Chapter I

Introduction
Heart is a muscular pump, which pumps about 14,000 litres of blood through the blood vessels everyday providing oxygen and essential nutrients to every cell in the body. Heart is also an electrical organ (Hoffinan, 1962) and an endocrine organ (Sagnella, 2002). Like any organ, heart and the cardiovascular system can have a variety of malfunction and disorders collectively called as cardiovascular diseases. Cardiovascular diseases (CVD) are the major cause of death globally. An estimated 17.3 million people died in 2008 representing 30% of all deaths (WHO 2011).

**What causes heart diseases?**

There are over 60 different types of cardiovascular diseases. Hence, the real cause of cardiovascular disease depends on the particular cardiovascular disease in question. A list of some of the cardiovascular diseases and their proximate cause is given in table 1.1

**Table 1.1 Causes of cardiovascular diseases**

<table>
<thead>
<tr>
<th>Cardiovascular diseases</th>
<th>Proximate cause</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angina</td>
<td>Temporary decrease in supply of oxygen to heart muscle mainly because of Coronary Artery Disease (CAD)</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>Not known</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>Atherosclerosis</td>
</tr>
<tr>
<td>Heart attack</td>
<td>Severely narrowed or completely blocked coronary artery due to blood clot</td>
</tr>
<tr>
<td>Stroke</td>
<td>Severely narrowed or completely blocked artery in the brain due to clot or rupture of a blood vessel in the brain.</td>
</tr>
<tr>
<td>High blood pressure</td>
<td>Not known</td>
</tr>
<tr>
<td>Congestive Heart failure</td>
<td>Damaged or weakened heart muscle mostly because of CAD, High BP or Diabetes.</td>
</tr>
</tbody>
</table>
Although different cardiovascular diseases have different risk factors, many of the risk factors are common. They are

- Age
- Gender
- Ethnicity
- Family history of cardiovascular diseases
- High blood pressure
- Diabetes
- Obesity
- Physical inactivity
- Cigarette smoking

These risk factors are classified as modifiable risk factors, such as cigarette smoking, physical inactivity and obesity and non-modifiable risk factors such as age, gender and ethnicity.

Since the actual cause of atherosclerosis is not known, several theories and hypotheses have been put forth to explain the origin and development of the atherosclerotic condition.

**The Diet-Heart Hypothesis:**

For over half a century the Diet-Heart hypothesis has dominated thinking about heart diseases. According to this theory, when we eat foods rich in saturated fats and cholesterol, the saturated fat is converted to cholesterol. The cholesterol is then deposited in the arteries in the form of plaque. This would result in blockage of the artery. If the blockage becomes severe, or if a clot forms and cannot go past the blockage, the heart becomes starved of oxygen and nutrients and a heart attack occurs.

This hypothesis was based on evidence that came from different experiments as follows:

1. Diet experiments reported that saturated fat increased total cholesterol levels.
2. A reported positive correlation between saturated fat intake of a country and the incidence of death from Coronary heart disease (CHD).

3. Presence of cholesterol in atherosclerotic plaques

4. Population surveys which showed that higher serum cholesterol levels correlated with higher incidence of death from CHD

5. Experiments with rabbits when fed large amounts of cholesterol developed high serum cholesterol levels and deposits of cholesterol in their arteries.

This theory was further expanded to propose that if total cholesterol is lowered by reducing saturated fat intake, protection against heart disease, or delay in the development of atherosclerosis or manifestation of CHD will result.

Although the Diet-Heart theory has been presented as a fact, it is still an unproven theory since much of the experiments on which it is based are controversial. This theory has not been proved by epidemiology, lipid biochemistry or post mortem pathology.

**Inflammation Hypothesis**

Inflammation is the nonspecific response of the immune system to trauma, infection and allergy. When a pathogen invades a host, the immune system will mount an attack on the pathogen which is characterized by redness, swelling, pain and heat. The immune system may also release proinflammatory molecules in order to overcome the threat. Once the threat has been removed, the healing process starts to repair any damage that may have been caused. The inflammation is turned off.

When the proinflammatory reactions are not completely turned off, continuing to stimulate proinflammatory cells results in acute inflammation. Acute inflammation has been attributed as the cause of many diseases like cancer, diabetes and cardiovascular diseases.

Several of the conventional risk factors for CVD have a strong influence on chronic inflammation.
i) **Smoking:** In addition to active smoking, passive smoking has been identified as a risk factor for CVD. (Pitsavos et al 2003, Rampach et al 2006) cigarette smoke contains free radicals. It has been estimated that one puff of cigarette smoke exposes the smoker to $10^{15}$ free radicals, which is a source of oxidation stress. (Pryor and Stone, 1993).

ii) **Stress:** Studies have shown that people with higher psychosocial stress and depression display elevated C-reactive protein and IL-6, both of which are markers of inflammation. Hence chronic psychosocial stress can lead to chronic inflammation.

iii) **Exercise:** While exercise is good over exercise is bad. Exercise increases systemic inflammation. Exercise increases shear stress on the arterial wall, which causes endothelium to be less permeable to LDL, produce more nitric oxide, which is a potent inhibitor of LDL oxidation. In general, it induces adaptive response. However over exercise can be proinflammatory.

iv) **Genetics:** Single nucleotide polymorphism in the IL-6 gene has been implicated in over expression of IL-6 genes during an inflammatory reaction.

v) **Visceral fat:** Visceral fat is treated by the innate immune system as an invader. Presence of macrophages has been demonstrated in visceral fat. The chemicals released by these macrophages get into circulation. Since the visceral fat cannot be completely handled by the innate immune system, there is a persistent chronic level of inflammation. Thus eating more, eating more often and change in the dietary patterns together lead to chronic state of inflammation.

vi) **Chronic infection:** The idea that chronic infection may be the cause of atherosclerosis is not new. It is the alternate theory to the Diet-Heart theory of heart disease.
Evolutionary cause of heart diseases

It is said that we are socially in the 21\textsuperscript{st} century, but genetically we are in prehistoric times. Consequently many of the genetic risk factors that were suppressed due to the life style then are now being expressed because of changed life style.

While the risk factors describe “proximate” cause of cardiovascular diseases, William and Nesse (1991) suggested the role of evolutionary biology in explaining the cause of diseases. This is often referred to as ‘Darwinian Medicine’ (William and Nesse 1991). Sequencing of human genome and primate genome has also provided important insights into human diseases (Nesse et al 2006).

Some of the candidate genes and their genetic role are shown in Table 1.2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Functions</th>
<th>Selection</th>
<th>Implication for CVD</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE2</td>
<td>Blood pressure</td>
<td>Increased nucleotide diversity</td>
<td>An insertion(^\prime)/deletion polymorphism is associated with hypertension</td>
<td>Wray et al 2008</td>
</tr>
<tr>
<td>Apo E</td>
<td>Lipid metabolism</td>
<td>Increased frequency of haplotypes carrying E3 allele</td>
<td>Ancestral allele E4 associated with CVD</td>
<td>Fullerton et al 2000</td>
</tr>
<tr>
<td>SCARB1</td>
<td>Lipid metabolism</td>
<td>Low levels of nucleotide diversity</td>
<td>Non Synonymous variant associated with high HDL-C and low LDL-C</td>
<td>Le Jossee et al 2004</td>
</tr>
<tr>
<td>ANGPTL 4</td>
<td>Lipid metabolism</td>
<td>Excess of rare allele</td>
<td>Loss of function alleles associated with lower plasma TG and higher HDL</td>
<td>Romeo et al 2007</td>
</tr>
<tr>
<td>TGF7L2</td>
<td>Energy metabolism</td>
<td>Long haplotypes in East Asians</td>
<td>Two polymorphisms associated with Type 2 Diabetes</td>
<td>Helgason et al 2007</td>
</tr>
</tbody>
</table>

In addition, work done in our lab has shown the association of a promoter polymorphism in the PON1 gene with increased risk of cardiovascular diseases (Jyothi et al 2011)

**Response to injury Hypothesis**

The response-to-injury hypothesis was proposed by Ross and Glomset (1973). The proposed initial step in atherosclerosis is endothelial dysfunction which leads to a number of compensatory responses. The initial injury enhances the adhesion of leukocytes and platelets to the endothelium, altering the physiology from an anticoagulant one to a procoagulant one. The adhering bioactive agents initiate an inflammatory response.

More recent studies have shown that endothelial dysfunction is not seen in atherosclerotic lesion. Hence, the original hypothesis has undergone refinement to state that endothelial dysfunction is sufficient to initiate atherogenesis through increased endothelial permeability of atherogenic lipoprotein (Ross, 1999)

Although this hypothesis appears quite reasonable, experimental evidence is not available to support the hypothesis. LDL leaks through the arterial wall even through the endothelium are normal. However accumulation of LDL takes place only at sites of atherosclerosis (Schenke and Zilversmit, 1998). Hence a modified version of the theory has emerged.

**Response to retention hypothesis**

According to this hypothesis, retention of LDL is the key event in development of atherosclerosis. This is mainly because of transendothelial lipoprotein delivery. However, it is limited to particles of size less than 70 nm in diameter. (Stoker and Keaney, 2004)

The retention of lipoprotein within the artery wall is linked to the presence of components on the extracellular matrix of the endothelial cells. The association between LDL and proteoglycans is mediated through specific residues on the Apo B protein. Even Apo B_{48} is also capable of such interaction (Boren et al 1998, Skalen et al 2002).
Introduction

The oxidative modification hypothesis

According to this theory, LDL is not the cause of atherosclerosis, but oxidized LDL is the real cause. The LDL-lipids are subject to oxidation and as a consequence the Apo B-100 gets modified such that the net negative charges on LDL increase. This is cleared by binding to scavenger receptors on macrophages, leading to the formation of foam cells. (Haberland et al 1984, Steinberg et al 1989)

LDL oxidation mechanism has been worked out invitro in order to understand the possible invivo process. LDL lipids can get oxidized without any modification of apo B100. This is called minimally modified LDL. The minimally modified LDL has been shown to induce the synthesis of monocyte chemotactic protein-1 (MCP-1), resulting in the recruitment of inflammatory cells (Navab et al 1991). More extensively oxidized LDL is termed “Ox-LDL” and is chemotactic to monocytes and T-lymphocytes. The recruitment of inflammatory cells may result in the continued oxidation of LDL leading to a formation of a full blown atherosclerotic lesion.

Each of these theories has attempted to explain the complex cellular events of atherosclerosis. Although each theory has its own initiating event for atherosclerosis, many of the steps in the progression of atherosclerosis follow a common path, involving common players in the process. This study focuses on the role of HDL in the initiation progression and prevention of atherosclerosis.
Atherosclerotic disease is highly prevalent throughout the world. Atherosclerosis is a complex disorder, the development of which is dependent on a broad array of histological, oxidative, inflammatory and thrombotic influences. Atherosclerosis begins at a young age and its rate of progression is significantly influenced by well-known risk factors, including age, dyslipidemia, hypertension, cigarette smoking, obesity, sedentary lifestyle, and diabetes mellitus. Since the early 1980’s, considerable investigations have shown that the control of risk factors through lifestyle modification and pharmacologic intervention slows or even reverses the course of the disease and decrease risk for such complications as myocardial infarction (MI), and stroke. Early identification and treatment of risk factors are crucial to the longterm prevention of cardiovascular disease given the fact that the number of coexisting risk factors, their severity, and the duration of exposure determine lifetime risk.

The Prevalence of CHD is rapidly increasing in India. It has become an “EPIDEMIC”. It is a major contributor for mortality and morbidity. In the year 1990 cardiovascular disease was responsible for 2.39 million deaths and the Nation incurred a loss of 28.59 million disability adjusted life years. It is expected that deaths due to HEART ATTACK will be 295 per 1, 00,000 population by the year 2015. Cardiovascular disease will account for 33.5% of total deaths by the year 2015, and would replace infectious diseases, as the number one killer in the Indian Population. The Prevalence was 1% in the urban India (Delhi) in the year 1960. Now the prevalence is 10% (Delhi) Rural India has also witnessed a similar increase. (Gupta R 1996, 2003).

HDL-cholesterol and coronary artery disease

The first indications of the protective role of HDL arose from epidemiological studies during the 1970s (Rhoads GG et al, 1976, Miller GJ et al. 1975). The Framingham study subsequently showed that HDL was the lipidemic factor with the
Chapter I

Introduction

strongest predictive capability for the development of coronary artery disease in men and women over 49 years of age (Castelli WP et al 1986). Newer studies confirmed these findings and showed that an increase in HDL by 1 mg/dl leads to a reduction in cardiovascular risk by 2% in men and by 3% in women (Gordon DJ et al. 1989). The findings of certain clinical trials also showed that therapeutic interventions aimed at increasing HDL levels reduced the risk of coronary heart disease. Thus, the Helsinki Heart Study showed that increasing HDL by 11% via administration of gemfibrozil reduced the risk of coronary events in men (Frick MH et al.1987). Moreover, in the recent Veteran Affairs HDL Intervention Trial, although the administration of gemfibrozil did not lower LDL levels, HDL increased by 6% and the risk of both death and non-fatal myocardial infarction was reduced significantly (Rubins HB et al. 1999). The role, therefore, of HDL-cholesterol in the prevention and treatment of atherosclerosis is coming to seem more and more important. In recent years this has led important international scientific bodies to issue treatment guidelines aimed at increasing HDL levels (Sacks FM et al 2002, The UK HDL-C Consensus group).

HDL and LDL are the two prominent lipoproteins that play a major role in heart diseases.

Lipoproteins

Lipoproteins have core of hydrophobic cholesteryl and triglycerides surrounded by a hydrophilic surface coat of phospholipids and apo-lipoproteins. Some of the surface apo-lipoproteins identify the different types of lipoproteins while others provide enzymatic ligands to facilitate the process of biochemical lipid balance and cellular function.

Each of the lipoprotein types has many functions, carrying various amounts of cholesterol and triglyceride, interacting with the endothelium and interacting with each other to maintain cellular homeostasis-maintaining it, that is, until our western lifestyle, genetic disorders, other diseases, or medications cause things to go terribly awry.
Chapter I

Introduction

Lipoproteins can be divided into two groups:

1. The atherogenic lipoproteins: these all have either an apo B-48 (Chylomicrons or chylomicron remnants) or an apo B-100 (VLDL, IDL, LDL and Lp (a)).

2. The anti-atherogenic lipoproteins (HDL): these all contain apo A-I or apo A-II.

Genetics, other medical conditions, medications, and lifestyle influence both the number and content of the lipoproteins, which in turn cause or prevent CVD.

Figure 1.1 Basic structure of lipoproteins

Each lipoprotein is characterized by specific surface apo-proteins, a specific lipid composition and size. These are indicated in the Figure (1.2).
The atherogenic lipoproteins

All of the atherogenic lipoprotein contain one apo B per lipoprotein: intestinally-produced apo B-48 on chylomicrons and chylomicron remnants; and hepatic-produced apo B-100 on VLDL, IDL, LDL, and Lp(a).

Chylomicron formation

The chylomicron is a large lipoprotein made in the wall of the jejunum. Chylomicrons carry triglycerides and cholesteryl ester from the gut. They deliver triglycerides for energy or fat storage to the peripheral adipose and muscle tissues, and then, as much smaller remnant, they deliver their cholesteryl ester to the liver. Under normal physiologic conditions, the chylomicron has a short half-life of 30 mins before the TG has been hydrolyzed away and the chylomicron remnant is taken up by the B/E receptors on the surface of the liver.

After a meal, the chylomicron is 90% TG and 10% cholesteryl ester. The outer wall of the chylomicron contains its identifying and gut-produced apo-lipoprotein apo B-48; apo E; the ligands apo C-II and apo C-III, which control the rate of hydrolysis; and apo A-I and A-II, which break away and let form HDL. After most of the TG is hydrolyzed, the apo E on the chylomicron remnant efficiently attaches to the E part of the B/E or LDL-Receptor of the liver.

The hepatic LDL-receptor is also called the LDL-R or LDL B/E receptor because it attaches to both apo B and apo E. In descending order of preferences, the LDL-R attaches to apo E3 and apo E4; then and with less efficiently, to apo B; and then poorly to apo E2.

LDL-R is a key player in the control of serum cholesterol and particle levels because the number of LDL-R determines the rate at which LDL-C and LDL are removed from chylomicrons, chylomicron remnants, VLDL, IDL and LDL.

Chylomicron catabolism

Hydrolysis of TG from chylomicron requires lipoprotein lipase (LPL), which is located on the endothelial lining of cardiac and skeletal muscles and adipose tissue.
Chapter I

Introduction

The hydrolysis process is controlled by the ligands apo C-II and apo C-III. Apo C-II activates and apo C-III deactivates hydrolysis by LDL. This turn-on-and-off process is necessary to ensure that TG is delivered to all parts of the body as the chylomicron tumbles through the circulation. As the chylomicron loses TG, it decreases to perhaps 10% of its original volume, becoming a chylomicron remnant. As the chylomicron loses volume and surface area, apo A-I, apo A-II and apo A-IV, break free to contribute to nascent HDL.

VLDL metabolism

VLDL is produced in the liver by the combination of apo B-100 proteins, which the liver produces in abundance, and free fatty acids (FFA), which are formed into TG by the enzyme DGAT (diglyceride acyl transferase). VLDL ferries TG to the systemic tissues for energy or storage. Under physiologic conditions, 80% of each VLDL is TG, the remainder is cholesteryl ester. Under the pathological states of insulin resistance, only 60% of each VLDL is TG.

When existing in the liver, each VLDL carries a large amount of TG and a small amount of cholesteryl ester surrounded by an outer wall of phospholipids, apo B-100, apo-CI, apo C-II, apo C-III and apo E.

Hydrolysis of the TG in the VLDL

The TG in the VLDL is initially hydrolyzed by lipoprotein lipase (LPL), the same enzyme that hydrolyzes the TG from chylomicrons into free fatty acids. As with chylomicrons, apo C-II activates and C-III inhibits LPL, ensuring that the VLDL tumbles through the blood stream to all parts of the body. As the VLDL is hydrolyzed, it shrinks and sheds some of its apo-lipoproteins. This intermediate size VLDL is a VLDL remnant or IDL.

This IDL can follow either of two pathways. It can be taken up by the hepatic LDL or further hydrolyzed by hepatic lipase HL. If the LDL is further hydrolyzed, it loses all surface apo-lipoproteins except apo B and becomes an LDL the only lipoprotein with only a single apoprotein on its surface: apo B. In general, half of all IDL is taken up by the liver LDL-R and half becomes an LDL-particle. VLDL has a
half life of a few hours before it is hydrolyzed to an LDL or taken up by the hepatic LDL-R.

VLDL and IDL turnover

Figure 1.3 Chylomicron and VLDL metabolism
Low Density Lipoprotein (LDL)

- Ninety percent of the atherogenic apo B particles are LDL.
- LDL under physiologic conditions contains 90 % cholesteryl ester and 10 % TG. This explains why LDL-C was a good marker for atherosclerosis-but why in an area of obesity and insulin resistance, LDL-C is less predictive.
- In some patients with insulin resistance, the LDL may only carry 60 % cholesterol, the rest having been replaced by TG through the action of CETP.
- The only surface apo-lipoprotein on LDL is apo B, an apo-lipoprotein that is least favoured by the hepatic LDL. This means that LDL is not efficiently removed from the circulation and circulates for two to three days.
- LDL stays in circulation longer than other apo B particles, and has the highest concentration of cholesterol ester making it a key cause of atherosclerosis.

LDL metabolism

LDL is the grandchild of VLDL, formed after LPL and HDL have hydrolyzed the TGs out of the VLDL and IDL. Under normal physiologic conditions, the LDL carries 90 % of the circulating cholesterol ester. We traditionally have called this LDL-C. This relationship falls apart in patients with insulin resistance or excess TG, where LDL may be filled with 60 % cholesterol and 40 % TG.

Lp(a). This is a complex and little understood lipoprotein. Lp(a) is modified apo B LDL-P with an apo-lipoprotein (a) attached. Numerous studies show that when both LDL-C and Lp(a) are elevated, the elevated Lp(a) is a risk factor for atherosclerosis. If the LDL-C is normal, Lp(a) does not appear to be atherogenic, even when elevated.

Lp(a) is genetically different from all other lipoproteins. First, Lp(a) represents a link between the atherogenecity of LDL-C and the thrombotic tendencies of plasminogen. Lp(a) has genetic components of both LDL and an apo(a) genetically similar to plasminogen. This places Lp(a) at the intersection of much of the pathology of atherosclerosis. Second, the level of Lp(a) is genetically determined. Neither
environmental factors nor pharmacology appears to impact the levels of Lp (a). Third, we do not yet understand the metabolism or the catabolism of Lp(a), or its real relationship to atherosclerosis.

**Non-atherogenic lipoproteins**

The term non-atherogenic lipoprotein means HD, a highly complex lipoprotein with multiple functions, some of which are just now being elucidated. HDL-P carries HDL from intimal macrophages back to the liver. Each HDL may have one to four surface apo A-I or A-II per particle.

**High Density Lipoprotein HDL**

- HDL is the HDL particle and HDL-C is the cholesterol carried within the HDL.
- The primary function of HDL is to carry cholesterol ester to the steroidogenic organs.
- HDL far outnumbers all other lipoproteins, but each HDL-P is small and carries only a small amount of cholesterol.

**HDL metabolism**

HDL is a complex lipoprotein that is usually—but not always antiatherogenic. The primary biologic functions of HDL-P are to deliver cholesterol ester to the steroidogenic organs (adrenal, testis and ovaries) and carry cholesterol from the arterial intima back to the liver for reprocessing into bile or back into a VLDL for another run through systemic circulation. This process is called the reverse cholesterol transport.

**HDL has many antiatherogenic functions**

HDL

1. Is responsible for reverse cholesterol transport from a cholesterol-laden macrophage in the intima back to the liver
2. Promotes vascular dilation
3. Preserves a well-functioning endothelium by increasing nitric oxide production

4. Is antioxidant

5. Is anti-inflammatory and inhibits thrombosis

Endothelial cell adhesion

Vascular and Cellular adhesion molecules.

**HDL is responsible for reverse cholesterol transport**

The most important anti-atherogenic function of HDL is reverse cholesterol transport (RCT). HDL starts as a nascent non-lipidated apo A-I molecule, may be produced in the jejunum or liver, formed as a break-away by-product of chylomicron hydrolysis or released and recirculated after hepatic delipidation of a mature HDL.

This nascent apo A-I comes in contact through the endothelium with a cholesterol-laden macrophage, which activates an ABCA-1 releases free cholesterol from the macrophage to the nascent A-I. As the cholesterol joins the apo A-I, the LCAT enzyme esterifies the cholesterol, moving the hydrophobic fat into the center of the now-maturing HDL, which then gathers surface phospholipid to make a hydrophilic surface coat. Other intimal, macrophage enzymes such as ABCA-4 directly contribute cholesterol ester to the enlarging HDL.

The HDL, which is now laden with cholesterol from vessel intima, then returns the cholesterol ester to the SR-B1 receptor on the surface of the liver. Here the HDL is delipidated and the apo A-I renters the circulation to repeat the process.
Figure 1.4 Formation of HDL and the reverse cholesterol transport process

The mature HDL can rid itself of cholesterol in one of two ways. First, the mature HDL can directly carry its cholesterol ester to either the SR-B1 receptor on either the liver or steroidogenic tissues. The SR-B1 receptor delipidates the cholesterol ester from HDL, freeing the apo A-I back into circulation, where the cycle is repeated. The enzyme CETP (Cholesterol ester transfer protein) exchanges the cholesterol ester in HDL for TGs in the VLDL. The cholesterol ester that moved from HDL to LDL, where has two possible outcomes; first, it may be carried back to the hepatic LDL-R and be removed or second, it can be carried back to the endothelium to cause more havoc.

The relationship between HDL and risk for CVD

The correlation between low HDL and increased risk for CVD is not a new concept. This relationship was first reported by Barr and coworkers in 1951 (Barr DP et al 1951) and was confirmed less than 10 years later in an Israeli cohort (Anderson GL et al 2004). Since the 1960’s, epidemiologic studies performed throughout the world have substantiated this finding and have shown that low serum levels of HDL-
C are an independent risk factor for CAD, stroke, peripheral arterial disease, premature atherosclerotic disease of the left main coronary artery, and sudden death (Weverling-ijnsburger AW et al 2003, Pearson TA et al 1979, Shah PK and Amin J 1992, Rizzo M et al 2008, Burke M et al 1999). Low HDL-C is also associated with more rapid rates of atheromatous plaque progression compared with patients with normal levels of this lipoprotein. In contrast, elevated levels of HDL-C appear to protect both men and women from developing atherosclerotic disease.

**Antiatherogenic functions of HDL and Reverse Cholesterol Transport (RCT)**

Cholesterol is an important modulator of cell membrane fluidity and is a precursor to steroid hormone and bile slats. Cholesterol can be catabolized to bile slats by hepatocytes. Peripheral somatic cells such as those found in arterial vessel walls cannot clear excess amounts of cholesterol by breaking it down into smaller byproducts. As cholesterol accumulates in macrophages to form foam cells, internalized lipid droplets will expand continuously until the cell dies unless it is stimulated to externalize excess cholesterol. HDL particles drive RCT, the process by which HDL promotes the mobilization and externalization of excess cholesterol and delivers back to the liver for disposal as either bile salts or biliary cholesterol. It is believed that RCT is among the most important antiatherogenic effects that HDL mediates (Toth PP 2001). Low serum levels of HDL may represent a state of reduced or inadequate for RCT, leading to an excess accumulation of cholesterol in the subendothelial space of blood vessels (Toth PP 2003, Toth PP 2004).

Apolipoprotein A-I (apo A-I) is secreted from both the jejunum and liver. Free or non-lipidated apo-AI can bind to phospholipids and form a hockey puck-like structure known as nascent discoidal high density lipoprotein (ndHDL) (Figure 1.5). Both Apo A –I and nHDL can bind to the surface of macrophages via the receptor ABCA 1, or ATP binding membrane cassette transport protein AI. When ABCA1 is bound by these molecular species, it transports cholesterol from the cytosol into the extracellular space (Brooks-Wilson A et al 1999). Externalized cholesterol is then esterified with a fatty acid to form cholesteryl esters by the enzyme lecithin cholesteryl acyl transferase (LCAT). Because cholesteryl esters are hydrophobic or poorly soluble in water, they become partitioned into the core of nHDL. As more and
more cholesteryl ester and phospholipids are incorporated into the particle, nHDL (nascent HDL) speciates and becomes progressively larger and more spherical, forming HDL3 and HDL2. Mutations that reduce the functionality of Apo A-I are associated with hypoalphalipoproteinemia and increased risk of CHD. Mutations in ABCA1 that reduce its capacity to bind to Apo A-I or translocate intracellular cholesterol result in hypoalphalipoproteinemia and increased risk of CHD (Vaisman BL et al 2001, Attie AD et al 2001, Hovingh GK et al 2004). Apo A-I and the nHDL that cannot be lapidated properly, are catabolised and cleared from the circulation.

HDL particles formed in this process undergo a number of fates. HDL can transport cholesteryl esters to steroidogenic organs and facilitate steroid hormone biosynthesis. HDLs can interact with cholesteryl ester transfer protein, an enzyme that exchanges cholesteryl ester in HDL for triglycerides in Apo B-100 containing lipoproteins, such as VLDL and LDL. The cholesteryl esters transferred into these lipoproteins can be delivered to the liver via the LDL receptor and the LDL receptor-related protein (Figure 1.5). This is known as indirect RCT.

Direct RCT involves the binding of HDL to other receptors on the hepatocyte surface. Two of these have been characterized. The first is scavenger receptor B-I (SR-BI), a receptor that mediates selective cholesteryl ester uptake. HDL binds to SR-BI via its Apo A-I moiety (Krieger GK et al 1999). After docking, cholesteryl esters are extracted, taken up into the hepatocyte, and the delipidated. HDL particle is extruded back into the circulation to begin another round of RCT. The second involves a protein that modulates holoparticle endocytosis of HDL (Martinez LO et al 2003). The entire HDL particle is taken up by the hepatocyte and is catabolized. The cholesterol delivered back to the liver by HDL can undergo a variety of fates. It can be repackaged into VLDL and secreted back into the circulation, or it can be catabolized to bile salts and it can be secreted into bile and the gastrointestinal tract unmodified. The process of RCT has been validated in both human and rodents (Eriksson M et al 1999, Naik SU et al 2006).

The HDLs are unique among lipoproteins because rather than driving the net deposition of cholesterol into vessel walls, they promote its extraction and delivery to the liver for catabolism and disposal. The HDLs are also unusual because they
participate in a variety of other reactions are believed to be antiatherogenic. HDL increases endothelial cell nitric oxide production, inhibits adhesion molecule expression, stimulates endothelial cell proliferation in areas of arterial injury and inhibits endothelial cell apoptosis (Ramet ME et al 2003, Levkau B et al 2004, Xia P et al 1999, Kimura T et al 2003). These changes are associated with increased vasodilation, reduced inflammatory tone, and integrity of endothelial cell layer. Oxidised LDL is the main substrate for foam cell formation; LDL is oxidized by a number of enzymes, such as myeloperoxidase and 5'-lipoygenase. HDL is able to reduce oxidized LDL. HDL is a carrier of paroxonase, glutathione peroxidase, and platelet activating factor acetylhydrolase (Aviram M et al 2000, Stremler K E et al 1991). These three enzymes reduce oxidatively modified components of LDL. HDL also exerts a variety of antiplatelet and antithrombolic effects. HDL stimulates endothelial prostacyclin production, which is both vasodilatory and antithrombotic (Vinals M et al 1999) HDLs decrease platelet thromboxane A2 production and platelet aggregability, and potentiate urokinase mediated fibrinolysis and the ability of proteins C and S to inactivate coagulation factor Va (Nofer JR et al 1998, Griffin JH et al 1999, Beitz J and Mest HJ 1986).
Antioxidative effects of HDL

1. Increases nitric oxide production and potentiate vasodilation and myocardial perfusion.
2. Suppress vascular cell adhesion molecule 1 (VCAM-1) and intercellular Adhesion Molecule 1 (ICAM-1) expression
3. Promote endothelial progenitor cell recruitment and engraftment
4. Stimulate endothelial cell proliferation and migration
5. Inhibit apoptosis by blocking activation of caspases 3 and 9.
6. There are two redox-active methionine residues in apo A-I that reduce oxidized phospholipids, lipid peroxides and hydroperoxides, and oxidized cholesterol esters, in LDL via the activity of three antioxidative enzymes:
   a. Paraoxonase (PON)
   b. Platelet activating factor acetylhydrolase (PAF-AH)
   c. Glutathione peroxidase.

Antithrombotic effects of HDL

Stimulates

1. Fibrinolysis
2. The ability of proteins C and S to inactivate coagulation factor Va
3. Prostacyclin production by activating cyclooxygenase

Inhibits

1. Thrombin-mediated platelet aggregation
2. Platelet activation
3. Platelet thromboxane A2 production
4. Tissue factor production.
5. And stimulates plaque regression in animals with atherosclerosis.
Antiatherogenic Activity of HDL

One of the mechanisms by which HDL may be antiatherogenic is its ability to protect LDL against oxidation. Several HDL-associated proteins have these antioxidant effects, including PAF-AH, PON, ceruloplasmin, and transferrin (Navab M et al 1998). PAF-AH catalyzes the hydrolysis of short-chained phospholipid products of oxidation. In rodents, LPS, TNF, and IL-1 increase plasma PAF-AH activity, primarily in HDL fractions (Howard KM et al 1997, Memon RA et al 1999). In persons infected with HIV, an increase in plasma PAF-AH activity is found in LDL, but not in HDL, fractions (Khovidhunkit W et al 1999). In contrast, rabbits injected with croton oil have a reduction in plasma PAF-AH activity (Van Lenten B J et al 1995). Whether the increase in PAF-AH activity is proatherogenic or antiatherogenic is unclear. While PAF-AH can protect lipoproteins from oxidation, a sustained increase in PAF-AH activity may be undesirable because PAF-AH activation can result in the production of LPC, which exhibits numerous proatherogenic effects. In addition to PAF-AH, PON, another HDL-associated protein, protects LDL from oxidative stress. PON, an enzyme initially known to hydrolyze organophosphate pesticides including paraoxon, was later recognized as being able to hydrolyze biologically active phospholipids in oxidized LDL. Therefore, it may have antiatherogenic functions. In contrast to PAF-AH, which is selective against phospholipids with short acyl chains, PON has a specific activity against oxidized phospholipids with longer acyl chains. Depletion of PON activity results in the loss of antioxidant function of HDL. Addition of PON to these HDL particles restores the protective function of HDL (Castellani LW et al 1997). An increased susceptibility to atherosclerosis in PON-deficient mice further supports its important role in preventing lipoprotein oxidation and atherogenesis (Shih D M et al 1998). During infection and inflammation, serum PON activity decreases in both humans and rodents (Feingold KR et al 1998, Van Lenten BJ et al 1995). With a reduction in serum PON activity, acute-phase HDL may lose its ability to protect LDL against oxidation. One study has shown that acute-phase HDL with decreased PON activity is unable to protect against LDL oxidation (Van Lenten BJ et al 1995). Acute-phase HDL, on the contrary, becomes prooxidant, further promoting the oxidative process.
and the migration of monocytes into a smooth muscle endothelial coculture. Ceruloplasmin is a copper-binding acute-phase protein that is also associated with HDL. During infection and inflammation, plasma ceruloplasmin levels increase. Ceruloplasmin is generally considered an antioxidant (Kunitake ST et al 1992, Samokyszyn VM et al 1989) however; recent studies show that ceruloplasmin can enhance LDL oxidation (Ehrenwald E et al 1994, Lamb DJ et al 1994). These conflicting results may be due to the integrity of its structure, since intact ceruloplasmin can oxidize LDL, whereas proteolytic cleavage of or removal of one of the seven copper atoms from intact ceruloplasmin (which may occur during isolation and/or storage) inhibits its oxidative action (Chisolm GM et al 1999). Whether ceruloplasmin is prooxidant or antioxidant in vivo is uncertain, but plasma ceruloplasmin level is a risk factor for CAD. Transferrin is another metal-binding protein associated with HDL (Manttari M et al 1994). Transferrin in HDL also has antioxidative effects since removal of HDL subpopulations that contain transferrin significantly reduces the ability of HDL to protect against LDL oxidation (Kunitake ST et al 1992). The reduction of transferrin that occurs during infection and inflammation may lead to less transferrin in HDL, making it less effective in preventing LDL oxidation. While the role of an increase in PAF-AH and ceruloplasmin during infection and inflammation in atherogenesis is uncertain, a reduction of PON and transferrin suggests that these changes affect the ability of acute-phase HDL to protect against LDL oxidation. Acute-phase HDL may even become proatherogenic, instead of being antiatherogenic.

The anti-atherogenic action of HDL-cholesterol can be attributed to the anti-inflammatory properties of its molecule. This anti-inflammatory action is directly connected with the antioxidative properties of certain compounds that help to make up the HDL molecule, given that an increase in the concentration of the products of lipid oxidation is the main stimulus that starts the inflammatory procedure that leads to the formation of atheromatous plaque. These compounds are apolipoprotein A-I, PON, PAF-AH and LCAT.
Apolipoprotein A-I

Apolipoprotein A-I has perhaps the greatest biological significance of all the ingredients of the HDL molecule. Apart from the fact that it plays a vital role in reverse cholesterol transport, it has clear antioxidative and anti-inflammatory properties. As Hyka et al. (2001) showed, insertion of the human apolipoprotein A-I gene into mouse DNA resulted in very high levels of apolipoprotein A-I in the animals’ plasma, sufficient to reduce LDL-cholesterol oxidation in vitro by 50%. Furthermore, recently Navab et al. (2000) published the results of a series of experiments that discovered a great deal of evidence about both the pathophysiological mechanism of LDL oxidation and the inhibitory effect of apolipoprotein A on that process. Those researchers proposed the view that LDL oxidation involves 3 stages. In the first stage the LDL molecule is combined with products of linoleic and arachidonic acid metabolism and with cholesterol hydroperoxide. In the second stage the LDL is trapped in the subendothelial region, while other products of oxidation arising from the action of 12-lipoxygenase, finally, in the third stage the HDL phospholipids are also oxidized and the oxidized lipids that result cause monocytes to connect to the endothelium, to enter the subendothelial region and to be transformed into macrophages. Apolipoprotein A-I appears to have an inhibitory effect on all three stages of LDL oxidation, removing oxidised lipids from its molecule. The same researchers found that the administration of human apolipoprotein A-I makes the LDL in both animals and humans resistant to the oxidative action of cells in the vascular wall. It should be noted, however, that the anti-inflammatory action of apolipoprotein A-I also extends beyond inhibiting the production of toxic oxidized lipids. Recent experimental studies have shown that HDL has an inhibitory effect on the production of adhesion molecules (σ-selectin, ICAM-1 and VCAM-1) by endothelial cells that have been exposed to the action of cytokines (Ashby DT et al. 1998, Cockerill GW et al. 1999). The central role in this action of HDL appears to be played by apolipoprotein A-I, but in combination with the phospholipids of the lipoprotein molecule (Baker PR et al. 1999). In addition, a new mechanism of action of apolipoprotein was discovered recently by Hyka D et al. (2001). These researchers found that apolipoprotein A-I inhibits the production of
inflammatory cytokines (interleukin-1, and TNF-α), blocking the connection between activated T-lymphocytes and monocytes in the blood. The practical significance of these findings is made more clear by data from animal studies such as the one by Shah PK et al (2001) who showed that the administration of a dose of recombinant apolipoprotein A-I to apolipoprotein E-deficient mice (which were thus susceptible to rapid development of extensive atherosclerotic lesions when put on a fatty diet) not only reduced the extent of the atherosclerotic plaques by 50% but also decreased their macrophage content by 29-36% over a 48-hour period. Furthermore, the implantation (Rong JX et al 2001) of the human apolipoprotein A-I gene in animals with extensive atherosclerotic lesions led to an 80% reduction in macrophages and a 300% increase in smooth muscle cells in the region of the lesion, suggesting that apolipoprotein A-I is likely to play a positive role in the stabilization of atheromatous plaque. Finally, in a study by Sampietro T et al (2002) who studied a population of 50 individuals with hypoalphalipoproteinemia (characterised by very low levels of HDL and apolipoprotein A-I), it was found that these subjects had very high values of C-reactive protein compared to normal individuals and that these values had a significant negative correlation with levels of both HDL and apolipoprotein A-I.

**Paraoxonase**

The antioxidative and anti-inflammatory action of the HDL molecule, however, is not related exclusively with the existence of apolipoprotein A-I. Another biologically active element of HDL is the paraoxonase enzyme, an esterase that is used to neutralize the toxic action of organophosphate compounds in the organism, mainly in the liver, and joins with the HDL-cholesterol molecule (Brealey CJ et al 1980). The gene responsible for the production of paraoxonase has been described and its location has been determined precisely. In fact, polymorphism of this gene has also been described. More specifically, the existence of the amino acid glutamine in position 192 characterises Isoenzyme Q, which has low paraoxonase activity. In contrast, replacement of glutamine by arginine leads to the creation of Isoenzyme R, which exhibits high paraoxonase activity (Humbert CJ et al 1993). Paraoxonase has a strong antioxidative action, given that it is capable of hydrolysing the oxidized
phospholipids that are formed during the third stage of LDL oxidation (Mackness B et al 1999). In a laboratory setting Shih D.M. et al (1998) created experimental animals that were completely lacking in the paraoxonase gene. The HDL that was isolated from the homozygote animals was unable to inhibit LDL oxidation in a cell culture model. In addition, the administration of a diet with high fat content led to clearly more extensive aortic atherosclerotic lesions in those animals compared to controls. In humans, too, it has been shown that both the levels and the potency of the enzyme are lower in patients with acute myocardial infarction (Ayub A et al 1999). Furthermore, studies of patients and controls have shown that polymorphism of the paraoxonase gene is related with a higher risk of coronary atherosclerosis in both diabetics (Ruiz J et al 1995) and non-diabetics (Serrato M et al 1995). In any event, according to many recent studies only a part of the antioxidative action of HDL is due to paraoxonase (Cao H et al 1999).

**PAF-AH**

Another portion of the antioxidative and anti-inflammatory action of HDL is attributable to the PAF-AH enzyme. Platelet activating factor (PAF) is implicated in the early phase of atherosclerosis, being produced by the endothelial cells under the influence of free oxygen radicals and products of lipid oxidation. PAF itself stimulates the macrophages, which produce peroxide anions, thus perpetuating the vicious circle of increased oxidative stress leading to an intensification of the inflammatory process and promotion of atherosclerosis (Evangelou AM et al 1994, Imaizumi TA 1995) In addition, as mentioned above; the third phase of LDL oxidation includes the formation of oxidized phospholipids, which also have PAF-like activity. PAF-AH is an enzyme that hydrolyses PAF and joins with molecules of both LDL and HDL (Tselepis AD et al 1995). Since the beginning of the 1990s studies has shown that PAF has a powerful inflammatory action and a little later Tjoelker (1995) et al. found that administration of PAF-AH to experimental animals inhibited the inflammation process. Subsequent studies connected the anti-inflammatory with the anti-atherogenic effects of PAF-AH. Theilmeir et al. (2000) used an adenovirus to implant the human PAF-AH gene in apolipoprotein E-deficient mice and found that the PAF-AH significantly reduced the tendency for macrophages to adhere to the
aorta in those animals. Furthermore, using similar methods Quark et al. (2001) showed that PAF-AH reduced the extent of atherosclerotic lesions by 42% and restenosis by 77% in apolipoprotein E-deficient mice. These findings have been confirmed by epidemiological studies showing that mutations of the PAF-AH gene are an independent risk factor for coronary artery disease, Yamada Y et al. (1998) while low levels of the enzyme have been found in patients with acute myocardial infarction (Serebruany VL 1998).

**LCAT**

LCAT, apart from its role in reverse cholesterol transport, also appears to have antioxidative properties. Studies have shown that the incubation of partially purified LCAT with LDL led to the inhibition of malonyldialdehyde formation (Vohl MC et al 1999) confirmed this finding, showing that LCAT that had been completely purified of other HDL ingredients was able to inhibit the accumulation of oxidized lipids in the LDL molecule. In any event, when oxidized LDL is formed, in its turn it inhibits the activity of LCAT, thus blocking the reverse cholesterol transport process (Bielicki JK et al 1999).

The mechanism for protective effect of HDL against CVD is not completely understood. Although the widely accepted mechanism comprises the ability of HDL to enhance reverse cholesterol transport, cholesterol-independent mechanisms have also been postulated. For example, lower HDL is associated with endothelial cell injury, which is involved both in the progression of atherogenesis and myocardial ischemia/reperfusion injury. The ability of HDL to inhibit endothelial adhesion molecule expression and to potentiate prostacyclin release from the endothelial cells further supports cholesterol-independent mechanism of HDL. Atherosclerosis is an inflammatory disease characterized by adhesion of circulating monocytes to activated endothelial cells followed by migration to the subendothelium with the help of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin. Consistent with these reports, there is an increased expression of adhesion molecules in atherosclerotic plaque and upregulation of adhesion molecules in the acute thrombotic process. Recently, an increased plasma concentration of soluble adhesion molecules has been described as a risk factor for
ischemic heart disease. The adhesion molecules are synthesized in the endothelial cells by the cytokines including interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α). An increased expression of TNF-α has been reported in human atheroclerosis. A recent study showed protection of endothelial cells from TNF-α induced apoptosis by HDL. In that study, the authors demonstrated that HDL prevented apoptosis of human umbilical venous endothelial cells (HUVECs) induced by TNF-α via an inhibition of protease activity. The incubation of HUVECs with TNF-α, significantly increased protease activity, and induced apoptosis. Prostacyclin (PGI₂), a vasodilator that contributes to the maintenance of vascular tone, may also function as an endogenous antiatherogenic molecule. The antiatherogenic property of PGI₂ is attributed to its ability to inhibit platelet aggregation and adhesion and to block leukocyte activation and adhesion. Several reports exist in the literature indicating PGI₂ release by HDL by a Cox-2– dependent mechanism although Cox-1 may also have some role. Cox-2 induction by HDL may be viewed as heart’s own effort to upregulate its own defence to limit the deleterious effects of ischemia and reperfusion. Reports have shown that HDL protection of isolated rat hearts from ischemia/ reperfusion injury by a mechanism that involves reduction of cardiac TNF-α and enhancement of prostaglandin release. Preperfusion of the isolated hearts with HDL improved post ischemic functional recovery and reduced creatine kinase release from the heart indicating cardioprotective effects of HDL. The rate-limiting enzyme for the prostaglandin synthesis is cyclooxygenase, which is present in two different forms. Cyclooxygenase-1 (Cox-1) is ubiquitously present in many tissues, whereas cyclooxygenase-2 (Cox-2) is usually absent in the cells, but induced upon stimulation by agents like cytokines and mitogens. A recent study demonstrated that HDL could induce PGI₂ release in Cox-2– dependent manner and that its synthesis is regulated by both transcriptional and translational machineries. The study showed several-fold increase in HDL-induced release of PGI₂, which was blocked by a selective Cox-2 inhibitor, (N-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide). Cycloheximide, actinomycin D, and dexamethasone down regulated HDL-induced PGI₂ synthesis, suggesting de novo synthesis of protein and mRNA of Cox-2 by HDL. HDL can differentially modulate cytokine-induced expression of E-selectin and Cox-2. Preincubation with HDL completely abolished E-selectin expression in the
endothelial cells in response to TNF-α. Transient cotransfection experiments determined that HDL could inhibit cytokine-induced expression of a reporter gene driven by the E-selectin proximal promoter. HDL did not influence the nuclear translocation or DNA binding of NF-κB or alter the kinetics of degradation and resynthesis of the inhibitory protein IκB. HDL synergized with cytokine to enhance the expression of Cox-2 and induce the synthesis of PGI2. A recent study showed that enhancement of the cytokine activated human primary monocytes production of matrix metalloproteinase-1 (MMP-1) by oxidized LDL can be inhibited by adding HDL in conjunction with oxidized LDL. MMPs are composed of a family of proteolytic enzymes that are capable of degrading extracellular matrix components. Recently, MMP-1 has been found to colocalize with monocytes/macrophages in regions of enhanced collagenolysis within rupture-prone plaques. Interestingly, the regulation of HDL and LDL-induced MMP-1 production in cytokine-activated monocytes is mediated, in part through Cox-2 and PGE2. Although the mechanism for HDL inhibition of the enhancement of MMP-1 is not clear, CD36 is likely to be involved because similar to oxidized LDL, HDL and LDL also possess high affinity for this scavenger receptor. It may be possible that high concentration of HDL saturate the binding sites of the CD36 receptor, thereby limiting oxidized LDL uptake and MMP-1 production. The crucial event in atherogenesis is endothelial activation by a variety of factors including cytokines such as TNF-α resulting in the expression of a variety of adhesion molecules. Sphingosine kinase signalling may play a significant role in this process because sphingosine-1-phosphate, generated by sphingosine kinase activation, is a key factor responsible for TNF-α induced adhesion molecule expression. A recent study demonstrated that HDL could inhibit TNF-α–mediated upregulation of sphingosine kinase activity in endothelial cells resulting in a reduction in sphingosine-1-phosphate production leading to a downregulation of adhesion protein expression. In concert, HDL reduced TNF-α–mediated activation of extracellular signal regulated kinases and NF-kB signalling cascades. Additionally, HDL enhanced the cellular levels of ceramide resulting in the inhibition of endothelial activation.
Thus, HDL appears to interrupt a sphingosine kinase signalling pathway, which is involved in endothelial cell activation and adhesion molecule generation. It appears that HDL provides cardioprotection through diverse mechanisms including reduction of adhesion molecules, increase in PGE\(_2\) and PGI\(_2\), inhibition of MMP-1, antioxidant action, and interruption of sphingosine kinase signalling pathway.

Figure 1.6 Inhibition of endothelial cell sphingosine kinase by HDL
Chapter I

HDL Modification

High density lipoproteins (HDL) are susceptible to structural modifications mediated by various mechanisms including oxidation, glycation, homocysteinylation or enzymatic degradation. Structural alterations of HDL may affect their functional and atheroprotective properties. Oxidants, such as hypochlorous acid, peroxyl radicals, metal ions, peroxynitrite, lipoxygenases and smoke extracts, can alter both surface and core components of HDL. The formation of lipid peroxidation derivatives, such as thiobarbituric acid reactive substances, conjugated dienes, lipid hydroperoxides and aldehydes, is associated with changes of physical properties (fluidity, molecular order) and of apoprotein conformation. Non-enzymatic glycation, generally associated with lipoxidation, leads to form irreversible complexes called advanced glycation end products. These HDL modifications are accompanied with altered biological activities of HDL and associated enzymes, including paraoxonase, CETP and LCAT. Homocysteine-induced modification of HDL is mediated by homocysteine-thiolactone, and can be prevented by a calcium-dependent thiolactonase / paraoxonase.

Oxidation

Several studies have demonstrated that HDL are readily modified in vitro by a variety of oxidants such as metal ions (Maziere JC et al 1993, Marcel YL et al 1989, Nagano Y et al 1991, Ferretti G et al 1993), peroxyl and hydroxyl radicals (Bowry VW et al 1992, Bonnefont-Rousselot D et al 1995), aldehydes (Maziere JC et al 1993, Salmon S et al 1992, Guertin F et al 1994, Mc Call MR et al 1995, Francis GA et al 1996), peroxidase-generated tyrosil radical (Francis GA et al 1996, Guertin F et al 1997), lipoxygenase (Garner B et al 1998), cigarette smoke (Mc Call MR et al 1994, Ueyama K et al 1998) and hypochlorous acid (Panezenboek U et al 1997). It has been hypothesized that oxidation of HDL invivo would take place in sequestered inflammatory microenvironments, like atherosclerotic lesions in the artery wall, rather than in circulating plasma. Interstitial fluid of arterial intima and other peripheral tissues are expected to be the major site of HDL oxidation invivo (Francis GA et al. 2000). Myeloperoxidase and superoxide anion radical (O$_2^-$) and H$_2$O$_2$, secreted by
activated phagocytes, may be potential candidates for generation of oxidized HDL in vivo, via intermediate formation of diffusible cytotoxic radicals such as hypochlorite (from chloride ion) and tyrosyl radical (from l-tyrosine) (Panezenboeek U et al 1997). Also peroxynitrite and lipoxygenase generated from nitric oxide and superoxide in intimal space, also appear to oxidize HDL in vivo. Moreover, several studies suggest that transition metal ions may also cause modification of lipoproteins within artery wall through peroxidation reactions on polyunsaturated fatty acids of phospholipids Esterbauer H et al. 1992). Using various models of oxidized HDL (Maziere JC et al 1993, Ferretti G et al 1993, Bonnefont-Rousselot D et al 1995, Marsche G et al 2002), it has been demonstrated that oxidation involves both surface (protein, phospholipid and cholesterol) and core (cholesteryl ester) components. Oxidation induces several structural and compositional modifications in lipids and apolipoproteins of HDL. Apo A-I, the main HDL protein, forms dimers, trimers or heterodimers with other apolipoproteins (e.g., Apo A-I to Apo A-II) and higher molecular weight aggregates (Maziere JC et al 1993, Marcel Y L et al 1989, Nagano Y et al. 1991, Garner B et al 1998, Ueyama K et al 1998, Mc Call MR et al 1995, Wang WQ et al 1998). These modifications are accompanied with a decrease of tryptophan fluorescence of apolipoproteins (Maziere JC et al 1993, Ferretti G et al 1993, Salmon S. et al 1992). Oxidation of HDL is followed by an increase in the levels of lipid peroxidation markers including conjugated dienes, lipid hydroperoxides, thiobarbituric acid reactive substances (TBARS), and aldehydes. Compositional changes are associated with alterations of physico-chemical properties of HDL, such as fluidity, molecular order and electric charges. Compositional and structural modifications are associated with several alterations of HDL biological activities. A decreased ability of HDL to promote the reverse cholesterol transport has been shown in HDL oxidized in vitro (Salmon S. et al 1992, Morel DW et al 1994, Rifici VA et al 1996, Cogny A et al 1996). Studies on cultured cells demonstrated that oxidatively modified HDL are potent inhibitors of cholesterol biosynthesis. Moreover, oxidized HDL have been shown to exert cytotoxic effect on cultured cells e.g., on macrophages, lymphoblastoid cells (Hurtado I, et al. 1996, Alomar Y et al. 1992). In addition, Girona et al (1997) have also demonstrated that oxidation impairs HDL anti-inflammatory properties. Several authors demonstrated that lipid peroxidation of HDL
alters the activity of enzymes associated with HDL. In fact, a significant reduction of paraoxonase (PON) activity has been observed in ox-HDL (Jaouad L et al 2003, Ferretti G et al 2004) and it has been suggested that PON inactivation in ox-HDL could be the cause of lower protective effect towards LDL and cell membrane oxidation (Ferretti G et al 2004). However, Garner et al. (Garner B et al 1998) have shown a role for methionine residues of apo A-I and apo A-II. In fact, oxidation of methionine residues to methionine sulfoxides does not decrease but rather increase the potential antiatherogenic properties of apo A-I (Panenbock U et al 2000, Panzenbock et al 1995). Other HDL proteins involved in lipoprotein metabolism such as LCAT (lechitin: cholesterol acyltransferase) (Maziere JC et al 1993, Mc Call MR et al 1995, Mc Call MR et al 1994) and phospholipid transfer protein (PLTP) activity are modified in oxidized HDL, suggesting alterations of lipoprotein metabolism of ox-HDL. Recent studies have demonstrated the presence of lipoprotein particles in human cerebro-spinal fluid (CSF). These particles display similar density and chemical composition with HDL isolated from human plasma. The functions of lipoproteins in cerebro-spinal fluid are only poorly understood in ex vivo experiments. HDL are transported across the blood brain barrier (BBB) in vitro without degradation, suggesting that they may play a role in cholesterol metabolism in the central nervous system (De Vries HE et al 1995). Various receptors exist in the nervous system modulating signals in the brain (Herz J et al 2002). CSF lipoproteins containing polyunsaturated fatty acids, the major substrate for lipid peroxidation, are susceptible to oxidative modifications and a similar time-course has been typically found in human plasma and CSF lipoproteins. Administration of highly or moderately oxidized HDL resulted in a dose and time-dependent increase in oxidative stress and death of cultured rat embryonic neurons (Keller J N et al 2000). Kivatinitz et al (1997) reported that HDL oxidized by UV irradiation or heating induced degeneration of neuronal cells, while native HDL produce a relatively reversible inhibition of neurite formation. These results suggest that aggregation is required for HDL to exert its neurodegenerative effects on cerebral neurons in culture. Aggregated HDL could participate in degeneration associated with oxidative stress in Alzheimer’s disease (AD). In the development of Alzheimer’s disease, Kontush et al. (2001) has shown increased production of β-amyloid induced by oxidative stress during aging. In a
similar manner, amyloid-peptide is normally secreted by neurons and can be found in low concentrations in cerebrospinal fluid and plasma. Resistance of human cerebrospinal fluid to oxidation is directly related to its \(\beta\)-amyloid content. In fact, Kontush et al. (2001) have found that the oxidative resistance is related to CSF levels of amyloid-\(\beta\) 1–40 and ascorbate, and inversely to levels of fatty acids. Amyloid-\(\beta\), which is an antioxidant, when present in elevated concentration, can become a prooxidant under the action of increased oxidative stress (Kontush A et al 2001) and contribute to Alzheimer’s disease. Several studies have suggested that oxidation of CSF lipoproteins might occur in vivo and play a role in the pathogenesis of neurodegenerative diseases (Bassett CN et al 2000, Keller JN et al 1999, Keller et al 2000). The breaking of blood brain barrier observed in Alzheimer’s disease and other neurological diseases could produce HDL levels higher than normal values in the brain, which could be susceptible to oxidation by free radicals. Moreover, a higher susceptibility to oxidation of lipoproteins isolated from CSF of patients affected by Alzheimer’s disease has been demonstrated (Schippling S et al 2000) and changes in chemical composition of CSF lipoproteins have been recently reported in Alzheimer’s disease patients suggesting a role of oxidation of CSF lipoproteins in the pathogenesis of neurodegenerative diseases.

**Glycation: Non-enzymatic glycation**

Several studies have reported alterations of plasma proteins and lipoproteins in hyperglycemic conditions (Mc Cance DR et al 1993, Lyons TJ et al 1993, Tames FJ et al 1992). It has been demonstrated that glucose induces a nucleophilic non-enzymatic reaction with proteins to form reversible products, Schiff bases and subsequently amadori compounds. Further reactions, rearrangements, dehydration and cleavage result in the formation of irreversible complexes called advanced glycation end products (AGEs). Glycation of apolipoproteins and lipids of lipoproteins and subsequent formation of glycated lipoproteins have been observed in plasma of diabetic subjects. A higher susceptibility to oxidation of glycated HDL has been reported. However, data concerning the effects of nonenzymatic glycation on HDL oxidizability are contradictory. Rashduni DL et al (1999) have shown that glycation of HDL does not increase its susceptibility to oxidation. On the contrary, Hedrick CC
et al (2000) have incubated for 1 week HDL in 25mM glucose and have noted significant increase in the glycation products, e.g., fructose lysine and an increase of lipid peroxidation products. Ferretti G. et al. (2001) have incubated HDL for 3 days with glucose (50 and 100 mM) and have shown significant increase in TBARS and conjugated dienes with respect to control HDL, confirming that lipid peroxidation accompanies HDL glycation in vitro (Gly-ox-HDL). Lipid compositional changes in HDL incubated with glucose are associated with modification of physico-chemical properties and apolipoprotein structure at HDL surface. Gly-ox-HDL show several functional alterations with a decrease in the activity of enzymes associated to its surface such as paroxonase (Jaouad L et al 2003, Ferretti G et al (2001), CETP and LCAT (Lemkadem B et al 1999). Furthermore, a lower ability of Gly-ox-HDL to mediate cholesterol efflux and a decreased ability to protect LDL from oxidative damage have been observed. Matsunaga T. et al. (2001) demonstrated that Gly-ox-HDL affect the functions of endothelial cells (EC) and suggest that they may induce EC apoptosis through a mitochondrial dysfunction. Therefore, HDL exposure to hyperglycemic conditions could contribute to the accelerated atherosclerosis in diabetic patients.

Homocysteinylation

Homocysteine is a product of folate metabolism. The mechanism by which Homocysteine appears to promote vascular disease is unclear. Elevated Homocysteine levels play a causative role in the production of arterial lesions, but deficiencies of other factors, such as vitamin B_{12} and folic acid, also may be involved, especially among the elderly. It has been proposed that Hcy-thiolactone could react with primary amines by nucleophilic addition of proteins (protein-N-Hcy) and damage protein structure (Jakubowski H et al 1999, Liu G et al 1997) and their functions. The interaction between Hcy-thiolactone and amino groups of Apo B lysyl residues of low density lipoproteins causes the formation of homocystamine-LDL (Hcy-LDL). This reaction induces LDL aggregation and a higher uptake of homocysteinylated-LDL by cultured macrophages. Moreover, Hcy-LDL induces functional alterations and oxidative damage in human endothelial cells (Ferretti G et al 2004, Vignini A et al
2004). Therefore, it has been suggested that homocysteinylation could represent an atherogenic modification of LDL.

Possible mechanism of increased risk is that hyperhomocysteinemia may impair release of nitric oxide from endothelial cells, stimulate proliferation of atherogenic smooth-muscle cells, and contribute to thrombogenesis through activation of protein C.

Homocysteine is derived from the sulphur containing amino acid methionine and is metabolized through pathways associated with folic acid, vitamin B<sub>6</sub>, and vitamin B<sub>12</sub> as cofactors.

Elevated plasma Homocysteine levels (greater than 15µ/L) confer an independent risk of vascular disease, according to cross-sectional and prospective case-control studies. The risk was first identified because of thromboembolic events, including MI and stroke, associated with homocystinuria, a rare disorder that involves Homocysteine levels greater than 100 µmol/L and is related to cystathione B-synthase deficiency.

**Tyrosylation**

Activated human phagocytes generate anion radicals, which are dismutated in hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Myeloperoxidase and H<sub>2</sub>O<sub>2</sub> together generate diffusible cytotoxic radicals including tyrosyl radical (from L-tyrosine). Moreover, Heinecke et al. (Heineke J.W et al. 1993) have found that phenolic coupling of free-tyrosine with dityrosine and protein–dityrosine crosslinking by myeloperoxidase occurs at physiological plasma concentrations of L-tyrosine. Studies in vitro have demonstrated that treatment with tyrosyl radical induces the tyrosylation of HDL, with formation of lipid peroxidation products and protein-associated dityrosine, and crosslinking of HDL proteins (Banka CL et al 1996). Moreover, tyrosylation of HDL induces the formation of dimers and trimers of Apo A-I and heterodimers (Apo A-I–Apo A-II) (Francis G. A. (1993). Studies by Suc I. et al. (2003) have demonstrated that oxidative tyrosylation of high density lipoproteins impairs cholesterol efflux from mouse J774 macrophages. It has been shown that tyrosylation of HDL enhances cellular cholesterol mobilization by increasing translocation of cholesterol from an
intracellular pool to sites on the cell surface available for removal by acceptors including Apo A-I suggesting that a similar action in vivo could enhance the lipidation of Apo A-I and the maturation of pre-β HDL thereby increasing plasma HDL levels.

**HDL modification induced by enzymes**

HDL treated with porcine pancreatic phospholipase A\(_2\) in the presence of albumin results in hydrolysis of 40–84% of HDL phospholipids. Consecutively, there is an increase in microviscosity and polarity of lipid regions in the surface monolayer of the particles (Gorshkova IN et al. 1996). These alterations appear to be similar to those associated with coronary atherosclerosis. Incubation of human HDL with monocyte-derived elastase causes selective proteolysis of apo A-II and apo A-I.

Elastase-digested HDL (ED-HDL) bind to J774-A1 murine macrophages with enhanced affinity and are internalized and degraded at a rate three-fold higher than that of native HDL. The uptake of ED-HDL lipoproteins does not affect the cellular lipid biosynthesis nor modify the cell lipid content. Liberation of elastase by lipid-laden macrophages is considered as an important event during atherogenesis. By enhancing the cellular uptake of HDL this process can lead to a local decrease of antiatherogenic HDL particles (Pirillo A et al 2000).

**Dysfunctional HDL**

HDL has many other activities aside from its role in RCT that can also contribute to its protective mechanism. These include the removal or detoxification of oxidized sterols and phospholipids, antiinflammatory activity, antioxidant activity, antithrombotic activity, and its protective and healing activities on endothelial cells. So, HDL-cholesterol is the “good cholesterol,” but there are many unresolved questions: are all HDL particles equally good, does HDL from different subjects perform the same; can HDL become dysfunctional; and, can “good cholesterol” be made into “better cholesterol. There are evidences for and causes of Dysfunctional HDL. Fogelman and colleagues developed an assay to measure the antiinflammatory activity of HDL by determining the extent to which HDL inhibits monocyte chemotaxis induced by LDL using an in vitro reconstituted artery wall model by the
coculture of smooth muscle cells and endothelial cells (Navab M et al 2000) Other assays for HDL function include cell free antioxidant activity, inhibition of endothelial cell adhesion molecule expression, (Cockerill GW et al. 1995) and the ability of HDL to act as an acceptor of cellular cholesterol. Navab, Fogelman, and colleagues using this artery wall model system have demonstrated several conditions in which HDL can lose its antiinflammatory properties, including during the acute phase response when serum amyloid A partially replaces apo A-I in HDL, during influenza A infection, and in apolipoprotein A-II transgenic mice (Van Lenten BJ et al 2001, Castellani LW et al 1997, Van Lenten BJ et al 1995). In a seminal study, Ansell et al discovered that HDL from CAD subjects who had high levels of HDL had less antiinflammatory activity, in this coculture assay, than HDL derived from healthy control subjects, thus indicating that HDL from CAD subjects is dysfunctional. In fact, HDL from many CAD patients was actually proinflammatory, thus increasing monocyte chemotaxis in response to LDL, unlike the HDL from healthy controls that reduced monocyte chemotaxis (Ansell BJ et al 2003). There are many possible alterations between this dysfunctional HDL and normal functional HDL. One possibility is a change in the protein composition of HDL. Human HDL particles are quite heterogeneous, encompassing a range of sizes and densities. Each HDL particle carries apo A-I and may also carry other apolipoproteins, such as apo A-II, apo A-IV, apo E, apo Cs. In addition, HDL is associated with a panoply of accessory proteins, including LCAT, phospholipid transfer protein (PLTP), paraoxonase 1 (PON1), myeloperoxidase (MPO), serum amyloid A1, (SAA1), and platelet-activating factor acetylhydrolase (PAF-AH). The HDL proteome is quite large, (Barter PJ et al 2007) and each HDL particle cannot accommodate all of the accessory proteins; thus, a minority of the HDL particles must be associated with each accessory protein. Changes in HDL protein composition attributable to infection, inflammation, or diabetes have been shown to be associated with decreased function. (Van Lenten BJ et al 2001, Castellani LW et al 1997, Kontush A et al 2008). An HDL proteomic study of HDL3 from different subjects found that the levels of PON1, PON3, and apoL-I correlate with HDLs antioxidant activity (Davidson WS et al 2009). Also, mouse models have been used to determine the effects of HDL protein composition changes, for example, HDL isolated from transgenic mice over expressing apo A-II or PLPT
show reduced function compared to control HDL (Castellani LW et al 1997, Moerland M et al 2007). Many studies have been performed to investigate the changes in HDL associated with inflammation. de Beer and colleagues have shown extensive HDL remodeling after treatment of mice with endotoxin, or in humans after surgery, leading to the so-called acute phase HDL, in which SAA and group IIa secretory phospholipase A2 (sPLA_{2}-IIa) are increased and apo A-I levels (and CETP levels in humans) are decreased (Van der Westhuyzen DR 2007). Because SAA itself can act as an acceptor for cellular cholesterol via ABCA1, SR-BI, and other pathways, the consequences of acute phase HDL on HDL function and RCT must be determined experimentally. Postsurgical human serum containing acute phase HDL maintains most of its ABCA1 and ABCG1 dependent cholesterol acceptor activity despite decreased HDL-C and apo A-I levels. The in vitro incubation of HDL with SAA, sPLA2-IIa, and CETP leads to the marked displacement of apo A-I from HDL generating lipid-free or lipid-poor apo A-I, expected to undergo more rapid catabolism, but also expected to be a good ABCA1-mediated acceptor of cellular lipids (Jahangiri A et al 2009). The over expression of SAA and SR-BI in mice via adenoviral vectors also leads to the production of small lipid poor apo A-I particles which are more rapidly cleared (de Beer MC et al 2009). Recently, Reilly’s laboratory demonstrated that LPS treatment in mice decreases RCT, although this was mediated primarily through the inhibition of liver sterol excretion associated with decreased expression of hepatic ABCG5, ABCG8, and ABCB11 (McGillicuddy FC et al 2009).

Another factor that might make HDL dysfunctional is a change in the HDL-associated lipids. HDL may acquire oxidized lipids from cells and by exchange with other particles, or HDL lipids may be oxidized in situ. Lipid peroxides may interfere with HDL antioxidant, antiinflammatory, and cholesterol acceptor activities. For example, treatment of HDL with 15-lipoxygenase, an enzyme forming lipid peroxides, reduces HDL cholesterol acceptor and antiinflammatory activities (Pirrillo A et al 2008, Pirrillo A et al 2006). HDL carries the bulk of lipid hydroperoxides in plasma (Bowry VW et al 1992). These lipid hydroperoxides can be converted into less reactive lipid hydroxides through specific methionine residues of apo A-I and apo A-II, with the concomitant formation of methionine sulfoxide (Garner B et al 1998). Fully functional HDL may promote lipid hydroperoxide metabolism and its uptake and
clearance by the liver, which would be protective. In contrast, dysfunctional HDL might promote the transfer of lipid hydroperoxides to apo B-containing lipoproteins and promote VLDL and LDL oxidation (McPherson PA et al 2007). A prominent factor that can lead to HDL dysfunction is the posttranslational modification of apo A-I. Copper-mediated oxidation of HDL leads to altered HDL migration on an agarose gel, apo A-I proteolysis, and decreased ability of HDL to unload cholesterol esters from cholesterol-loaded macrophages (Nagano Y et al 1991). Malondialdehyde modification of HDL leads to similar changes in regard to HDL structure and function and is associated with the loss of lysine and tryptophan residues and apo A-I polymerization. HDL from diabetic subjects has evidence of glycated apo A-I and apo A-II, and this glycated apo A-I has altered structure and lipid binding activity (Calvo C et al 1992, 1998). Similarly, HDL incubated with high glucose results in a reduction in paraoxonase activity and decreased antiinflammatory activity (Hedrick CC et al 2000). Szapacs ME et al 2008). Lipid peroxides added to human plasma can covalently modify apo A-I. Also, incubation of HDL with activated neutrophils was shown to render apo A-I more negatively charged and decrease its cellular cholesterol acceptor activity (Cogny A et al 1996, 1994).

Myeloperoxidase Modification of Apo A-I

Myeloperoxidase (MPO) is an enzyme found in neutrophils, monocytes, and some macrophages that uses hydrogen peroxide to generate chlorinating and nitrating oxidants, which play an important role in killing microorganisms. However, these same reactive species can also modify host proteins and lipids. MPO is enriched in human atheroma, (Daughery A et al 1994) and its presence may promote lesion progression, by increasing LDL oxidation, and block plaque regression, by modifications of apo A-I/HDL that impair RCT. Bergt et al (1991) demonstrated that incubation of HDL with MPO, in a chlorinating reaction (MPO/H$_2$O$_2$/Cl) that produces HOCl (chlorine bleach), or with reagent HOCl (at a very high 100:1 ratio of HOCl: apo A-I) leads to a loss of unsaturated fatty acids in phospholipids and cholesterol esters, and the loss of cholesterol acceptor activity. Bergt et al (1999) also showed that modification of lipid-free apo A-I at an HOCl:apo A-I ratio of 25:1 led to the loss of apo A-I methionine residues, consistent with the high sensitivity of
methionine to MPO/H$_2$O$_2$/Cl (Panzenbock U et al 2000) Subsequently, it was shown that selective apo A-I methionine oxidation does not disrupt its structure, and actually leads to an increase in lipid binding and cellular lipid acceptor activities, although apo A-I Met-148 modification by MPO is associated with loss of LCAT activation (Shao B et al 2008). Bergt et al (2000) also used mass spectroscopy to identify specific alterations caused by reagent or MPO generated HOCl, and they confirmed the disappearance of the methionine-containing tryptic peptides with concomitant production of methionine sulfoxide-containing peptides. The cholesterol acceptor activity of recombinant HDL (rHDL) made by cholate dialysis using apo A-I and phosphatidylcholine is much more sensitive to the HOCl treatment, with loss of activity starting at an HOCl:apo A-I ratio of 5:1. In addition to decreased lipid acceptor activity, Panzenboeck also showed that MPO- or HOCl-modified HDL was more susceptible to uptake and degradation by macrophages, thus turning HDL from a lipid-accepting lipoprotein to a lipid-loading lipoprotein. (Panzenboeck U et al 1997). Although methionine and cysteine residues (apo A-I has no Cys residues) are the most sensitive to MPO generated oxidants, tyrosine, lysine, tryptophan, and other residues are also targets, with 3-chlorotyrosine being used as a fingerprint for MPO oxidants, as HOCl uniquely forms this adduct. (Nightingale ZD et al 2000, Hazen Sl et al 1997). Since 2004, the combined Smith, Hazen, and Kinter labs at the Cleveland Clinic and the combined Heineke and Oram labs at the University of Washington have each examined the MPO-induced molecular alterations in apo A-I and their functional significances. Both teams fundamentally agree that MPO modification of apo A-I is physiologically relevant by demonstrating that:

1) apo A-I in plasma and more so in arterial lesions is a selective target of MPO modification that leads to the nitration and chlorination of specific apo A-I tyrosine residues;

2) plasma apo A-I nitro-and chloro-tyrosine levels are higher in coronary artery disease patients than in control subjects; and

3) apo A-I tyrosine chlorination, whether in endogenous plasma or after in vitro MPO-mediated modification, is associated with the specific loss of ABCA1-mediated cholesterol acceptor activity, such that apo A-I cholesterol acceptor

Both groups using mass spectroscopy studies identified apo A-I Tyr192, within helix 8, as the most sensitive site to MPO mediated chlorination. Furthermore, Bergt et al (2004) demonstrated, using short synthetic peptides, that tyrosine chlorination is maximally stimulated when the tyrosine is 3 residues away from a lysine residue, which is the case for the sensitive Tyr192. The University of Washington group claimed that MPO impairment of the ABCA1-mediated cholesterol acceptor activity of apo A-I is through methionine oxidation and site-specific chlorination of Tyr192, as treatment of MPO modified apo A-I with the enzyme methionine sulfide reductase could partially restore the cholesterol acceptor activity of apo A-I, particularly in a recombinant apo A-I (r-apo A-I) isoform with Try192 replaced by Phe. On the other hand, the Cleveland Clinic group made r-apo A-I with all 7 Tyr residues replaced by Phe (7YF isoform), and found that it is equally susceptible to MPO or HOCl loss of cholesterol acceptor activity as the wild-type isoform (Peng DQ et al 2005). Furthermore, replacement of the 3 Met residues in apo A-I with Val residues actually increased susceptibility to low doses of H2O2 in the complete MPO chlorination system, showing that apo A-I Met residues can absorb HOCl oxidation without loss of function, hence protecting the other residues, (Peng DQ et al 2008) and in agreement with the prior finding. Thus, the site-directed substitution and cholesterol efflux data indicate that neither methionine nor tyrosine serve as the oxidant sensitive residue involved in MPO-induced apo A-I inactivation.
Chapter I

Introduction

Proinflammatory HDL

The displacement of paraoxonase by serum amyloid A may explain in part the proinflammatory nature of HDL in the acute phase impaired, proinflammatory HDL. Systemic infection, stress related to surgery, coronary artery disease, diabetes mellitus, autoimmune disease, and a diet high in saturated fat impair HDL’s ability to perform its key roles and even cause it to enhance the oxidative and inflammatory processes that lead to plaque formation.

Proinflammatory HDL

- was less able to perform reverse cholesterol transport
- had lower apo A-I levels
- had impaired reverse cholesterol transport and antioxidative and anti-inflammatory roles
- Increased production of MCP-1 and cellular adhesion molecules (ICAM-1, VCAM-1)

HDL from healthy people is most often anti-inflammatory (Ansell NJ et al 2003). However, when systemic inflammation is present, HDL can become proinflammatory as part of an acute phase response. Surgery is a good example of this effect. Van Lenten showed that HDL obtained several days after surgery was less able to suppress chemotaxis (as assessed by the monocyte chemotaxis assay) than was HDL obtained before surgery. This dysfunctional HDL reverted to a normal anti-inflammatory role once the patients had recovered from surgery. Influenza also appears to make HDL proinflammatory (Van Lenten BJ et al 2001). Sepsis has been reported to have dramatic effects on the composition of HDL, including a marked reduction in levels of the major apolipoprotein within HDL, apolipoprotein A-I (apo A-I) (Van Lenten HJ et al 2003). Chronic systemic inflammation is a more protracted state of impaired HDL function in some patients. This has been observed in patients with coronary heart disease, in people with coronary risk equivalents, and in patients on haemodialysis (Ansell NJ et al 2003, Hasselwander O et al 1999). In one relatively unusual cohort of patients who developed symptomatic coronary disease despite very high HDL-C levels (≥ 84 mg/dL), HDL uniformly increased monocyte chemotaxis (as
assessed by the monocyte chemotaxis assay) and phospholipid oxidation (as assessed by the cell-free assay), while HDL from healthy matched controls had opposite effects. HDL from a second cohort of patients with known coronary disease or coronary risk equivalents who had more typical HDL-C levels (mean ± 57 mg/dL) also showed proinflammatory tendencies on the same tests. Recent research has also produced evidence of dysfunctional HDL in patients with chronic nonvascular inflammation, such as in rheumatologic diseases. McMahon and colleagues (McMahon M et al 2006) reported that 44% of patients with systemic lupus erythematosus and 20% of those with rheumatoid arthritis had proinflammatory HDL as assessed by the cell-free assay, compared with only 4.1% of controls. These observations may help explain why women with lupus have a risk of myocardial infarction 50 times higher than normal, (Manzi S et al 1997) and why rheumatoid arthritis doubles the risk of coronary disease, even when conventional risk factors have been controlled for (Solomon D H et al 2003).

**How does HDL become proinflammatory?**

Systemic inflammation can promote specific changes within HDL particles that can hinder their antiatherosclerotic effects. One such target within HDL is apo A-I, a protein critical to HDL’s reverse cholesterol transport, antioxidant, and anti-inflammatory roles. Apo A-I levels can fall in response to inflammation and can be displaced from HDL particles by acute phase reactants such as serum amyloid A. Apo A-I can also be chemically damaged by the white blood cell enzyme myeloperoxidase (Zheng L et al 2004). Inflammation and associated oxidative stress can also lead to a reduction in levels of protective antioxidant enzymes such as paraoxonase, as well as to a build up of oxidized phospholipid molecules within HDL. These changes lead to an increase in oxidized LDL particles, which in turn promote a variety of inflammatory processes within the blood vessel wall (Navab M et al 2004).
Deficiency diseases

Tangier disease

Tangier disease is an inherited disorder characterized by significantly reduced levels of high-density lipoprotein (HDL) in the blood. HDL transports cholesterol and certain fats called phospholipids from the body's tissues to the liver, where they are removed from the blood. HDL is often referred to as "good cholesterol" because high levels of this substance reduce the chances of developing heart and blood vessel (cardiovascular) disease. Because people with Tangier disease have very low levels of HDL, they have a moderately increased risk of cardiovascular disease. Additional signs and symptoms of Tangier disease include a slightly elevated amount of fat in the blood (mild hypertriglyceridemia); disturbances in nerve function (neuropathy); and enlarged, orange-colored tonsils. Affected individuals often develop atherosclerosis, which is an accumulation of fatty deposits and scar-like tissue in the lining of the arteries. Other features of this condition may include an enlarged spleen (splenomegaly), an enlarged liver (hepatomegaly), clouding of the clear covering of the eye (corneal clouding), and type 2 diabetes.

Isolated apo A-I defects

More than 50 apo A-I mutations (mainly structural variants) have been identified to date. Most of the apo A-I structural variants are associated with neither low HDL-C nor with premature CHD, and the carriers are clinically asymptomatic. A reduction in HDL-C has been described for some apo A-I point mutations, but no increased risk of premature vascular disease could be firmly identified in the bearers of these mutations. Several apo A-I null alleles have been identified; where the synthesis of apo A-I is prevented by deletion or insertions, these are often associated with premature development of atherosclerosis and increased CHD risk.

LCAT deficiency and Fish eye disease

In normal individuals, 80% of LCAT is localised in HDL particles and 20% in apo B-containing proteins (mainly LDL) (alpha- and beta-LCAT activity, respectively). Reflecting this dual enzymatic activity are two manifestations of LCAT
deficiency, Fish eye disease and complete LCAT deficiency. The association of these diseases with atherosclerosis is not clear.

Fish eye disease is associated with:

- extensive corneal opacification;
- absence of cholesterol esterification from HDL substrate (alpha-LCAT activity);
- HDL-C deficiency and decreased apo A-I levels; and
- Close to normal total cholesterol-to-unesterified cholesterol ratio in plasma.

Complete LCAT deficiency is associated with

- corneal opacification, nephropathy, xanthomas and haemolytic anaemia;
- marked deficiency of cholesterol ester formation;
- decreased total cholesterol-to-unesterified cholesterol ratio in plasma;
- HDL-C deficiency and decreased apo A-I levels; and
- Presence of typical LDL particles.

Therapies to increase HDL

Lifestyle modification on serum HDL

Saturated fat raises ICAM-1 and VCAM-1; unsaturated fat decreases them. In one study, the fat composition of a diet markedly affected HDL’s impact on ICAM-1 and VCAM-1 expression on endothelial cells. Nicholls SJ et al (2006) found that HDL collected from patients 6 hours after eating a meal high in saturated fat increased the levels of these adhesion molecules 50% to 80% from baseline. In contrast, HDL from patients who ate a meal high in unsaturated fat inhibited ICAM-1 and VCAM-1 expression by 50% to 70%. These change occurred without significant changes in HDL-C levels. In this study, then, saturated fat promoted qualitative changes in HDL that could potentially increase monocyte adhesion to the arterial wall.
1. Weight loss

HDL-C decreases as function of raising weight or body mass index and, as a general rule of thumb, for every 1 Kg m\(^2\) rise in fat mass, HDL-C decreases by 1mg/dl (Lamon-Fava S et al 1996, Dattilo AM and Kris-Etherton PM 1992). The opposite is also true: weight loss results in an increase in HDL-C. HDL-C will typically be higher than at baseline. Increased ingestion of trans fat and carbohydrate or decreased saturated fat intake lowers HDL-C. A Mediterranean diet characterized by increased intake of legumes, olive oil, fruits and vegetables has been shown to decrease insulin resistance and increase HDL-C.

2. Exercise

Increased exercise is always a key feature of any lifestyle modification plan, and regular exercise is associated with reduced risk for CVD. Roberts and colleagues (2006) studied the effects of a 3-week intervention consisting of a high fiber, low-fat diet and exercise (45–60 minutes of walking fast on a treadmill) in obese men with metabolic syndrome. The lifestyle changes significantly improved the ability of the men’s HDL to inhibit monocyte chemotaxis compared with baseline. Before the intervention the men’s HDL was proinflammatory as assessed by the monocyte chemotaxis assay, with a mean inflammatory index of 1.14 plus or minus 0.11 (1.0 is neutral); afterward, their HDL was anti-inflammatory, with a mean index of 0.94 plus or minus 0.09 (P < .05). This study suggests that therapeutic lifestyle changes may affect HDL function, in addition to exercise’s well-recognized effect of raising HDL-C levels.

3. Smoking:

Smoking cessation can increase HDL-C by up to 20 %, an elevation that is on par with most efficacious pharmacologic interventions we currently have available (Moffatt RJ 1990).

4. Alcohol:

It has been known for some time that increased alcohol consumption is associated with reduced risk for CVD. Alcohol increases HDL-C by (i) stimulating hepatic Apo A-I secretion and (ii) inhibiting CETP activity. However, alcohol
consumption can also increase the serum TG concentration and, in excessive quantities, is associated with increases in various health risks. The relative benefits and risks associated with increased alcohol consumption is an issue that would have to be weighed on a patient basis.

**Pharmacological treatments to raise HDL**

The discovery of novel drugs and bimolecular to increase serum levels of HDL-C constitutes a substantial focus in contemporary cardiovascular medicine. The capacity of currently available medications to raise HDL is summarised in Table 1.3.

**Table 1.3 Medications to raise HDL-C**

<table>
<thead>
<tr>
<th>Drug</th>
<th>% increase in HDL-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statins</td>
<td>3-15</td>
</tr>
<tr>
<td>Fibrates</td>
<td>10-15</td>
</tr>
<tr>
<td>Niacin</td>
<td>10-30</td>
</tr>
<tr>
<td>Thiazolidinediones</td>
<td>5-24</td>
</tr>
<tr>
<td>Estrogen</td>
<td>10-25</td>
</tr>
</tbody>
</table>

Statins have also shown the ability to modify HDL’s proinflammatory and anti-inflammatory properties. Ansell BJ et al (2003) conducted a study in which patients with coronary heart disease or risk equivalents were treated with simvastatin (Zocor) 40 mg for 6 weeks. Over the treatment period, their HDL changed from mostly proinflammatory to mostly antiinflammatory, as assessed by both the monocyte chemotaxis assay and the cell-free assay. More recently, Charles-Schoeman et al (2007) showed that HDL from patients with active rheumatoid arthritis became more antiinflammatory after the patients were treated with atorvastatin 80 mg daily for 12 weeks.

**Experimental modulators of HDL function**

A new class of drugs known as apo A-I mimetics shows promise as a way to modulate HDL function, as tested in animal models. These drugs are relatively small peptides. An example is D-4F, which is 18 amino acids long and has a helical
structure similar to the active sites of apo A-I and a dextro orientation that makes it resistant to gastrointestinal enzymatic degradation and therefore active when taken orally. Mice and monkeys who were fed D-4F produced more anti-inflammatory HDL as assessed by the monocyte chemotaxis assay (Navab M et al 2005). Interestingly, statins and D-4F appear to work synergistically in promoting aortic lesion regression in monkeys when they are given together compared with the effects of low doses of the two agents alone (Navab M et al 2005). In preliminary studies in humans, 28 D-4F was well absorbed when taken orally, and the subjects’ HDL showed subsequent improvement in anti-inflammatory and antioxidant effects. It is clear, then, that HDL is a dynamic molecule, and that whether it is anti-inflammatory or proinflammatory depends on circumstances such as inflammatory stimuli and on therapeutic interventions.
Aim and scope of the present study

In recent years the concept of “dysfunctional HDL” is emerging. HDL is a complex particle having a wide range of sizes densities, composition and function. A close relationship between inflammation, oxidative stress dyslipidemia and atherosclerosis suggests that alterations in the size, density and composition of HDL may play an important role in transforming the normal HDL into a dysfunctional HDL.

The current dogma is that lipoprotein oxidation is necessarily atherogenic. However this may not be true concerning HDL oxidation. Hence all oxidation is not bad. HDL is a major carrier of oxidized lipids in the plasma. It also carries oxidized cholesteryl esters to liver for excretion. ApoA1 appears to reduce lipid hydroperoxides to alcohols, which may reduce their atherogenicity.

One of the factors attributed to dysfunctional HDL is oxidative stress. Oxidation has been shown to impair the RCT function of HDL. This is attributed to specific modification of two to three methionine residues of ApoA1 that occurs during the initial stages of HDL oxidation. Another modification is the Tyrosine of the proteins, which results in cross linking of proteins.

In contrast there are several reports which show that oxidation of HDL enhances its function rather than inhibiting them, particularly the lipid efflux. This apparent controversy, that mild oxidization of HDL enhances its function, whereas extensive oxidation inhibits it, merits further investigation. In view of the conflicting roles of oxidation of lipoproteins, this study was taken up to evaluate the role of oxidation on HDL function.
Objects of the present investigation:

The overall objective of the study was to investigate the role of oxidized HDL, and to find out the changes taking place in its antiatherogenic properties. In this study the focus has been on the HDL-associated enzyme PON1.

The specific objectives are as follows:

1. To study the effect of oxidation on the substrate specificities of PON1.
2. To study the effect of oxidation of HDL on its antioxidant properties.
3. To raise antibodies to oxidized HDL and use it in assay of oxidized HDL in serum, and to use it to distinguish between oxidized and unoxidised HDL.
4. To evaluate the anti-inflammatory properties of HDL after oxidation.
Chapter I

Introduction

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Introduction


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Chapter I

Introduction


Chapter I

Introduction


Introduction


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