binding affinity of LDLs isolated from individuals carrying mutations of the apoB gene, and possibly other functionally deficient species of apoB. As this method can be used for determining the delivery of cholesterol into cells, other lipophilic bioactive substances carried in LDL particles could also be delivered into cells in the same way. Therefore, in this study, we emphasize this system for studying the cellular effects of lipophilic soy isoflavones carried in LDL particles (**Study II**).

6.2. Mechanism of 17 β-estradiol esterification

Sterols such as cholesterol esterified with fatty acids in the body, have been known about for a long time (reviewed by Hochberg 1998), whereas the existence of naturally occurring fatty acid esters of steroids has been recognized only relatively recently. The findings of testosterone esterification and adrenal lipoidal derivatives of the Δ^5-3β-hydroxysteroids increased the possibility that other biologically active steroids, especially E_2, may also be metabolized in a similar way (Kishimoto 1973, Belanger et al. 1990). Researchers from Hochberg´s group for the first time detected the lipoidal derivatives of E_2 in many tissues (Schatz and Hochberg 1981, Mellon-Nussbaum et al. 1982) and human blood plasma (Janocko and Hochberg 1983). These metabolites were further demonstrated that they are a family of fatty acid esters. Several lines of evidence support that the esterification of E_2 occurs only at C-17 but not at the C-3 phenolic hydroxyl group (Larner et al. 1993). First of all, the metabolite was protected from oxidation, suggesting to have a free hydroxyl group. In addition, estrone with similar structure as E_2 but lacking a hydroxyl group at C-17, was not enzymatically converted into a lipoidal derivative. Furthermore, mass spectra confirmed that the ester group is at C-17 but not at the C-3 hydroxyl position (Mellon-Nussbaum et al. 1982). The E_2 esters are a heterogenous mixture of several fatty acid esters, predominantly unsaturated (86%). The composition of these E_2 esters varies in tissues such as blood, fat, ovarian follicilar fluid, breast cyst fluid. For example in the ovarian follicular fluid, the distribution of E_2 esters (% from all E_2 esters) is as follows: E_2 17-linoleate (43%), E_2 17- palmitate (20%), E_2 17-arachidonate (19%), E_2 17-oleate (14%), and E_2 17-stearate (4%) (Larner et al. 1993), while in bovine endometrial tissue, E_2 17-arachidonate is the most abundant component (Mellon-Nussbaum et al. 1982). E_2 was esterified at the highest rate of the steroids tested and is the most potent estrogen with a long lasting effect. One may ask if the E_2 esters are secreted by tissues or enzymatically synthesized in situ. This question was answered by Jones and James (1985). Their studies demonstrated that the esterification of E_2, but not testosterone, is catalyzed by LCAT in blood. Further investigations have found that in follicular fluid, the E_2 esters are also synthesized by LCAT (Roy and Belanger 1989b, Pahuja et al. 1995).

In a recent study, Shwaery et al. (1997) observed that physiological levels of E_2 could be esterified in plasma in vitro and they postulated that the nonpolar forms of E_2 were fatty acid esters. LCAT is supposed to be the enzyme necessary for E_2 esterification in plasma. We repeated these experiments using non-radioactive E_2 as well as E_2 17-stearate and a quantification method based on GC-MS. In line with the findings of Shwaery et al. (1997, 1998), we observed accumulation of esterified E_2 in LDL following plasma incubation, a phenomenon which was inhibited in the presence of sulphydryl reagent, DTNB also known to inhibit LCAT. Moreover, we also found that some esterification of free E_2 occurred in HDL during incubation in Celite dispersion, suggesting that some of the LCAT enzyme remained associated with HDL. Based on these studies, we postulate that the esterification of E_2 is catalyzed by LCAT which is associated with HDL in blood. Further studies are needed to explore the meaningful physiological role of E_2 esters.

6.3. Binding characteristics of isoflavone and 17 β-estradiol esters to LDL

Using the Celite transfer system, we demonstrated that incorporation of the oleic acid esters of genistein and daidzein into LDL (molar ratio 2-7:1) was comparable to that of α-tocopherol (molar ratio 6:1) (Esterbauer et al. 1992) whereas the stearic acid esters of genistein were less effectively incorporated, as were also the free isoflavones. The ineffective incorporation of unesterified isoflavones into LDL can be explained by their relatively low lipid solubility (Cunningham et al. 1997). The ineffective incorporation of genistein stearates (containing no double bonds) is in line with a previous study suggesting preferential incorporation into LDL of esters of unsaturated fatty acids containing at least one double bond (Krieger et al. 1979), possibly because the protein, phospholipid component of LDL, or both, have a specific ability to interact with long chain fatty acyl groups with double bonds permitting more efficient penetration of the lipoprotein surface. Preliminary structural studies by molecular modeling (Still et al. 1992) show that in the distearates the fatty acid chains at the opposite ends of the isoflavone nucleus may be folded back shielding the same face of the aromatic ring system whereas in the dioleates, the isoflavone nucleus is apparently sandwiched between the fatty acid chains. This configuration could possibly have important repercussions in terms of their incorporation into LDL particles. Therefore, the binding efficacy of isoflavones to LDL depends on their lipid solubility and fatty acid substitutions.

Unesterified E_2 was not significantly incorporated into lipoproteins under any of the incubation conditions (plasma, Celite dispersion) used, presumably because of its limited fat-solubility. The increased lipid-solubility caused by blocking the 17β-hydroxyl group with a fatty acyl residue markedly facilitated incorporation of E_2 into lipoproteins. We speculate that E_2 17-esters could become orientated on the lipoprotein surface in the same way as free cholesterol which has the carbon side chain directed towards the lipoprotein core and the ring structure reaching the surface. In analogy, the lipophilic fatty acid carbon chain of the E_2 17-esters could stick into the lipoprotein core with the antioxidative structure (A ring with 3-hydroxyl group) being positioned on the surface.

E_2 could be esterified in many tissues and also in plasma (Hochberg 1998). The fatty acid esters of E_2 are a unique family of long-acting estrogens that circulate in blood. In circulation, almost all of the E_2 17-fatty acid esters are bound to lipoproteins (Larner et al. 1987) and the transfer between lipoproteins (LDL, HDL) was not supposed to be mediated by CETP as CEs usually do (Provost et al. 1997, Hesler et al. 1988). Another LDL-associated plasma protein, lipid transfer inhibitor protein (LTIP or apoF), has also been reported to regulate the transfer of CE from HDL in plasma (Serdyuk and Morton 1999). We do not yet know if this protein plays any role in the regulation ot the transfer of estrogen esters. Based on their studies, Leszczynski and Schafer (1990, 1991) suggested that incorporation of E_2 was dependent on its esterification by LCAT which was associated with HDL. In contrast to the continuous LCAT-induced accumulation of lipophilic E_2 in HDL, the E_2 uptake into LDL was limited reaching an equilibrium after 10 hours.

These studies demonstrate that esterified derivatives of isoflavones and E_2 are relatively readily able to bind to lipoproteins in contrast to their unesterified forms, suggesting that esterification is the prerequisite to facilitate the incorporation of these substances into lipoproteins.

The binding of E_2 esters to lipoproteins may provide a unique pathway by which the E_2 esters are delivered into cells via a lipoprotein receptor-mediated mechanism and a critical contribution

to their long-acting estrogenic effect and metabolic characteristics. Their physiological and pharmacological roles might be important in humans.

6.4. Influence of LDL carrying isoflavones on U937 cell proliferation

U937 cell proliferation was markedly inhibited with a concentration-dependent manner by direct addition of genistein or daidzein to the cell culture medium, although low concentrations of genistein (0.1-0.5 μmol/L) caused a temporary boost in cell proliferation. The inhibitory effect may not be related to estrogen receptors since these cells do not have them (Danel et al. 1985), but may be explained by the genistein-induced increase of LDL receptor gene expression (Liu et al. 1993, Kanuck and Ellsworth 1995) facilitating delivery of LDL cholesterol into cells. At higher genistein concentrations, this effect is suppressed by increasing amounts entering the cells. The mechanisms by which genistein suppresses cell proliferation probably involved inhibition of tyrosine kinases (Akiyama et al. 1987, Fotsis et al. 1993) and DNA topoisomerase (Okura et al. 1988).

The small amounts of free isoflavones and their stearates incorporated into LDL in Celite dispersion did not influence cell proliferation compared with control LDLs which were processed in the same way except in the absence of isoflavones. However, LDLs containing 7-oleates or 4´,7-dioleates of genistein and daidzein exhibited a significant inhibitory effect, while the strongest effect was shown by daidzein 4´,7-dilinoleate (Study II). In contrast, the 4´-oleates of genistein and daidzein did not reduce cell proliferation although high incorporation was achieved. The common feature of these antiproliferative isoflavones is the presence of a fatty acid residue at C-7, suggesting that the antiproliferative efficacy was somehow dependent on the presence of this esterified oleyl or linoleyl residue. Comparison of the inhibitory effects on U937 cell proliferation demonstrated that only much smaller concentrations of isoflavone equivalents (<0.5 μmol/L) (Section 5.4) carried in LDL were needed to cause similar degree of inhibition compared with free genistein and daidzein added directly to the medium (IC_{50} for genistein 16.0 μmol/L and for daidzein 39.6 μmol/L) (Study II, Fig.2). This indicates that intracellular delivery of isoflavone esters via the LDL receptor pathway was a more effective way of influencing cell functions compared to addition directly to the medium. On the other hand, the water-soluble unesterified isoflavones in the medium may have entered the cells by a less efficient mechanism.

Based on this interesting finding, we may speculate that even when only very small amounts of isoflavones are esterified and incorporated into lipoproteins, they could be delivered by LDL into target cells via LDL receptor pathway exerting their biological effects intracellularly. Theoretically, this could be achieved pharmacologically by administration of synthetic isoflavone esters subcutaneously. However, more clinical data are needed before the amounts can be prescribed.

6.5. Antioxidant function of isoflavone and 17 β-estradiol esters enriched LDL

Studies on the oxidation resistance of LDLs containing lipophilic isoflavone derivatives such as 4´,7-dioleates of daidzein and genistein, and 7-oleate of daidzein demonstrated statistically significant prolongations of lag time. A trend prolongation of lag time could also be brought about by LDL-bound genistein 7-oleate. However, none of the 4´-oletaes of genistein and daidzein contained in LDL increased oxidation resistance, suggesting the antioxidant property of isoflavones is structure-related. Moreover, there was no correlation between the concentrations

of any of the above mentioned isoflavone esters and lag times, suggesting that these antioxidants behave differently from typical lipid peroxyl scavengers such as butylated hydroxytoluene (BHT). One possible explanation is that isoflavones might have themselves promoted lipid oxidation under our experimental conditions. Flavones and isoflavones may act as antioxidants against peroxyl and hydroxyl radicals but may also serve as prooxidants in the presence of Cu^{2+} (Cao et al. 1997). For example, it has been demonstrated for several flavones that their hydroxyl radical absorbing capacity increased proportionally to their concentration at low concentrations, but after reaching a maximum value, decreased with increasing concentrations (Cao et al. 1997), due to Cu^{2+} initiated prooxidant activity at high concentration.

Hydroxyl groups are supposed to be necessary for isoflavones and flavones to have antioxidant activity (Rice-Evans et al. 1996). For some flavonoids with similar basic structures the peroxyl radical scavenging capacity is proportional to the number of free hydroxyl groups on their structure (Cao et al. 1997). This raises the question how it was possible that significant antioxidant activity was present despite blocking of the active hydroxyl groups simultaneously at 4´ and 7 positions leaving only the 5-OH group free in genistein and none in daidzein (**Fig. 6**). One possibility is that the C-4 carbonyl group and the double bond between C-2 and C-3 of the C ring exert antiperoxidative activity (Cook et al. 1996). Also, keto-enol tautomerism at the C-4 position (Chang et al. 1995) has been suggested in urinary daidzein metabolites after soy feeding in humans (Kelly et al. 1993) providing a potentially active OH group in the enol form. One may assume that isoflavone molecules are positioned at the surface of the LDL particle in such a way that the fatty acyl residues are interspersed with the fatty acyl residues of the phospholipid monolayer. The ring structure with the unsaturated C-ring on the lipoprotein surface facing the aqueous phase would be anchored to the lipid layer by the fatty acyl carbon chains.

Other quite different mechanisms could, in theory, be involved in the antioxidant activity. For example, it is known that binding of Cu^{2+} to apoB of LDL initiates lipid peroxidation (Esterbauer et al.1992, Kuzuya et al. 1992, Gieseg and Esterbauer 1994). Isoflavone phytoestrogens are known to bind tightly to soybean protein (Pratt and Birac1979), and it is conceivable that esterified isoflavones could also become bound to apoB or in some other way cause steric hindrance blocking the apoB binding sites to which Cu^{2+} is normally attached, thus inhibiting lipid peroxidation.

The mechanisms involved in the antioxidative protection of estrogens on lipoproteins are not fully understood. It was assumed that E_2 may inhibit LDL oxidation by regenerating or maintaining endogenous antioxidants such as α-tocopherol in LDL particles (Mukai et al. 1990, Esterbauer et al. 1987). However, this mechanism is not supported by other studies (Shwaery et al. 1997, Ayres et al. 1998). Ayres et al. (1998) postulated that the antioxidative protection of E_2 could be due to inhibition of the generation of superoxide ($O^{\bullet}_2$), H_2O_2, or $OH^{\bullet}$ radicals, thus further preventing chain propagation.

The way in which estrogen esters contained in lipoprotein particles might inhibit oxidation could depend on their positioning in the lipoprotein particle. The phenolic A ring with a free hydroxyl group has been proposed to be necessary for the antioxidant effect (Nakano et al. 1987, Ruiz-Larrea et al. 1994, Miller et al. 1996, Mukai et al. 1990). Lipid peroxidation is thought to start from the PUFA of the PL on the surface of LDL and then propagate to core lipids (Witztum 1994). We speculate that E_2 17-esters could become orientated on the lipoprotein surface in the way that the lipophilic fatty acid carbon chain of the E_2 17-esters could stick into the lipoprotein

core with the antioxidative structure (A ring with 3-hydroxyl group) being positioned on the surface. This positioning might be important since unesterified estrone (E_1), although incorporated more effectively into LDL than E_2, did not influence LDL oxidation resistance (Shwaery et al. 1998).

The antioxidant capacity was observed only when the concentration of esterified E_2 in LDL had risen to 500 pmol/mg LDL (0.27 molecules /LDL particle or 1 E_2 equivalent per 3 LDL particles) or more as demonstrated in our **study IV** (**Table 5**). One possibility is that the intact phenolic hydroxyl groups may directly intercept free radical species inhibiting Cu^{2+}-mediated oxidation of LDL. A recent report suggests that E_2 inhibits the effects of hydrogen peroxide, superoxide and hydroperoxyl radicals, thereby acting as a chain-breaking antioxidant (Ayres et al. 1998). Based on their findings that physiological concentrations of E_2 in plasma protected LDL from Cu^{2+}-induced oxidation, Shwaery et al. (1997) speculated that the presence of an E_2 derivative on the surface of the LDL particle could catalyze a process promoting stable modifications of other LDLs and interfere with Cu^{2+} binding for initiation of the oxidation process.

6.6. Relevance of the results with regard to atherosclerosis

Our **Study I** demonstrates that cholesterol can be taken up by myelomonocytic U937 cells via effective binding affinity of LDL-apoB to LDL receptors. Accordingly, other lipophilic substances carried in LDL particles could also be delivered into cells via the LDL receptor pathway. Therefore, this cell assay provides a model system to detect apoB gene mutations but also to study the effects of biosubstances delivered by LDL into cells on lipid metabolism and cellular functions.

Cell proliferation is one of the characteristics of atherosclerotic lesion formation (Ross 1993). Cells found within the lesion include SMCs, monocytes/macrophages, and lymphocytes (Raines and Ross 1995). Studies have demonstrated that soy isoflavone phytoestrogens, particularly genistein, can inhibit all vascular cell proliferation presumably involved in atherosclerotic lesion formation (Wilcox and Blumenthal 1995, Fotsis et al. 1993, Shimokado et al. 1994, 1995). Although we have demonstrated that lipophilic phytoestrogen esters carried in LDL inhibit U937 cell proliferation via LDL receptor pathway (Study II), we do not know if these effects could also be observed in SMCs and such investigation will no doubt expand the meaningfulness of isoflavones.

Evidence exists that Ox-LDL plays an important role in the initiation and progression of atherosclerosis (Heinecke 1998, Ylä-Herttuala 1998, Witztum and Steinberg 1991, Steinberg 1997a) and that the oxidation of LDL must occur in the arterial wall rather than in the blood which is indicative of a potential antioxidant defense system. Based on the oxidation theory, it is plausible that antioxidant treatment should be effective in the prevention and retardation of atherosclerosis.

Isoflavone phytoestrogens and human endogenous estrogens are potent antioxidants in vitro (Hodgson et al. 1996, Ruiz-Larrea et al. 1997, Nakano et al. 1987, Mukai et al. 1990, Huber et al. 1990, Maziere et al. 1991, Rifici and Khachadurian 1992, Subbiah et al. 1993) and ex vivo (Tikkanen et al. 1998, Kanazawa et al. 1995, Sack et al. 1994, Wakatsuki et al. 1998, Wilcox et al. 1997). We speculate that lipoproteins which carry lipophilic phyto- and endogenous

estrogens in vivo might migrate into the arterial wall where LDL oxidation occurs, delaying the oxidation occurrence. Our in vitro studies (III, IV) provide evidence that both esterified phytoestrogens and estrogens could be incorporated into lipoproteins to protect them against oxidation. As known, estrogens can be esterified in plasma and the lipoidal derivatives can be incorporated into lipoproteins. In view of the structural similarity between phytoestrogens and estrogens, the conversion of the esterified form of isoflavone phytoestrogens and incorporation into lipoproteins may also exist. However, this in vivo possibility is still under investigation. Even though the physiological content of these lipophilic derivatives may be small or undetectable in vivo, this can be raised in a pharmacological way. For example, the esterifid form of phytoestrogens or estrogens could be administered subcutaneously to be taken up by lipoproteins. When a certain concentration is reached, they might prevent lipoprotein from oxidation and thus have a significant impact on atherosclerosis. In principle, these antioxidants could also be incorporated into LDL in vitro during LDL-alpharesis and then retransfused to the human body to protect LDL against oxidation in blood circulation and arterial wall. The following (**Fig. 6**) is a schematic figure depicting the possible role of isoflavones and estrogens carried in LDL in protection against atherosclerosis.

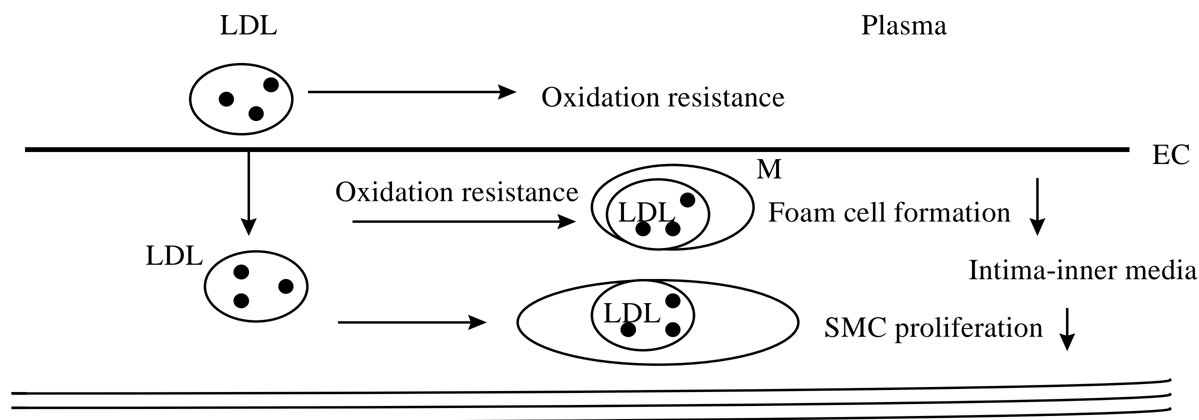

Figure 6. Possible role of isoflavone and estrogen esters on atherosclerosis.
⑦ Represents isoflavone or E_2 esters

7. SUMMARY AND CONCLUSIONS

LDLs transport about 75% of total cholesterol in plasma and play a major role in the development of atherosclerosis and CHD. LDL is a spherical particle, 22-28 nm in diameter. Its oily core is composed of CE and TG, and shielded by a layer of PL, FC, and an apoB-100. In theory, other lipophilic substances could also be incorporated into the LDL in addition to lipoprotein lipids. The lipophilic bioactive substances contained in LDL may be delivered into cells via LDL-receptor pathway influencing its metabolism and cellular functions. The lipophilic antioxidants carried in LDL could protect it against oxidation. Our findings can be summarized as follows:

I LDL can deliver cholesterol into cells for their growth via LDL-receptor pathway. The U937 cell proliferation is proportional to the cholesterol uptake. Defective binding of LDL-apoB to its receptors can affect cholesterol uptake and further impair cell proliferation. This cell proliferation assay can be used not only for determining the binding affinity of LDL but also for studying the delivery of lipophilic biosubstances carried in LDL into cells via the LDL receptor pathway and their biological effects.

II Isoflavone fatty acid esters can be incorporated into LDL in vitro and delivered into cultured U937 cells via the LDL-receptor pathway. LDL containing 7-oleates or 4´,7-dioleates of genistein and daidzein as well as daidzein 4´,7-dilinoleate remarkably reduced the cell proliferation.

III Esterification with fatty acids rendered soybean derived isoflavones fat-soluble enabling them
to become incorporated into LDL particles. Some isoflavone oleic acid esters (i.e., 7-oleates or 4´,7-dioleates of genistein and daidzein) contained in LDL particles increased oxidation resistance of this lipoprotein.

IV Human endogenous estrogens, mainly in esterified form, can be incorporated in vitro into both LDL and HDL. E_2 can be esterified by LCAT in plasma. The amounts of E_2 esters incorporated by incubation in plasma did not influence oxidation resistance of these lipoproteins in our study. Greater amounts of estrogen incorporated (> 500 pmol/mg LDL protein) by using the Celite transfer system increased the oxidation resistance. Therefore, lipophilic esterified estrogens carried in lipoproteins can protect them against oxidation.

In conclusion, these studies have demonstrated that LDL participates in the delivery of bioactive substances (e.g., lipophilic esterified isoflavones) into target cells influencing their functions. In addition, bioactive substances such as lipophilic antioxidants (isoflavones and estrogens) contained in lipoproteins may protect them against oxidation. However, the degree of protection of lipophilic antioxidants to LDL or growth inhibition to the cells of biosubstances delivered by LDL may not be sufficient to protect individuals at physiological levels and their in vivo effects need further investigation.

These findings provide the possibility to make isoflavone phytoestrogens and exogenous estrogens fat-soluble by esterification so that these substances can be incorporated into LDL. The lipophilic substances carried in LDL could then be delivered specifically into target cells via the LDL receptor pathway and influence the cellular functions or prevent the lipoprotein from oxidation. This would be a more effective and efficient way for drug therapy. In principle, synthetic esters of these substances could be administered percutaneously, and if taken up by lipoproteins, they might exert antiproliferative and antioxidative as well as weak estrogenic effects. Enrichment of bioactive substances in plasma lipoproteins such as isoflavones and other antioxidants could be achieved by diet supplementation. It would also be of interest, in the future, to further explore how to catalyze these biosubstances to be esterified in vivo, facillitate the binding to lipoproteins, and study their physiological role.

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