CHAPTER II

LITERATURE REVIEWS

1. ATHEROSCLEROSIS

Atherosclerosis and its relationship to cardiovascular disease

Atherosclerosis is characterized by the accumulation of cholesterol deposits in macrophages in large- and medium-sized arteries (Keaney, 2000). This deposition leads to a proliferation of certain cell types within the arterial wall that gradually impinge on the vessel lumen and impede blood flow. This process may be quite insidious lasting for decades until an atherosclerotic lesion, through physical forces from blood flow, becomes disrupted and deep arterial wall components are exposed to flowing blood, leading to thrombosis and compromised oxygen supply to target organs such as the heart and brain. The loss of heart and brain function as a result of reduced blood flow is termed "heart attack and stroke", respectively. These two clinical manifestations of atherosclerosis are often referred to as coronary artery disease (CAD) and cerebrovascular disease (CBVD) which, are commonly referred to collective term *"cardiovascular disease (CVD)"*.

Mechanism of atherosclerosis

Atherosclerosis involves the cellular infiltration of several cell types, including monocytes, T lymphocytes and mast cells. Monocytes interact with the endothelial layer, attach firmly to the endothelium, and migrate into the subendothelial space and differentiate to macrophage. Then, oxidized low density lipoprotein (Ox-LDL) is accumulated in the macrophages and turn into foam cells. Macrophages and foam cells can secrete growth factors, which lead to cell migration and proliferation of smooth muscle cells (SMC) and matrix production in the intima of the artery. Thus, both macrophages and foam cells contribute to lesion growth and may also contribute to instability and thrombotic events (Libby, 2002) (Figure 1), which lead to manifested clinically as CVD and peripheral arterial disease (PAD) (Stocker and Keaney, 2004).

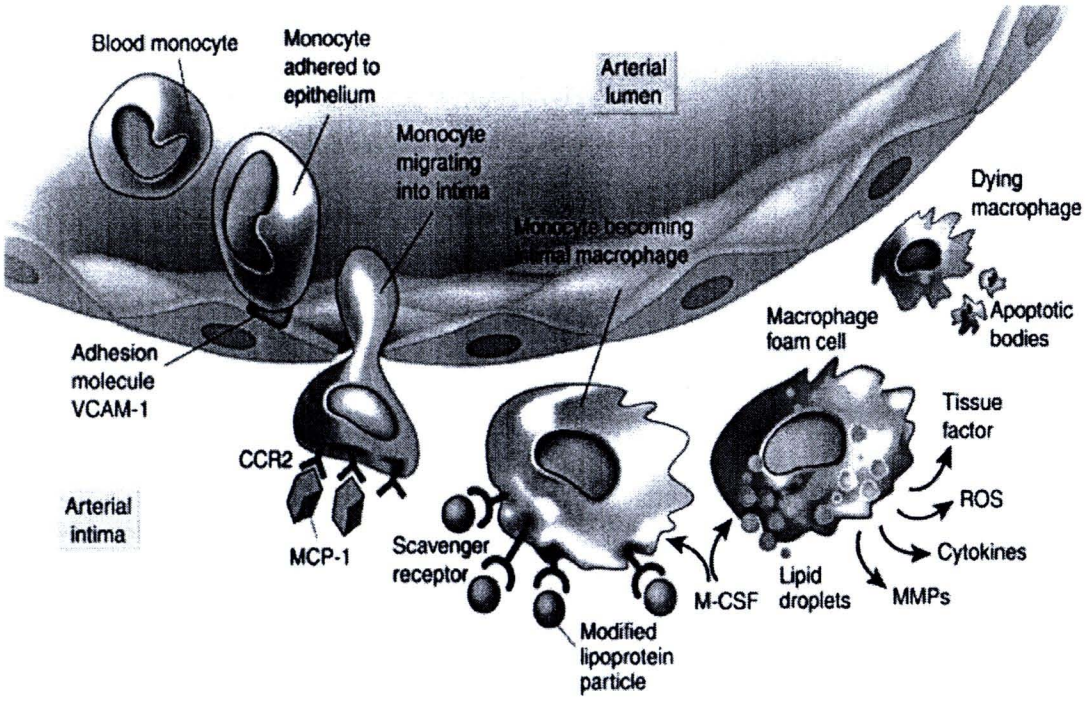

Figure 1 Mechanism of atherosclerosis (Libby, 2002)

Epidemiology and risk factors

Atherosclerosis is considered to be the number one cause of mortality in Western societies and elsewhere in the world. Mechanisms contributing to atherosclerosis are multiple and complex. A number of theories including the role of dyslipidemia, hypercoagulability, oxidative stress, endothelial dysfunction, inflammation and infection by certain pathogens have been propounded from time to time to explain this complex phenomenon (Mallika et al., 2007). Recently, it has been suggested that atherosclerosis is a multi-step disease and multi-factorial. Major differences are between different countries, mainly because of the variation of risk factors for atherosclerosis between populations. Over 300 risk factors have been associated with atherosclerosis. However, between 70% and 90% of the risk of atherosclerotic disease can be explained by different associations between conventional risk factors, such as age, gender, obesity, cigarette smoking, hypertension, diabetes mellitus and abnormal lipids (Vinereanu, 2006) as following areas:

A) AGE. Age is among the most important risk factors for predicting incidence of CVD. In the National Cholesterol Education Program-Adult Treatment

Panel III (NCEP-ATP III), age (men ≥45 years; women ≥55 years) is regarded as a coronary heart disease (CHD) risk equivalent (Expert panel NCEP-ATP III, 2001).

B) GENDER. Numerous observational studies have indicated that male exhibit excess risk for CVD compared with age matched women (Barrett-Connor and Bush, 1991).

C) OBESITY. There is now a growing appreciation that obesity, defined as an excess body weight with an abnormal high preponderance of body fat, is a condition that increases the incident risk of CVD. The exact mechanism(s) to explain this phenomenon, however, are controversial. A number of other risk factors for CVD, such as hypertension, low high density lipoprotein-cholesterol (HDL-C) and diabetes mellitus, often coexist with obesity (Wilson et al., 1999). This relation between obesity and CVD has become of considerable concern as the prevalence of obesity in the developed world is increasing at an alarming rate.

D) CIGARETTE SMOKING. Smoking increases atherosclerotic disease by more than 50% and doubles the incidence of CHD (US Department of Health and Human Services, 1989). This excess risk of smoking on CHD is readily reduced through smoking cessation. In fact, the risk of heart attack in ex-smokers declines to almost that of non-smokers over two years (Gaziano et al., 1996).

E) HYPERTENSION. Hypertension is defined as a systolic blood pressure (SBP) in excess of 140 mmHg or a diastolic blood pressure (DBP) above 90 mmHg (Chobanian et al., 2003). There appears to be an approximately linear relation between blood pressure elevation and the increased incidence of atherosclerotic vascular disease, with an increase of 7 mmHg DBP corresponding to a 27% increase in myocardial infarction (MI) and 42% increase in stroke (MacMahon et al., 1990). The causal nature of this association is supported by numerous studies demonstrating that both heart attack and stroke are significantly reduced in hypertensive patients with the institution of antihypertensive therapy (He and Whelton, 1999).

F) DIABETES MELLITUS. Atherosclerotic coronary disease is a major complication of diabetes mellitus (DM). Approximately 17 million people in the United States, or 6.2% of the population, carry the diagnosis of DM (Cowie et al., 2003). In patients with diabetes the risk of coronary atherosclerosis is 3-5 folds greater than non-diabetics despite controlling for other risk factors (Bierman, 1992). A number of other known risk factors for coronary disease such as hypertension and

abnormal lipids are also more common in diabetics than the general population (Bierman, 1992). Despite this association, no more than 25% of the excess coronary atherosclerosis risk from diabetes can be attributed to these known risk factors (Nishigaki et al., 1981). Thus, diabetes represents a major contributing factor to atherosclerosis.

G) SERUM CHOLESTEROL. The association between cholesterol and atherosclerosis is unequivocal. Some cholesterol is often termed "*good*" and some often termed "*bad*". A higher level of HDL-C is considered "*good*" and gives some protection against heart disease. Higher levels of low density lipoprotein-cholesterol (LDL-C) are considered "*bad*" and can lead to heart disease. A lipoprotein profile can be done to measure several different forms of cholesterol including total cholesterol (TC), HDL, LDL and triglyceride (TG) in the blood. In general, approximately 50% of all Americans between the ages of 20 and 74 have cholesterol levels that exceed 200 mg/dL (Lehr et al., 1999). The excess risk of increased LDL-C appears to be linear with a 1% increase in serum cholesterol corresponding to a 2-3% increase in the risk of CHD (Manson et al., 1992). In contrast to the situation with LDL-C, the relation between HDL-C and atherosclerosis is an inverse one (Gordon et al., 1977; Manson et al., 1992).

The anti- atherogenic properties of HDL

HDL is a class of heterogeneous lipoproteins containing approximately equal amounts of lipid and protein (Gordon and Rifkind, 1989). HDL particles are characterized by high density (> 1.063 g/mL) and small size (diameter between 5 to 17 nm). The various HDL subclasses vary in quantitative and qualitative content of lipids, apolipoproteins (apo), enzymes, and lipid transfer proteins, resulting in differences in shape, density, size, charge, and antigenicity. The most abundant protein of HDL is apo A-I, apo A-II, apo A-IV, apo C, apo E and apo J are found in lower amounts. Some proteins associated with HDL have enzymatic activity. The best-known are lecithin-cholesterol acyltransferase (LCAT), cholesterol ester transfer protein (CETP), phospholipid transfer protein (PLTP), acetyl-PAF hydrolase, and paraoxonase. The epidemiological studies have demonstrated that HDL-C levels are inversely and independently related to the incidence of CHD, which the data showed 1% decrease in HDL increases the risk for CHD by 2-3% (Manson et al., 1992).

Cardiovascular mortality was 36% higher in man with low HDL compare with normal HDL. This effect was amplified in subjects with combination of low HDL and diabetes producing 65% increase in CHD mortality (Genest et al., 1991; Rubins et al., 1995). However, the mechanisms involved in HDL protection against atherosclerosis are uncertain, but the beneficial effect of HDL might be the consequence of its properties. The best known of them is related to the anti-atherogenic properties of HDL. These include: (1) inhibition of chemotaxis of monocytes; (2) inhibition of monocyte adhesion to endothelial cells; (3) inhibition of LDL oxidation; (4) inhibition of Ox-LDL-induced endothelial dysfunction and apoptosis; (5) stimulation of endothelial cell proliferation; (6) stimulation of endothelial synthesis of prostacyclin and natriuretic factor C (CNP); (7) stimulation of cholesterol efflux from macrophages and foam cells; (8) stimulation of SMC proliferation; (9) inhibition of platelet activation; (10) inhibition of factor X activation and stimulation of activated protein C as shown in Figure 2 (Nofer et al., 2002). Interestingly, paraoxonases (PONs), which is an enzyme system that associated with HDL, might be related to the those anti-atherogenic properties of HDL.

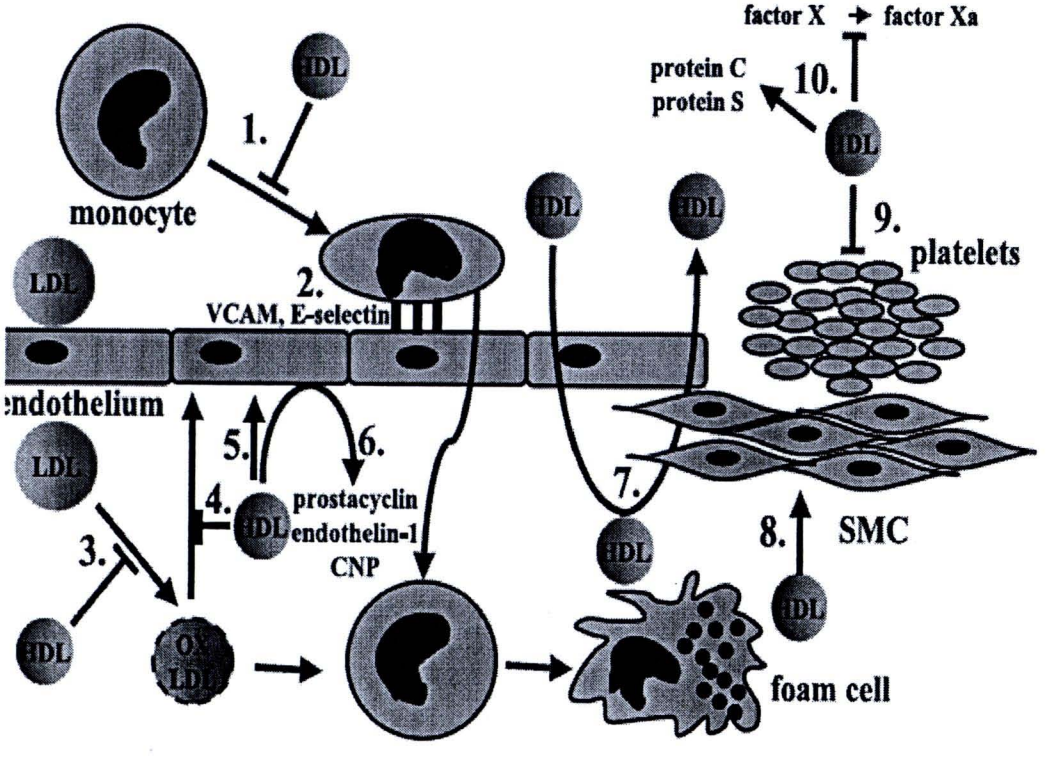

Figure 2 Pleiotropic effects of HDL in the vessel wall (Nofer et al., 2002)

2. PARAOXONASES (PONs) FAMILY

History of paraoxonase

In 1946, Abraham Mazur was the first to report the presence of an enzyme in animal tissue which was able to hydrolyse organophosphate compounds (Mazur, 1946). During the 1950s, Aldridge was the initial identification of the human serum paraoxonase (PON1) enzyme (Aldridge, 1953). PON1 was named after its ability to hydrolyse the organophosphate substrate paraoxon (paraoxonase activity, EC 3.1.8.1), which is the toxic metabolite of the insecticide parathion. Because PON1 could also hydrolyse aromatic esters, such as phenyl acetate (arylesterase activity, EC 3.1.1.2), the term 'A-esterase' was introduced for the enzyme hydrolyzing both compounds. The following years, much discussion during the following years as to whether one enzyme or two were responsible for the paraoxonase and arylesterase activity, but finally, conclusive evidence was delivered that both paraoxonase activity and arylesterase activity were properties of PON1. Mackness and colleagues (1991) demonstrated that PON1 could prevent the accumulation of lipoperoxides in LDL (Mackness et al., 1991), thus linking PON1 to CVD, the scientific interest in PON1 increased immensely. Despite the boom in research, to date the exact physiological function of PON1 is still unclear.

Paraoxonases (PONs) family

The PON gene family consists of three members, PON1, paraoxonase2 (PON2), and paraoxonase3 (PON3), located adjacent to each other on the long arm of chromosome 7 between q22.3 and q23.1 in humans (Primo-Parmo et al., 1996). These three members appear to be clustered together as shown in Figure 3 (Hong-Liang et al., 2003).

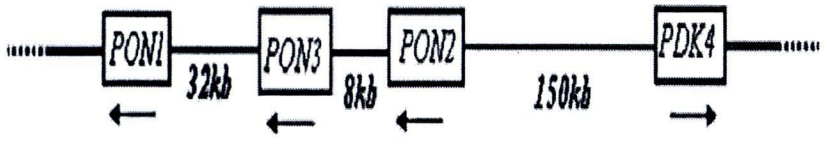

Figure 3 Paraoxonases (PONs) family (Hong-Liang et al., 2003)

The three PON genes share approximately 65% similarity at the amino acid level and approximately 70% similarity at the nucleotide level (Mackness et al., 2002). From an evolutionary standpoint, PON2 appears to be the oldest member, followed by PON3, and then PON1 (Draganov et al., 2004). PON1 and PON3 are expressed primarily in the liver and excreted in the blood where they are associated with the HDL particle. Furthermore, unlike PON1, significant PON3 mRNA level is also detectable in the kidneys (Reddy et al., 2001). In contrast, PON2 is not present in blood and undetectable in HDL and LDL particles (Mochizuki et al., 1998; Ng et al., 2001), but is expressed widely in a number of tissues, including the heart, liver, kidney, lung, placenta, small intestine, spleen, stomach and testis (Figure 4). In addition, PON2 message is also detected in the cells of the artery wall, including endothelial cells, smooth muscle cells, and macrophages (Ng et al., 2001)

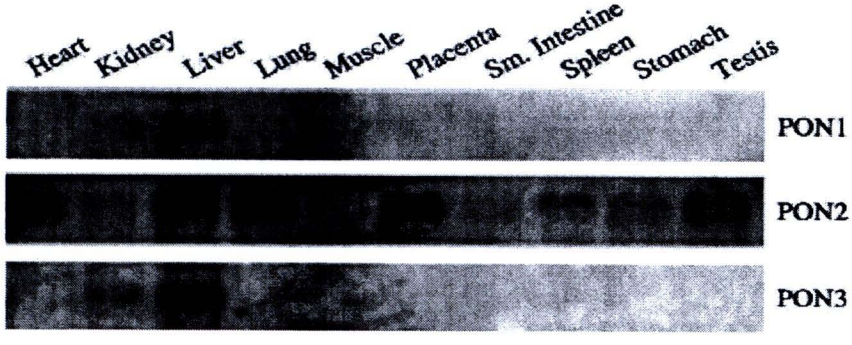

Figure 4 Tissue distribution of the human PONs family (Ng et al., 2005)

Paraoxonase 1 (PON1)

PON1 is a calcium-dependent esterase consisting of 354 amino acids with a molecular mass of approximately 45 kDa (Primo-Parmo et al., 1996). Initially characterized for its ability to hydrolyze organophosphates, the name PON1 reflects its ability to hydrolyze paraoxon, a metabolite of the insecticide parathion. General structure of human PON1 contains as many as three carbohydrate chains, three cysteine (Cys) residues, a single disulfide bond between Cys-42 and Cys-353, with a single free cysteine only one at position 284 which has a free sulfhydryl group. PON1 has an extremely hydrophobic N-terminal end that could anchor it to HDL lipids, however, PON1 is not present in LDL or very low density lipoprotein (VLDL),

indicating a specific interaction with HDL by association with apo A-I (Sorenson et al., 1999) as shown in Figure 5.

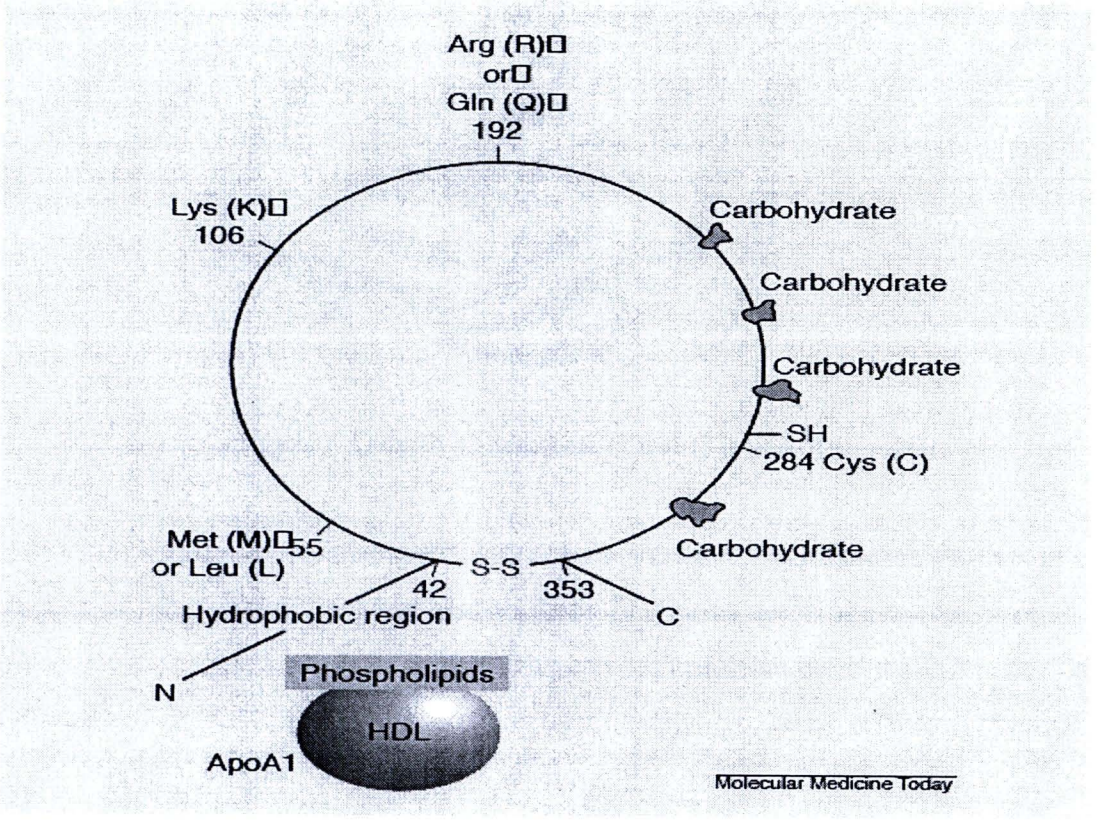

Figure 5 General structure of human serum paraoxonase1 (PON1) (Aviram, 1999)

PON1 consists of six bladed (1-6) beta propellers and each blade contains four strands (A, B, C and D), which the top of the propeller is, by convention, the face carrying the loops connecting the outer beta strand of each blade (strand D) with the inner strand (A) of the next blade, and the two calcium ions ($Ca^{2+}1$ and $Ca^{2+}2$) in the central tunnel of the propeller as seen in the Figure 6 (Michal et al., 2004).

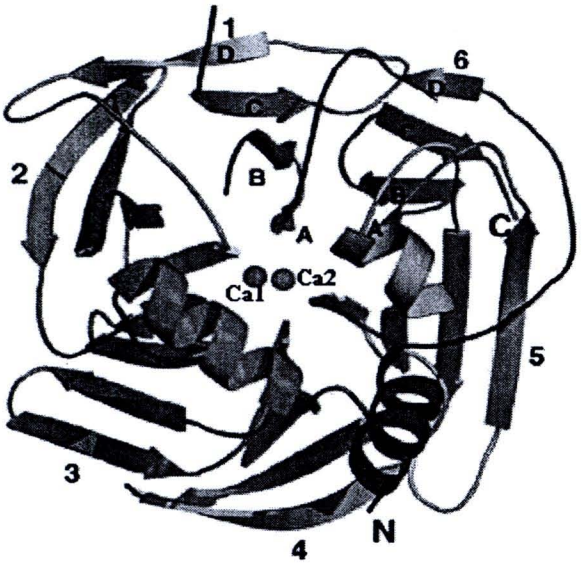

Figure 6 Overall structure of PON1 (Michal et al., 2004)

In addition to paraoxon, PON1 has been shown to hydrolyze metabolites of a number of other insecticides and also to detoxify various nerve agents (Costa et al., 2005). However, evidence gained in recent years suggests that the name PON may be a misnomer, as PON2 and PON3 lack any significant paraoxonase activity (Draganov et al., 2000; Ng et al., 2001; Reddy et al., 2001). PON1, PON2, and PON3 do, however, share an ability to hydrolyze aromatic and long-chain aliphatic lactones, and thus the term lactonase may be more appropriate (Draganov et al., 2004). Nonetheless, the physiological substrates for these proteins have not been identified and are currently under investigation. Interestingly, Draganov and colleagues (2005) characterized the enzymatic activities of the purified recombinant human PONs as shown in Table 1.

Table 1 Specific enzymatic activities of the purified recombinant human PONs (Draganov et al., 2005)

Substrate	PON1	PON2	PON3
Organophosphatase activity (U/mg)			
Paraoxon	1.94 ± 0.11	ND	0.205 ± 0.05
Chlorpyrifos oxon (0.32 mM)	40.9 ± 0.9	ND	ND
Diazoxon	113 ± 5	ND	ND
Arylesterase activity (U/mg)			
Phenyl acetate	1,120 ± 50	0.086 ± 0.0013	4.1 ± 0.3
p-NO_2-acetate	15.0 ± 0.03	0.7 ± 0.07	39.0 ± 4.1
p-NO_2-propionate	13.6 ± 0.04	0.96 ± 0.06	20.7 ± 3.2
p-NO_2-butyrate	1.3 ± 0.015	1.4 ± 0.03	11.4 ± 0.7
Lactonase activity (U/mg)			
Dihydrocoumarin	129.9 ± 8.30	3.1 ± 0.2	126.1 ± 12
2-Coumaronone	135.7 ± 10.3	10.9 ± 0.4	40.7 ± 3.8
Homogentisic acid lactone	329.5 ± 13.1	ND	ND
γ-Butyrolactone	32.1 ± 2.73	ND	0.81 ± 0.1
γ-Valerolactone	45.0 ± 3.7	ND	6.2 ± 0.4
γ-Hexalactone	51.7 ± 4.2	ND	23.9 ± 3.2
γ-Heptalactone	57.2 ± 2.3	ND	27.7 ± 2.7
γ-Octalactone	69.2 ± 4.3	ND	25.6 ± 3.2
γ-Nonalactone	144.7 ± 11.3	ND	30.9 ± 2.7
γ-Decanolactone	173.8 ± 14.7	ND	45.6 ± 3.6
γ-Undecanolactone	127.6 ± 10.5	ND	71.4 ± 3.1
∝-Angelica lactone	183.0 ± 16	ND	20.7 ± 3.2
γ-Phenyl-γ-butyrolactone (0.5 mM)	63.0 ± 3.1	0.68 ± 0.08	11.4 ± 0.7
α-Valerolactone	671 ± 14	ND	14.5 ± 0.7
δ-Hexalactone	72 ± 2.3	ND	11.7 ± 1.2
δ-Nonalactone	150 ± 12.3	ND	11.1 ± 0.9
δ-Decanolactone	251 ± 13	ND	44.3 ± 3.2
δ-Undecanolactone	287 ± 17	ND	84.4 ± 2.7
δ-Tetradecanolactone (0.5 mM)	154 ± 24	ND	22.7 ± 2.2

Substrate	PON1	PON2	PON3
5-HETEL (10 μM)	75.4 ± 8.36	1.83 ± 0.08	27.5 ± 3.6
DL-3-Oxo-hexanoyl-HSL (250 μM)	0.0334 ± 0.0031	0.2683 ± 0.0384	ND
L-3-Oxo-hexanoyl-HSL (250 μM)		0.5080 ± 0.0661	
DL-Heptanoyl-HSL (25 μM)	0.0036 ± 0.0004	0.0311 ± 0.0026	0.0049 ± 0.0023
DL-Dodecanoyl-HSL (25 μM)	0.0167 ± 0.0005	0.4588 ± 0.0371	0.0877 ± 0.0014
DL-Tetradecanoyl-HSL (25 μM)	0.0035 ± 0.0013	0.4239 ± 0.0204	0.0255 ± 0.0003
Lovastatin (25 μM)	ND	ND	0.0266 ± 0.022
Spironolactone (25 μM)	ND	ND	0.011 ± 0.001
Canrenone (25 μM)	ND	ND	0.013 ± 0.001
Lactonizing activity (U/mg)			
Coumaric acid (100 μM)	0.047 ± 0.004	ND	0.013 ± 0.0007
4-HDoHE (10 μM)	1.51 ± 0.16	0.52 ± 0.03	13.7 ± 2.0

5-HETEL, (±)5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid 1,5-lactone; HSL, homoserine lactone; ND, not detectable under these assay conditions. Data are averages from two to four measurements ± SD or range. One unit = 1 μmol of substrate metabolized per minute. All substrates were at 1 mM final concentration unless indicated otherwise.

Modulation of PON1

PON1 can be modulated by environmental chemicals, drugs, smoking, alcohol, diet, age, and disease condition of which have been shown to modulate PON1 activity in either direction as shown in Figure 7. PON1 hydrolyzes several organophosphate compounds used as insecticides, as well as nerve agents, thus PON1 plays a protective role in organophosphate toxicity. It metabolizes toxic oxidized lipids associated with both LDL and HDL. These actions suggested a role of PON1 in CVD and atherosclerosis. In addition, it can hydrolyze a number of lactone-containing pharmaceutical compounds (Costa, 2005) (Figure 7).

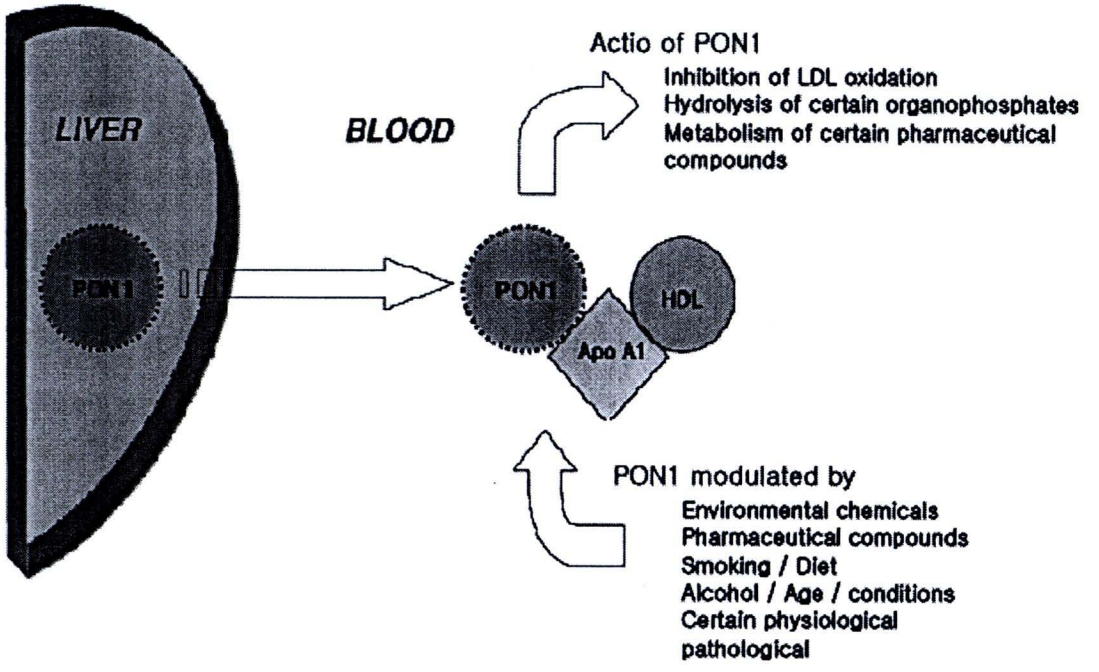

Figure 7 Biological effects and modulation of PON1 (Costa, 2005)

PON1 as anti-atherosclerosis

Mackness and colleagues (1991) found that PON1 might play an important physiological role in lipid metabolism and protect against the development of atherosclerosis resulting in on intensive study of PON1 (Mackness et al., 1991). Throughout the past decade, effects of PON1 has been clarified especially the antioxidant properties that protect LDL against oxidation and reverse the biological effects of Ox-LDL as well as preserve the function of HDL by inhibiting its oxidation (Aviram, 1999; Durrington et al., 2001). An additional important clue to a physiological function of PON1 was reported by Shih et al. that PON1 null mice developed atherosclerosis when fed an atherogenic diet, and in contrast to wild-type, their HDL fails to prevent LDL oxidation in cultured artery wall cells (Shih et al., 1998). Furthermore, when PON1 null mice were crossed with apoE null mice, PON1/apoE null mice developed significantly larger lesions of atherosclerosis than apoE null mice. LDL freshly isolated from PON1/apoE null mice had higher levels of biologically active phospholipids relative to LDL from apoE null mice, suggesting higher levels of oxidative stress in the double knockout mice. Similar to PON1 null mice, HDL from PON1/apoE null mice failed to protect LDL against oxidation (Shih

et al., 2000). Thus, results from those studies were correlated to the hypothesis that PON1 protects against atherosclerosis and is importantly contributing HDL of the antioxidant capacity. Furthermore, in human PON1 trangenic mice on the background of apoE null mice, a decreased lesion size of atherosclerosis was observed (Tward et al., 2002). These information of the *in vivo* studies underscore the potential of PON1 as a therapeutic agent to prevent atheroma. Although, many clinical papers have been published in the past decade, most supporting, but some excluding, a relationship between the PON1 and anti-atherosclerosis which possible mechanisms of PON1 to protect against atherosclerosis was shown in Table 2

Table 2 Evidence for human PON1 antiatherogenicity (summarized from Aviram, 1999; Durrington et al. 2001)

1. Protects HDL against oxidation and preserves its functions
   ↑ Cellular cholesterol efflux from macrophages
2. Protects LDL against oxidation
   ↓ Lipid peroxides
3. Ameliorates effects of Ox-LDL
   ↓ Inflammatory and cytotoxic oxidized phospholipids
   ↓ LDL uptake by macrophages
   ↓ monocyte transmigration induced by Ox-LDL
4. Decreases lipid peroxides in atherosclerotic lesions

Mechanism of PON and its relationship to anti-atherosclerosis

The association between PON1 and anti-atherogenic properties are shown in Figure 8. PONs act as an anti-atherogenic agent at several steps along the atherogenic pathway: (a) PON1 associated HDL can hydrolyze specific lipid peroxides in Ox-LDL and convert this atherogenic lipoprotein to a non-atherogenic lipoprotein (LDL). Under oxidative stress, lipid peroxidation takes place in the arterial wall, affecting not only arterial LDL, but also arterial cells, including macrophages. (b) PON1 associated HDL can hydrolyze lipid peroxides in oxidized macrophage (Ox-MQ). (c) PON1

associated HDL protects HDL from oxidation and promotes HDL mediated cholesterol efflux from macrophage foam cells (Aviram, 1999).

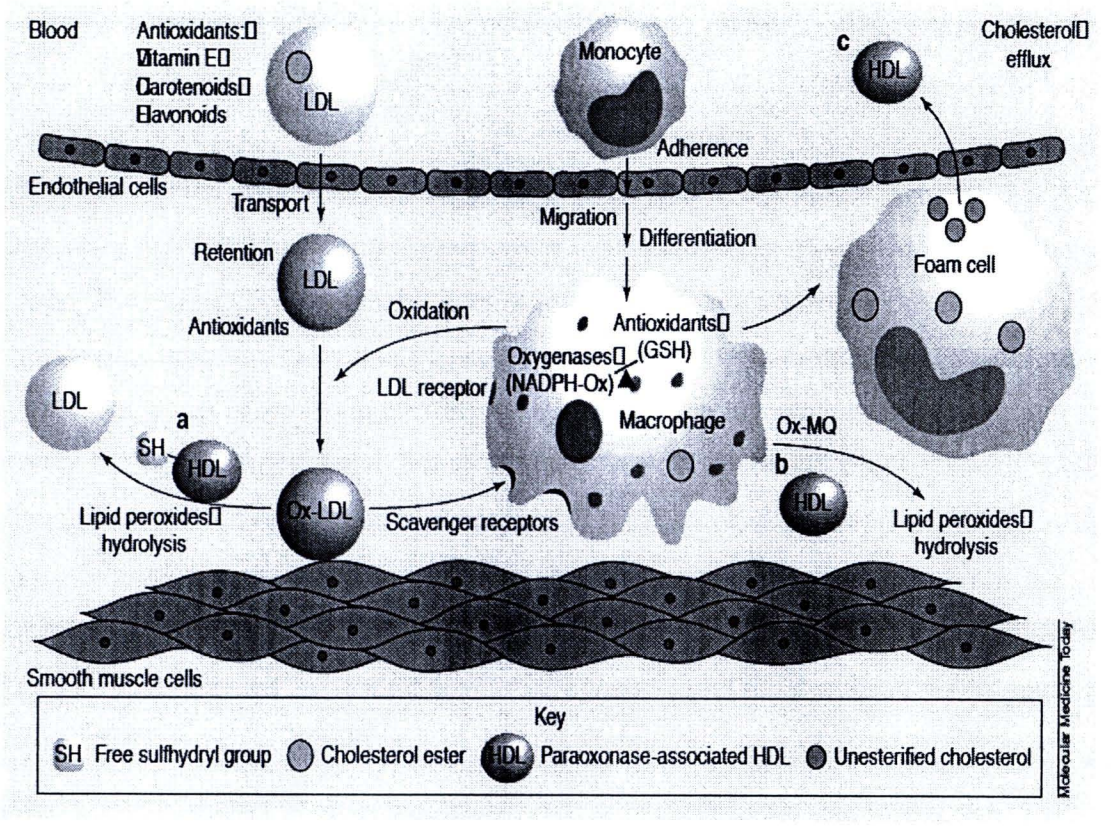

Figure 8 PON1 and macrophage foam cell formation (Aviram, 1999)

Paraoxonase 2 (PON2)

PON2 is a widely expressed intracellular protein with a molecular mass of approximately 44 kDa, which is expressed in various tissues and cells. PON2, unlike PON1 or PON3, does not associate with HDL fractions in the circulation but remains intracellular, associated with the membrane fractions. Furthermore, PON2 is widely expressed in a number of tissues and cell types, including cells of the artery wall, whereas human PON1 and PON3 expression is limited primarily to the liver, (Ng et al., 2001). These unique properties of PON2 make it an interesting candidate to study the physiological or pathophysiological function of PONs. However, to date, little is known about the functional role of PON2 in the pathogenesis of CVD. *In vitro* studies suggested that PON2 possesses anti-oxidative properties (Ng et al., 2001). Over-expression of PON2 in HeLa cells reduced cellular oxidative status and decreased the

ability of the cells to Ox-LDL (Ng et al., 2001). Rosenblat and colleagues have also demonstrated that incubation of purified recombinant PON2 with mouse peritoneal macrophages (MPM) isolated from the atherosclerotic apo E deficient mice decreased cellular oxidative status (Rosenblat et al., 2003). In addition, LDL incubated with stably transfected cells over-expressing PON2 had lower levels of lipid hydroperoxides, and was less able to induced monocyte transmigration through endothelial cells. Moreover, PON2 was able to retard the oxidation of preformed minimally modified low density lipoprotein (MM-LDL) (Ng et al., 2001). In humans, lower level of PON2 message was seen in monocyte derived macrophages of hypercholesterolemic individuals relative to individuals with normal cholesterol levels (Rosenblat et al., 2004). Interestingly, administration of atorvastatin was able to reduce both cellular oxidative stress and cholesterol content leading to up-regulated macrophage PON2 expression and activity (Rosenblat et al., 2004). Recently, Horke and colleagues investigated the expression, regulation, and potential anti-oxidative functions of PON2 in 3 major cell types of the human vasculature. The results showed that PON2 expressed at similar levels in human endothelial cells, smooth muscle cells, and adventitial fibroblasts, which implied that PON2 represented an endogenous defense mechanism against vascular oxidative stress and unfolded protein response induced cell death, thereby contributing to the prevention of atherosclerosis (Horke et al., 2007). In contrast, PON2 expression increased in monocytes during differentiation into macrophages, and this effect was shown to be mediated via the transcription factor activated protein-1 (AP-1) (Shiner et al., 2007). Thus, one function of PON2 may be to act as a cellular antioxidant, protecting cells from oxidative stress, which it inhibits LDL lipid peroxidation, reverses the oxidation of MM-LDL and inhibits its ability to induce monocyte chemotaxis. Cellular oxidative stress and PON2 activity show a biphasic U-shape relationship (Shiner et al., 2006).

Paraoxonase 3 (PON3)

PON3 enzyme is a 40-kDa glycoprotein with calcium-dependent esterase activities. Thus, PON3 are able to catalyze the hydrolysis of a broad range of substrates including aryl-esters, lactones, and many pharmacological agents. PON3 shows high similarity in structure and functions with PON1. Similar to PON1, PON3 are expressed primarily in the liver and then secreted into the serum where they are

closely associated with HDL. However, significant PON3 mRNA level is also detectable in kidney (Reddy et al., 2001). PON3, like PON1, contains the N-terminal hydrophobic peptide and share three conserved cysteine residues: Cys-42, Cys-284, and Cys-353, and possesses similar properties in structure and activities. Many evidences suggested that PON3 is important in the prevention of atherosclerosis and seems to be a candidate in PONs family (Aviram and Rosenblat, 2004). Purified rabbit PON3 possesses the ability to protect LDL against *in vitro* copper-induced oxidation with approximately 100 times more potent than the rabbit PON1 in protecting LDL against oxidation (Draganov et al., 2000). Recently, it was also found that over-expression of human PON3 in mice reduced atherosclerotic lesion (Ng et al., 2007). In contrast to PON1, PON3 has very limited arylesterase activity and no paraoxon activity, but it can rapidly hydrolyze lactones as shown in Table 1 (Draganov et al., 2000; La Du et al., 2001; Ng et al., 2001). Under oxidative stress, the expression of PON1 is down-regulated while the expression of PON3 is unchanged (Reddy et al., 2001). Thus, PON3 may act by a mechanism similar to that of PON1 but play a key role different from PON1 in the prevention of atherosclerosis. Its contribution against LDL oxidation and in the prevention of atherosclerosis might be as great as that of PON1. So, PON3 may have the potential to be a therapeutic agent to prevent atheroma. Furthermore, unlike PON2, PON3 message remains unchanged in mouse peritoneal macrophages exposed to various oxidative stress inducing agents, although PON3 lactonase activity decreased whereas PON2 expression and enzymatic activity increased (Rosenblat et al., 2003). Draganov and colleagues (Draganov et al., 2005) reported the expression of PON1, PON2 and PON3 from human tissue by a baculovirus-mediated system. Insect cells used in conjunction with the baculovirus expression vector system (BEVS) are gaining ground rapidly as a platform for recombinant gene expression, especially for proteins that are insoluble in *Escherichia coli* or are covalently modified while Lu et al. (Lu et al., 2005), which amplified PON3 cDNA from Human Fetal Liver Marathon-Ready cDNA and expressed it in baculovirus-mediated Sf9 cells at high level. The results shown that the purified PON3 from Sf9 cells could inhibit LDL oxidation *in vitro*, and in contrast to the results from Draganov et al. (Draganov et al., 2005).

3. Statins

History of statins

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, more commonly known as the statins. Statins were first introduced into clinical practice in the late 1980s after the chance discovery of their lipid-lowering effects in 1976 when Endo et al. isolated the first statin from *Penicillium citrinum*, called mevastatin (Endo et al., 1976). Clinical trials of mevastatin quickly discontinued because intestinal tumors were found in dogs. In general, statins are regarded as a remarkably safe and well-tolerated class of drugs. It must be stressed that on August 8, 2001, Bayer AG voluntarily withdrew cerivastatin from the world pharmaceutical market, after 31 patients died by acute renal failure caused by rhabdomyolysis. As a result, currently there are seven statins in clinical use: lovastatin, simvastatin, pravastatin, fluvastatin, atorvastatin, pitavastatin and rosuvastatin (Davidson, 2002).

Classification of statins

There are a number of classification criteria for statins, including: (1) How they are obtained, lovastatin, pravastatin and simvastatin are natural products of fungal origin, while atorvastatin, cerivastatin, fluvastatin, pitavastatin and rosuvastatin are fully synthetic compounds (Davidson, 2002). (2) Liver metabolism, all statins have the liver target organ. For their liver metabolism, lovastatin, simvastatin and atorvastatin are metabolized via the cytochrome P450 (CYP) 3A4 pathway. Fluvastatin is metabolized by the CYP 2C9 and cerivastatin is metabolized by dual CYP 2C9 (or 2C8) and 3A4 pathways. Pravastatin, pitavastatin and rosuvastatin are not significantly metabolized by the CYP450 pathways (Davidson and Toth, 2004). (3) Physico-chemical properties, atorvastatin, fluvastatin, lovastatin, cerivastatin and simvastatin are relatively lipophilic compounds, while pravastatin and rosuvastatin are extremely hydrophilic as a result of a polar hydroxyl group and methane sulphonamide group, respectively (McTaggart et al., 2001). (4) Specific activity, lovastatin and simvastatin are administered as lactone prodrugs, and are enzymatically hydrolysed *in vivo* to their active, hydroxyl acid forms in the liver (Corsini et al., 1995). The other statins such as atorvastatin, cerivastatin, fluvastatin and pravastatin are administered as active drug (Corsini et al., 1999).

Mechanism for the action of statins

Statins are oral systemic agents that suppress cholesterol synthesis by competitive inhibition of the HMG-CoA reductase, the rate-limiting step in the conversion of HMG-CoA to mevalonate, a precursor of cholesterol (Almuti et al., 2006). Inhibition of cholesterol biosynthesis leads to up-regulation of LDL receptors on the hepatocyte cell surface, which results in increased extraction of LDL-C from the blood and decreased circulating LDL-C concentration as shown in Figure 9 (Law et al., 2004). Statins also have beneficial effects on other lipid parameters, including increases in HDL-C concentration and decreases in TG concentration (Expert panel NCEP-ATP III, 2001).

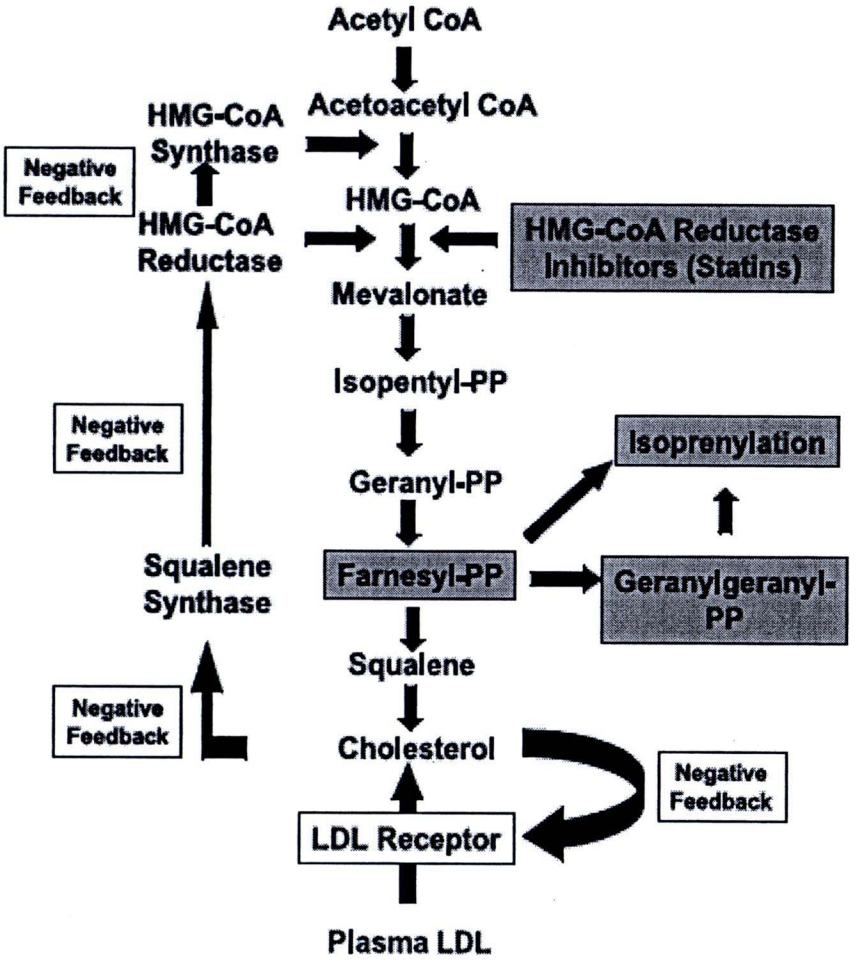

Figure 9 Cholesterol biosynthetic pathway and its inhibition by statins (Law et al., 2004)

Anti-atherosclerosis mechanisms of statins

Atherosclerosis results from a complex interplay between many factors and processes such as lipid metabolism, inflammation, endothelial dysfunction, plaque instability, thrombosis and smooth muscle proliferation (Ross, 1999). The mechanisms for anti-atherosclerosis of statins, include reduction of LDL oxidation and uptake of Ox-LDL into macrophages, inhibition of monocyte-endothelial cell adhesion, reducing of vascular inflammation, inhibition of vascular smooth muscle proliferation and/or migration, inhibition of platelet aggregation and arterial intimal thickening, induction of apoptosis of vascular smooth muscle cells and inhibition of cholesterol esterification (Figure 10).

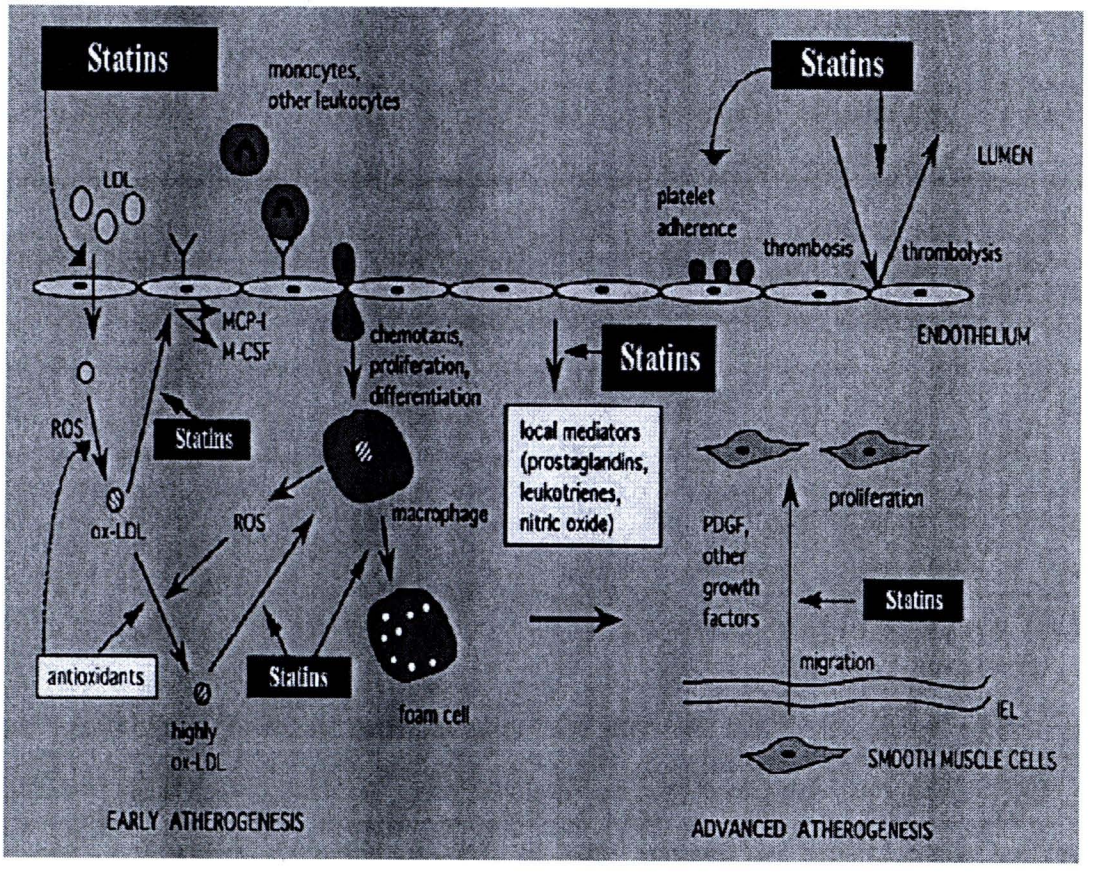

Figure 10 Mechanisms for anti-atherosclerosis of statins (Stancu and Sima, 2001)

The effect of statins on paraoxonases

Statins are widely used as lipid lowering drugs in patients with disturbed lipid metabolism. Apart from reducing both plasma total cholesterol and LDL-C, an increased HDL-C level is also found. Statins have additional beneficial effects on

other processes involved in the atherosclerotic process. Interestingly, elevated levels of HDL-C are predictive of protection against atherosclerosis. Thus, statins may favourable effect on HDL-associated enzyme PONs. Up to now the effect of statins on PONs was extensively investigated both *in vitro* and *in vivo* studies. The effect of statins on PONs associated-HDL can also act as an anti-atherosclerosis in circulation and at several organs such as liver, artery wall cells and contribute to macrophage. For effect of statins on PON2 which not associated with HDL may protect cells from oxidative stress in macrophage (Rosenblat et al., 2004) as shown in Figure 11.

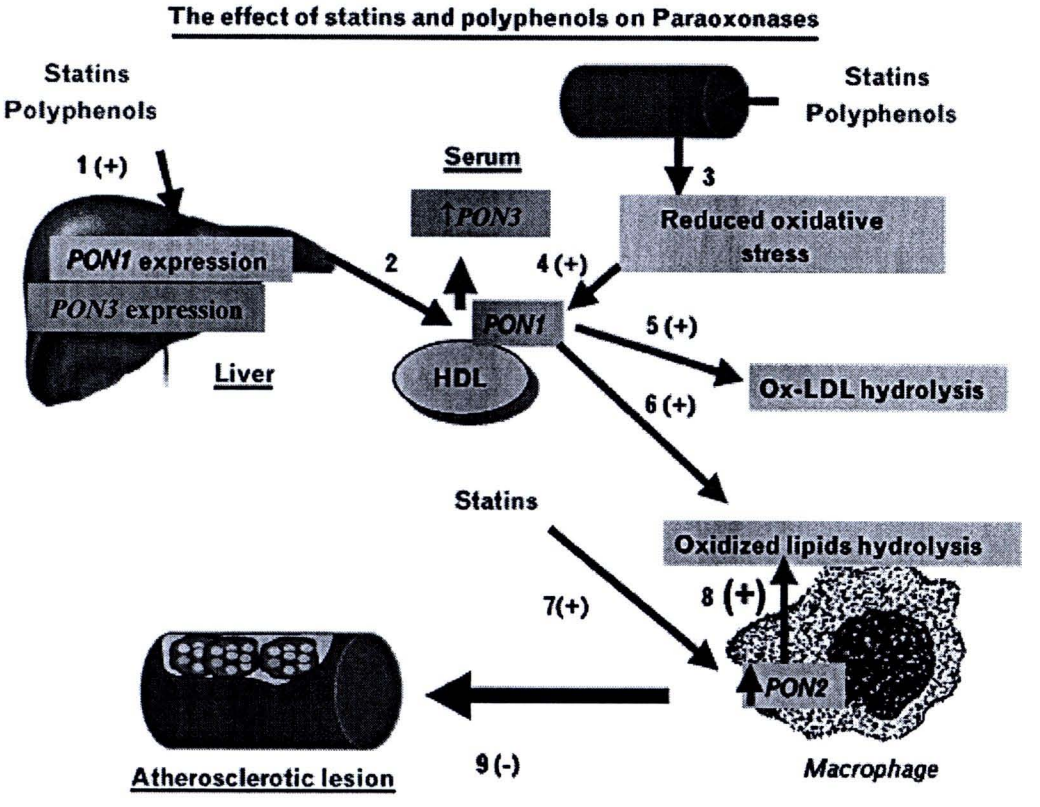

Figure 11 The effects of statins on PONs (Modification from Aviam et al., 2005)

Simvastatin

Simvastatin is a hypolipidemic drug belonging to the class of pharmaceuticals called "statins". Simvastatin, a synthetic derivative from a fermentation product of *Aspergillus terreus*, was introduced in the late 1980s. The molecular formula of simvastatin is $C_{25}H_{38}O_5$ and its molecular weight is 418.57. Its structural formula is:

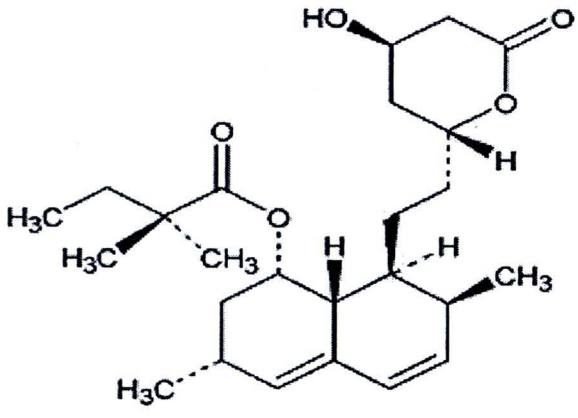

Figure 12 Chemical structure of the simvastatin (Schachter, 2004)

Pharmacodynamic properties of simvastatin

Simvastatin, after oral ingestion which hydrolysis in the liver to form the active [beta]-hydroxy acid metabolite, acts as a potent reversible, competitive inhibitor of HMG-CoA reductase to mevalonate, which is an early and rate limiting step in the biosynthesis of cholesterol. As a result, *in vitro* cell culture studies show that simvastatin is a potent inhibitor of cholesterol synthesis (Nagata et al., 1990). Various studies *in vivo* in rats and dogs have confirmed the ability of simvastatin to reduce plasma cholesterol levels (Vickers et al., 1990) and there is some evidence showing that the drug reduces the production of atherosclerotic lesions in rabbits (Bocan et al., 1994; Nachtigal et al., 2005). In human, simvastatin decreased plasma total cholesterol (TC) and LDL-C and TG levels but elevated HDL-C level (Strano et al., 1989; Lu, 1993; Vaughan et al., 2004).

Pharmacokinetic properties of simvastatin

Simvastatin is a lactone prodrug that is readily hydrolyzed *in vivo* to the corresponding [beta]-hydroxy acid, a potent inhibitor of HMG-CoA reductase. Simvastatin is absorbed rapidly following oral administration, with time to reach peak concentrations (T_{max}) of within 1.3-2.4 hr. As a result undergoes extensive first-pass metabolism in the liver which the systemic bioavailability of the drug is low at 5%. However, food intake dose not affect absorption by co-administration with simvastatin. Repeated administration of normal therapeutic doses of simvastatin has no accumulation. Ninety-five percent of simvastatin are bound to protein in human

plasma. Since simvastatin is predominantly metabolized by cytochrome P450 (CYP) 3A4 system, a potential for drug interactions exists. Elevated HMG-CoA reductase inhibitory activity has been observed when simvastatin was administered concurrently with cyclosporin, possibly increasing the risk of myopathy and subsequent rhabdomyolysis which are associated with simvastatin use. Simvastatin has also been shown to potentiate the effects of warfarin. The excretion of simvastatin mainly via the bile in feces at 58%, as elimination half-life is short (2-3 hr) (Corsini et al., 1999; Schachter, 2004; Shitara and Sugiyama, 2006).

Dosage administration of simvastatin

The dosage range is 5-80 mg/day which the recommended usual starting dose is 20 to 40 mg once a day in the evening. Doses should be individual according to baseline LDL cholesterol levels and the LDL goal recommended by the NCEP. For patients at high risk for a CHD event due to existing CHD, diabetes, peripheral vessel disease, history of stroke or other cerebrovascular disease, the recommended starting dose is 40 mg/day. For lipid determinations and adjustments of dosage should be performed after 4 weeks of therapy and periodically thereafter. However, dosage recommendations should be considered for patients requiring smaller reduction in special populations such as adolescents and renal insufficiency or for patients receiving concomitant therapy such as cyclosporin, diltiazem, verapamil and gemfibrozil (Lacy et al., 2002).

Adverse effects of simvastatin

Simvastatin is generally well tolerated. The most important adverse effects are liver and muscle toxicity. For definitions of potential muscle adverse experiences due to statins are shown in Table 3. The risk of muscle toxicity with statin therapy may be increased by factors that predispose the patient to myopathy, such as increasing age, female sex, renal insufficiency, hepatic dysfunction, hypothyroidism, diet (grapefruit juice) and polypharmacy (Rosenson, 2004; Bays, 2006). However, the properties of the statins themselves could also influence the risk of myopathy and may be worth considering during treatment selection to maximize the benefit-risk ratio.

Table 3 Definitions of potential muscle adverse experiences due to statins (Bays, 2006)

Potential Muscle Adverse Experience	Definitions Used in this Overview
Myalgias	Muscle ache, pain, or weakness with or without CK elevation
Myopathy	Otherwise unexplained elevations in CK≥10X the ULN, associated with muscle symptoms (myalgias)
Rhabdomyolysis	Marked CK elevation, typically substantially >10X the ULN and with creatinine elevation (usually with brown urine and urinary myoglobin). Elevations in other muscle enzymes may also occur, as well as the following: ▪ Hyperkalemia ▪ Hypocalcemia ▪ Hyperphosphatemia ▪ Hyperuricemia ▪ Metabolic acidosis ▪ Renal failure ▪ Death ▪ Symptoms of muscle weakness may be present, but perhaps only 50% of the time.

CK = creatine kinase; ULN = upper limit of normal.

Contraindications and precautions of simvastatin

The contraindications of simvastatin are including: hypersensitivity to any component of this preparation, active liver disease or unexplained persistent elevations of serum transaminases, pregnancy, lactation and myopathy secondary to other lipid lowering agents (Moghadasian, 1999). For precaution, simvastatin is

avoided in patients taking potent CYP 3A4 inhibitors such as human immunodeficiency virus (HIV)-protease inhibitors and azole antifungal agents. In addition, the dose of simvastatin should be limited when using certain drugs that elevate circulating simvastatin concentrations such as gemfibrozil, and verapamil (Davidson and Toth, 2004).

Clinical studies: simvastatin

In 1994, the Scandinavian Simvastatin Survival Study (4S) established clearly that hypolipidaemic therapy was safe and reduced morbidity and mortality in hypercholesterolaemic patients with ischemic heart disease (IHD) (Scandinavian Simvastatin Survival Study Group, 1994). Subsequently, Heart protection study (HPS) firmly establishes the benefit of simvastatin therapy in preventing adverse events in patients at high risk of atheromatous disease, regardless of initial lipid levels (Heart Protection Study Collaborative Group, 2002). In 2004, Phase Z of the A to Z trial was important to remember that acute coronary syndrome (ACS) patients also benefit clinically from high dose simvastatin therapy (De Lemos, 2004). However, using simvastatin in clinical practice was found to demonstrate a risk of causing adverse effects (Grundy et al., 2004).

Simvastatin treatment on paraoxonase

The information about the effect of simvastatin on human PON activity still ambiguous untill now. Tomas and colleagues (2000) found that a 4-month simvastatin might have important antioxidant properties through increasing serum PON1 activity, perhaps as a consequence of reducing oxidative stress (Tomas et al., 2000). Deakin et al. also indicated that simvastatin can modulate expression *in vitro* of the antioxidant enzyme paraoxonase and associated with increased serum paraoxonase concentration and activity (Deakin et al., 2003). In contrast, Balogh et al. (2001) found a non-significant decreased in PON1 activity after one month of simvastatin treatment in patients with types IIa and IIb hyperlipidemia (Balogh et al., 2001). Paragh and colleagues investigated the effects of simvastatin and atorvastatin on PON1 activity in hyperlipidemic patients at 3-month treatment and found that a short term administration of simvastatin did not influence the activity of PON1, while atorvastatin significantly increased PON1 activity (Paragh et al., 2004). From the

reasons for these differences could be attributed to the diverse study populations, different types of diseases and various dosages of statins.

4. *Curcuma comosa* Roxb.

Curcuma comosa (*C. comosa*) Roxb. (Figure 13), is a plant in family Zingiberaceae. It is an indigenous plant of Thailand with a common name in Thai as Waan Chak Mod look (Smitinand, 2001). Rhizomes of *C. comosa* has been used extensively in Thai traditional medicine as an anti-inflammatory agent particularly for the treatment of postpartum uterine bleeding, peri-menopausal bleeding and uterine inflammation. A number of different active principles of *C. comosa* (Suksamrarn et al., 1994, 1997) such as:

1. Diarylheptanoids: *trans*-1,7-diphenyl-5-hydroxy-1-heptene, *trans*-1,7-diphenyl-6-hepten-3-one-5-ol, *trans*-1,7-diphenyl-3-acetoxy-6-heptene, *trans*-1,7-diphenyl-6-heptene-3-one, *trans,trans*-1,7-diphenyl-1,3-heptadien-5-ol, *trans,trans*-1,7-diphenyl-4,6-heptadien-3-one, 1,7-diphenyl-1(*1E,3E,5E*)-heptatriene, 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(*1E*)-1-heptene), 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene.
2. Acetophenones: 4,6-dihydroxy-2-*O*-(*β*-D-glucopyranosyl) acetophenone.

(A) **(B)**

Figure 13 *Curcuma comosa* Roxb. plant (A) and rhizome (B)

Pharmacological effects

Pharmacological effects and potential toxicities of *C. comosa* have been reported and relationship with cardiovascular disease as following:

1. Choleretic effect

The choleretic effect of *C. comosa* has been investigated in rats. The butanol and ethyl acetate extracts of *C. comosa* showed the greatest stimulatory effects on bile flow rate in rats. Intra-duodenal administration of the ethyl acetate extract caused a dose-dependent stimulation of bile secretion. A high dose (1000 mg/kg) of these extracts increased bile flow rate with decreased bile salt concentration, whereas low doses (20-200 mg/kg) increased the bile flow rate without altering bile salt concentration. Furthermore, the high dose of ethyl acetate extract increased biliary cholesterol output. Concurrent with increased biliary cholesterol secretion, *C. comosa* caused a decrease in plasma cholesterol level. These suggested that the increase in biliary cholesterol excretion was, at least, a factor which might have led to the lower level of plasma cholesterol (Piyachaturawat et al., 1996). Subsequently, a phloracetophenone glucoside, [4,6-dihydroxy-2-*O*-(*β*-D-glucopyranosyl) acetophenone] was isolated from the ethyl acetate and n-butanol extracts. And has been identified as the choleretic principle of the plant by Suksamrarn and colleagues (Suksamrarn et al., 1997). Study in rat model indicated that the aglycone part of compound, phloracetophenone (2,4,6-trihydroxyacetophenone, THA), induced a dose-dependent increase in bile flow rate after a single intra-duodenal administration of THA at doses of 10–150 mg/kg. The increase in bile flow was associated with increased biliary secretion of bile acid, decreased secretion of cholesterol and phospholipid, and lowered bile lithogenic index. However, THA at a dose of 100 mg/kg induced a maximal increase of bile flow rate and bile acid output. A considerable decreased in plasma cholesterol was also observed which may attributed to the great choleretic activity with enhancement of biliary bile acid secretion. These results suggested that THA may have potential for reduced plasma cholesterol (Piyachaturawat et al., 1998). In addition, the study which explored the relationship between the chemical structure and choleretic activity of phloracetophenone (2,4,6-trihydroxyacetophenone) was undertaken. The study indicated that among 14

acetophenone analogues, 2,4,6-trihydroxyacetophenone possessed the most potent choleretic effect, which induced both high blood flow rate and a high bile salt output and led to lower plasma cholesterol levels in rats (Piyachaturawat et al., 2000). Although, Piyachaturawat and colleagues did not found significantly alter total hepatic cholesterol content but observeed significantly increased the excretion of both bile acids and cholesterol into the intestinal lumen for elimination after THA treatment in hypercholesterolemic male hamsters (Piyachaturawat et al., 2002a). Correlate with the increasing in bile acid excretion, THA caused a seven-fold increase in hepatic cholesterol 7∝-hydroxylase activity (Piyachaturawat et al., 2002a).

2. Hypolipidemic effect

The hypolipidemic effect of *C. comosa* had been found in many studies. From the choleretic effect of *C. comosa* rhizome extract and it remarkably stimulated bile secretion and enhanced biliary excretion of bile salt and cholesterol which consequently led to a decrease in plasma cholesterol (Piyachaturawat et al., 1996). *C. comosa* has hypolipidemic action by decreased plasma lipid levels of both triglyceride and cholesterol in rats (Piyachaturawat et al., 1998). Subsequently study by administration of *C. comosa* ethyl acetate extract at a dose of 0-500 mg/kg/day for 7 days in hypercholesterolaemic hamsters, caused a decrease of both plasma cholesterol and triglyceride levels in a dose-dependent manner (Piyachaturawat et al., 1999). Additionally, phloacetophenone (THA), the aglycone part of a glucoside from *C. comosa* enhanced biliary excretion of bile acids and decreased plasma cholesterol in rats (Suksamrarn et al., 1997; Piyachaturawat et al., 1998). In addition to administration of THA (300–600 μmol/kg) twice a day for 7 days decreased both plasma cholesterol and triglyceride level in hypercholesterolaemic hamsters (Piyachaturawat et al., 2002a).

3. Anti-inflammatory effect

The hexane extract of *C. comosa* has been found to possess a strong anti-inflammatory activity in activated microglia (Jantaratnotai et al., 2006). Recently, it has been found that *C. comosa* hexane extract, ethanol extract and two diarrylheptanoids (5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1E)-1-heptene and 7-

(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1E)-1-heptene of *C.comosa* significantly decreased the release of pro-inflammatory cytokines, tumor necrosis factor α (TNF-α) and interleukin-1β, from phorbol-12-myristate-13-acetate (PMA)-stimulated PBMC and U937 cells (Sodsai et al., 2007). In PMA-stimulated U937 cells, both *C. comosa* diarylheptanoids reduced the expression of TNF-α, suppressed expression of IκB kinase and the activation of nuclear factor kappa B (Sodsai et al., 2007).

4. Anti- oxidative effect

The anti-oxidative effect of crude ethanol extract of *C. comosa* has been revealed recently. The results clearly demonstrated that 4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl) acetophenone possess highly antioxidant power (Niumsakul et al., 2007).

Toxicological effects

Acute toxicity of THA was dependent on species and route of administration. For subacute toxicity study, THA at high doses (150 and 300 mg/kg/day) significantly increased plasma concentrations of alanine and aspartate aminotransferase, bilirubin, blood urea nitrogen (BUN), and hepatic triglyceride content (Piyachaturawat et al., 2002b). Chivapat and colleagues investigated a subchronic toxicity of ethanolic extract of *C. comosa* rhizome in Wistar rats by administration of *C. comosa* ethanolic extract at the doses of 100, 200, 400 and 800 mg/kg/day for 90 consecutive days. The result showed that at the dose of 800 mg/kg/day, *C. comosa* caused a significantly decrease of hematocrit, red blood cell (RBC) and hemoglobin in male rats while female rats receiving the extract at 200 mg/kg/day possessed a significantly decrease of mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCH) but a significant increase of platelet number at 800 mg/kg/day of the extract. In addition, at this highest dose (800 mg/kg/day), a significant increase of these following blood chemistry parameters were found: alkaline phosphates (ALP), alkaline aminotransferase (ALT), total protein and albumin. Triglyceride levels were significantly decreased in male rats treated with 400 mg/kg/day and female rats treated with 200 and 400 mg/kg/day of the extracts. Morever, *C. comosa* extract at 200 mg/kg/day increased stomach weight while other higher doses increased liver

weight as well as hyperplasia and hyperkeratosis of gastric epithelium (Chivapat et al., 2003).