CHAPTER I: INTRODUCTION

1.1 Statement and significance of problem

Atherosclerosis-associated cardiovascular disease is the leading cause of death in the world, and also in Thailand societies. Diabetes, cigarette smoking, age, sex, hypertension, hypercholesterolemia and modifications of LDL are associated with a marked increase risk of atherosclerotic cardiovascular disease (Steinberg D and Witztum JL, 1990). The oxidation of LDL is an important role in the pathogenesis of atherosclerosis. An elevated level of plasma low density lipoprotein cholesterol (LDL-C) is an independent risk factor for atherosclerosis and coronary heart disease (CHD). The pathological hallmark of the early human atherosclerotic lesion is the appearance of macrophage-derived, lipid-laden foam cells in the subendothelial space of the vascular wall (Koenig W, 2001). Macrophages have the capacity to accumulate modified LDL in contrast to native LDL. Free radical–mediated oxidation has been proposed as a mechanism by which LDL becomes modified in the vascular wall, leading to increased uptake by macrophages scavenger receptor pathways (Steinberg D, 1997). The lipid hydroperoxides subsequently decompose to aldehydic products that can covalently modify apolipoprotein B-100. leading to an increased net negative surface charge of the LDL particle and altered receptor recognition. The subsequent accumulation of cholesterol-loaded macrophages (foam cells) in the subendothelium leads to the formation of fatty streaks and atherosclerotic plaques. LDL oxidation may promote atherosclerosis by further additional mechanisms, such as chemoattraction of monocytes and smooth muscle cells (SMC), cytotoxicity, inhibition of endothelium-derived relaxing factor, and stimulation of SMC proliferation (Steinberg D, *et al.*, 1989). LDL oxidation also may modulate thrombosis and fibrinolysis by stimulating endothelial cells (EC) synthesis of procoagulant tissue factor and plasminogen activator inhibitor-1 (Zhang W, *et al.*, 1994).

Diabetes mellitus increase risk of atherosclerotic cardiovascular disease as they have higher levels of harmful LDL cholesterol, which is more easily oxidized than other forms (Steiner G, *et al.*, 1985). The rate of LDL oxidation also appears to be higher in diabetic. This combination increases the risk of damage to arteries. There are various compounds and processes that lead to diabetic complications. Prolonged periods of high blood sugar cause glucose

molecules to become attached to proteins, glycation, causing changes in structures and functions of that proteins.

A large body of experimental evidence supports the hypothesis that oxidation of LDL contributes to the development of atherosclerosis. Moreover, it is postulated that inhibition of LDL oxidation by antioxidants might protect against the development of atherosclerosis (Diaz MN, *et al.*, 1997). Thus, dietary antioxidants such as α-tocopherol he chemically and biologically active form of vitamin E and ascorbic acid or vitamin C can protect LDL from oxidation (Losonczy KG, *et al.*, 1996).

The capacity of LDL particles to resist oxidation can be increased or even regained by increasing the amount of antioxidants in the particles as α-tocopherol (Palinski W, *et al.*, 1989). Another possible way to protect LDL against oxidative stress would be increasing the concentration of antioxidants in the different tissue fluids. Thus, ascorbic acid, a water-soluble antioxidant, and α-tocopherol, a lipid-soluble antioxidant will protect LDL from oxidation apparently by maintaining the endogenous antioxidants in the reduced state (Reaven PD, *et al.*, 1992). Furthermore, ascorbic acid and α-tocopherol can prevent the oxidation of LDL by binding covalently to the apo B-100 component of LDL particles, thus inhibiting interaction between the LDL component and copper ions (Retsky KL, *et al.*, 1993).

This paper interested in whether a Thai herb, turmeric curcuminoids could have inhibition effect on LDL oxidation. Curcuminoids are natural phenolic coloring compound found in rhizomes of *Curcuma longa* Linn., commonly called turmeric. Curcuminoids content in turmeric is about 1-5 %, and it has been identified as the major yellow pigment in turmeric (Srinivasan KR, 1953). It has been widely used as a spice, a coloring agent for cheese and butter and as an ingredient in cosmetic and medicinal preparations. Curcuminoids have a wide range of biological and pharmacological activities, including antioxidant, anti-inflammatory agent, anti-mutagenic activity, anti-carcinogenic effects, hypocholesterolemic effects and hypoglycemic effects (Ammon HPT and Wahl MA, 1991).

The oxidatively modified low density lipoprotein (ox-LDL) could contribute to the atherosclerotic process by its cytotoxic effect, uptaking by macrophage scavenger receptor. In this study, the oxidative susceptibility of LDL in healthy with normolipidemic and diabetic with hyperlipidemic groups were investigated for the ability effect of curcuminoids to inhibit Cu^{2-}-

induced oxidative modification of LDL in comparison with α-tocopherol or ascorbic acid using cellular LDL uptake by U937 cells, conjugated diene formation and thiobarbituric acid reactive substances (TBARs) formation *in vitro*.

1.2 Literature reviews

1.2.1 Plasma lipoproteins

Lipids play very important roles in maintaining the structure of cell membrane (cholesterol, phospholipids), cell growth (cholesterol), steroid hormone synthesis (cholesterol), and energy metabolism (triglycerides or triacylglycerol). Since lipids are highly hydrophobic, need to be packed into lipoproteins as water-soluble particles in blood circulation. Lipoprotein is a particle consisting of a core of hydrophobic lipids, triglycerides (TG), cholesteryl esters (CE), surrounded by a polar layer of phospholipids (PL), unesterified or free cholesterol (FC), and apolipoprotein (Ginsberg HN, 1990 and Beisiegel U, 1995) (Figure below). Plasma lipoproteins are usually classified into five major subfractions based on their densities (d), particle sizes, floatation rate (Sf), and electrophoretic mobility which are summarized in Table at Page 5.

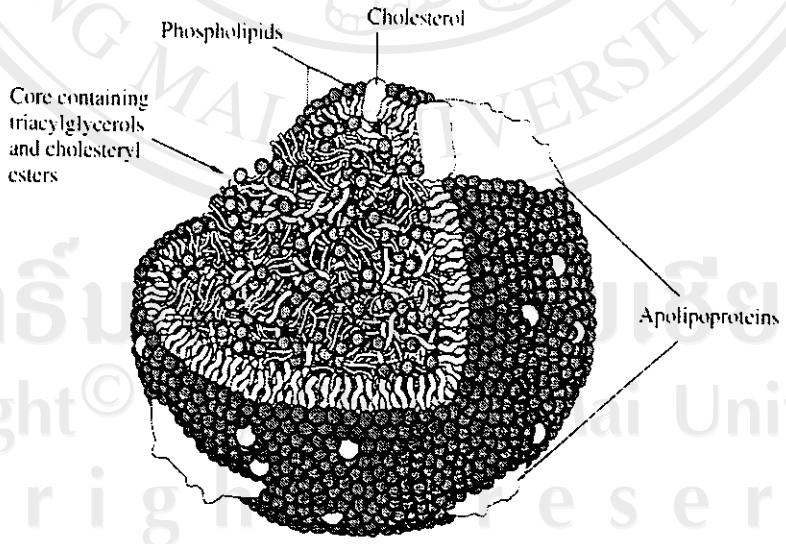

Figure. Schematic model of lipoprotein particle

(http://cwx.prenhall.com/horton/medialib/media_portfolio/text_images/FG16_05.JPG)

Chylomicron (CM)

Chylomicrons are synthesized in the small bowel, and function is the transportation of dietary triglycerides and cholesterol. They constitute the largest molecules and by ultracentrifugation remain suspended at a density below 0.95 g/mL. Ninety percents of their lipid content is dietary triglycerides, and the remaining 5% are cholesterol and phospholipids. Apo B-48 is their main protein structure. During modification, they may contain apo A, apo A-II, apo A-IV, apo C-I, apo C-II, apo C-III, and apo E.

Very low density lipoproteins (VLDL)

The liver assembles triglyceride-riched, very low-density lipoproteins (VLDL) and secretes them into the circulation. By ultracentrifugation, VLDL can be separated within a density range of 0.95 to 1.006 g/mL. Their lipid part contain approximately 60% of triglycerides, 20% of cholesterol, and the rest is constituted by phospholipids. Their protein structure is formed originally by apo B-100, and then apo C-I, apo C-II, apo C-III, and apo E were transferred from HDL for their metabolism. The main function of VLDL is to supply tissues with endogenous triglycerides which are captured and hydrolyzed to free fatty acids by the lipoprotein lipases placed on the surface of endothelial cells for further oxidation giving ATPs.

Intermediate density lipoproteins (IDL)

They result from the partial catabolism of VLDL with density in a range between 1.006 and 1.019 g/mL. For each degraded molecule of VLDL, a molecule of IDL is produced, which full transference of apo B-100, while other apolipoproteins such as apo C and apo E progressively disappear with increasing hydrolysis of triglycerides. These lipoproteins are smaller than VLDL, with higher amount of cholesterol and lower content of triglycerides. IDL have a short half-life and are removed from the circulation within hours.

Low density lipoprotein (LDL)

LDL, the end product of degradation of VLDL, with a smaller molecule than IDL rich in esterified cholesterol and constituted almost exclusively by apo B-100. LDL has a density range of 1.019 to 1.063 g/mL and is the main carrier of free and esterified cholesterol, these lipids are the predominant components of the foam cells. The cholesterol of LDL is used by the cells to integrate the structure of their membranes and by specialized cells for biosynthesis of steroid

hormones. Normally LDL binds to specific receptors of the cell membranes, which will be later described.

High-density lipoproteins (HDL)

They represent a heterogeneous group of particles that can be separated by ultracentrifugation in a density range of 1.063 to 1.210 g/mL. Approximately 50% is constituted by apolipoproteins, mainly apo A-I. Cholesterol constitutes about 40% of its lipid composition, and phospholipids and trace of triglycerides form the remaining 60%. HDL is synthesized in the liver and intestinal mucosal cells having a function of removing cholesterol from peripheral tissues to the liver for converting to bile acids and then is secreted into the duodenum, or being precursor of steroids synthesis.

Table. Properties and apolipoprotein composition of the major human plasma lipoproteins (Brewer HBJr. *et al.*, 1988)

Lipoprotein class	Density (gm/mL)	MW (Da)	% Weight lipid/protein	Major core lipid	Major apoprotein
Chylomicron	< 0.95	> $0.4x10^9$	98/2	Dietary TG	B-48, C-I, C- II, C-III
VLDL (pre β)	0.95-1.006	$5\text{-}10x10^6$	92/8	Endogenous TG	B-100, E,C-I, C-II, C-III
IDL (β)	1.006-1.019	$4\text{-}4.8x10^6$	85/15	CE,TG	B-100, E, C-I, C-II,C-III
LDL (β)	1.019-1.063	$2.8x10^6$	79/20	CE	B-100
HDL (α)	1.063-1.210	$3.6x10^5$	50/50	PL,CE	A-I, A-II

CE = Cholesterol ester, PL = Phospholipid, TG = Triglyceride, VLDL = Very low-density lipoprotein, IDL = Intermediate-density lipoprotein, LDL = Low-density lipoprotein, HDL = High-density lipoprotein

1.2.2 Lipoprotein metabolism

Chylomicron (CM) is derived from dietary lipids (exogenous pathway) and assembled in the intestinal mucosal cells. TGs are the major constituents of the CM particles. The TGs in CM are hydrolyzed in the peripheral tissues by lipoprotein lipase (LPL) to form the CM remnants which are taken up by the liver in a process that probably involves apolipoprotein E (apo E) on the surface of the remnants and a hepatic receptor called LDL receptor-related protein (LRP) (Beisiegel U, 1995). VLDL particles are synthesized in the liver (endogenous pathway). They are the main liver-derived TG-rich lipoproteins and in circulation. TGs are hydrolyzed by LPL and IDL (Gotto AM Jr, *et al.*, 1986). About half of the IDL particles are taken up by the liver via LDL receptor and remnant receptor, whereas the other half are converted into LDL by hepatic lipase (HL) (Taskinen M-R, and Kuusi T, 1987).

HDLs consist of apo A-I and apo A-II as the main apolipoprotein constituents and carry about 50% cholesterol, most of which are CE. HDLs are synthesized in the liver and intestine (Franceschini G, *et al.*, 1991). HDLs can also be generated following the lipolysis of TG-riched lipoproteins whereafter plasma phospholipid transfer protein (PLTP) facilitates the transfer of phospholipids and some cholesterol into HDL pool (Eisenberg S, 1984). In addition, the lecithin-cholesterol acyltransferase (LCAT) has a crucial role in the maturation of HDL particles. LCAT catalyzes the formation of CE which are then incorporated into the core of discoidal nascent HDL. HDLs play a major role in the transport of cholesterol from peripheral tissues to the liver, a process known as reverse cholesterol transport. HDL-CE are transfered by cholesteryl ester transfer protein (CETP) to apo B-containing particles which are finally removed from the circulation by the liver (Tall AR, 1993 and Tall AR, 1998). In addition, HDLs can be taken up by class B scavenger receptor (SR-BI)-mediated process in certain cells where this receptor mediates selective CE uptake leaving the HDL particles largely intact, or directly removed by the liver (Figure at Page 7.) (Acton S, *et al.*, 1996).

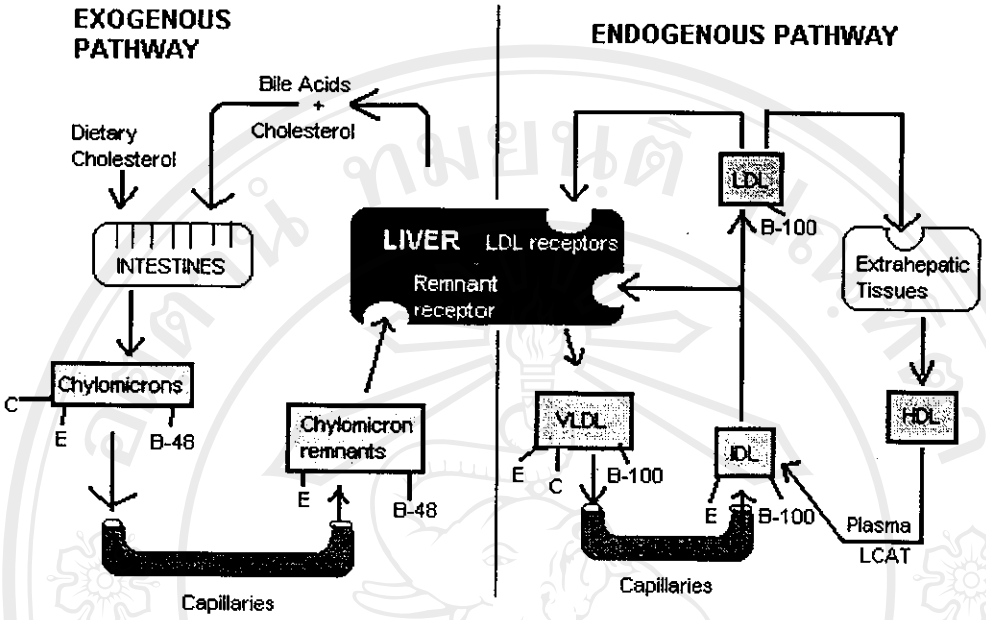

Figure. Lipoprotein metabolism

(http:// hsc.usf.edu/2005/ lipoprotmet.jpg)

1.2.3 Low density lipoproteins

1.2.3.1 LDL particle structure

In blood circulation, TG and CE are packed into LDL particles forming a hydrophobic core surrounded by a surface monolayer of polar PL together with unesterified cholesterol (FC) and apoB as shown in Figure at Page 8. LDL normally also contains lipophilic antioxidants, mainly Vitamin E and β-carotene. LDL is a large spherical particle, molecular weight of about 3 x 10^6 Da, with a diameter of 22-28 nm and density between 1.019-1.063 g/mL. The core is composed of some 1,600 molecules of CE (long chain fatty acid) and 170 molecules of TG. The CE is the main lipid of the lipoprotein core with the most fatty acyl chain in these esters being linoleate. This core is shielded by a layer of PL (700 molecules), FC (600 molecules), and apo B-100 (1 molecule). In the percent mass composition, each LDL particle consists of 35-45% CE, 7-10% FC, 7-10% TG, 15-20% PL, and 20-25% protein (Deckelbaum RJ, 1987).

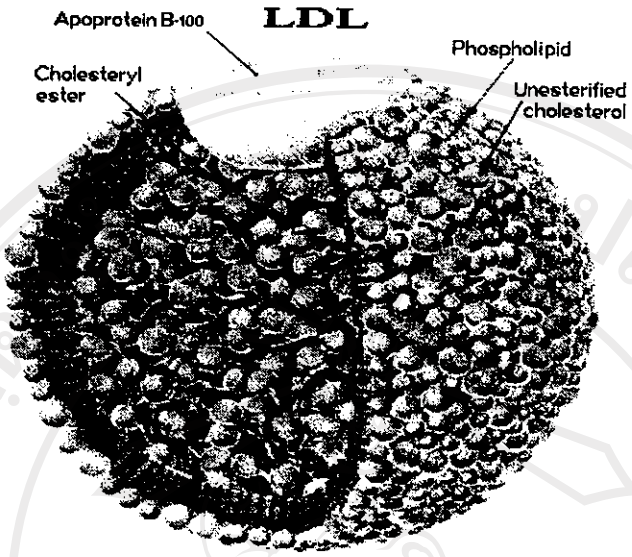

Figure. Schematic model of LDL particle

(http://www.biochem.szote.u-szeged.hu/astrojan/Prot/Ldl.jpg)

1.2.3.2 LDL receptors and receptor mediated metabolism

LDLs are the principal plasma carriers of cholesterol-delivering lipoprotein from the liver (*via* hepatic synthesis of VLDL) to peripheral tissues, primarily the adrenals and adipose tissue. LDL also return cholesterol to the liver. The cellular uptake of cholesterol from LDL occurs following the interaction of LDLs with the LDL receptor (also called the apo B-100 receptor). The sole apoprotein presents in LDLs is apo B-100, which is required for interaction with the LDL receptor. An extracellular domain is responsible for apo B-100 binding. The intracellular domain is responsible for the clustering of LDL receptors into regions of the plasma membrane termed coated pits. Once LDL binds the receptor, the complexes are rapidly internalized (endocytosed). ATP-dependent proton pumps lower the pH in the endosomes, which results in dissociation of the LDL from the receptor. The portion of the endosomal membranes harboring the receptor are then recycled to the plasma membrane and the LDL-containing endosomes fuse with lysosomes. Acid hydrolases of the lysosomes degrade the apoproteins and release free fatty acids and cholesterol. As indicated above, the free cholesterol is either incorporated into plasma

membranes or esterified by acylcholesterol acyltransferase (ACAT) and stored within the cell (Goldstein J and Brown M, 1977 and Jones A, *et al.*, 1984).

The receptor-mediated removal of LDL cholesterol occurs mostly *via* classical LDL receptors that have been observed in all mammalian cells tested except erythrocytes. The liver plays a crucial role in receptor mediated uptake of LDL approximately 75% of the LDL particles removed from the circulation are mediated by the liver, in which 3/4 of the clearance is LDL receptor-mediated, the remainder is by a nonspecific, receptor-independent low affinity process (Pittman RC, *et al.*, 1982). Also scavenger receptor B1(SR-B1) mediates LDL binding but only CE is selectively delivered to the cell especially in non-placental steroidogenic tissues (Van Berkel TJ, *et al.*, 1995).

The LDL receptor is presented on both hepatic and extrahepatic cells. The high binding interaction between LDL apo B-100/apo E and the LDL receptor is responsible for the receptor-mediated uptake and clearance of LDL from the circulation. The apo E on apo E-containing lipoproteins (VLDL, IDL) is also capable of interacting with the LDL receptors and regulating the metabolism of these lipoproteins. After LDL binds to its receptors, it is internalized and delivered into lysosomes where its CE is hydrolyzed. The liberated cholesterol is then used by the cell for the synthesis of plasma membranes, bile acids, and steroid hormones, or stored in the ester form. The level of intracellular cholesterol is regulated through cholesterol-induced suppression of LDL receptor synthesis and cholesterol-induced inhibition of cholesterol synthesis. The increased level of intracellular cholesterol that results from LDL uptake will activate ACAT, allowing excess cholesterol storage within cells. Then, LDL receptor synthesis is suppressed and the rate LDLs and IDLs removing from the serum also decrease as shown in Figure at Page 10. Excess circulating levels of cholesterol and cholesteryl esters when the dietary intake of fat and cholesterol exceeds the needs of the body tends to be deposited in the skin, tendons and (more gravely) within the arteries, initiate atherosclerosis processing (Mahley RW,1990).

Apo B-100 is a large (513 kDa), single chain glycoprotein composed of 4,536 amino acid residues with a coding gene residing on the short arm of chromosome 2. There is only one apo B-100 molecule in each LDL particle (Tikkanen MJ and Schonfeld G, 1985). Apo B-100 is not transferred between lipoprotein particles during the metabolic conversion of VLDL into LDL. It

is presumed that the apo B-100 binding site resides in the carboxyterminal portion of the molecule.

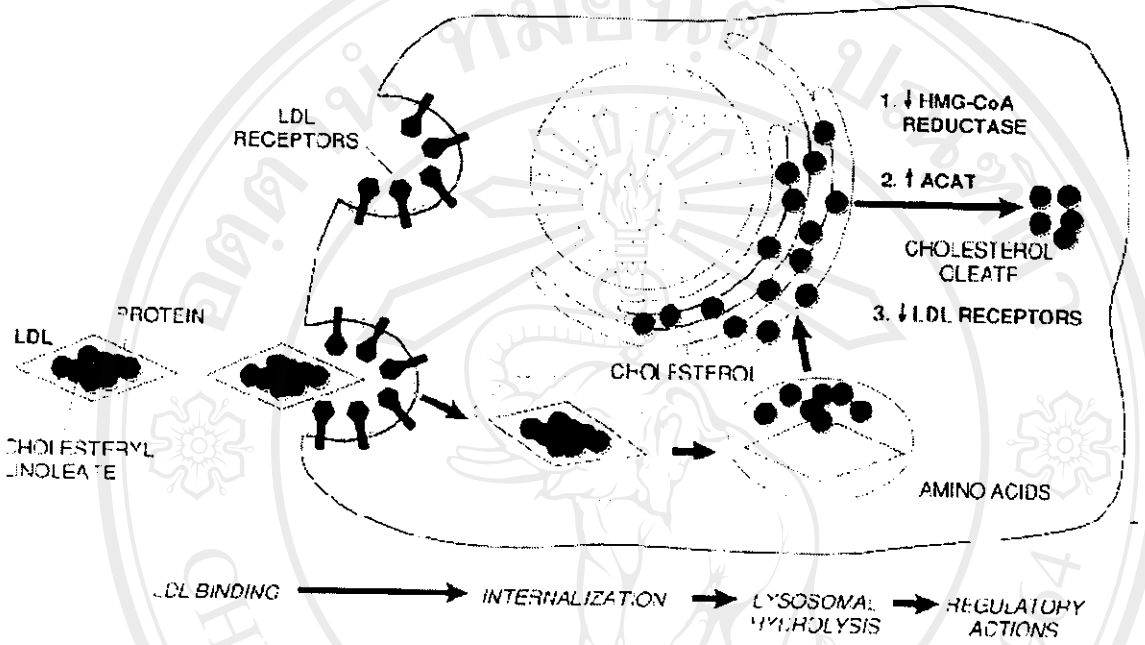

Figure. Cellular pathway of LDL receptor

(http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/L/LDLendocytosis.gif)

1.2.4. LDL and atherogenesis

LDL transports about 75% of the total cholesterol in blood circulation. Evidence exists that LDL cholesterol is a critical atherogenic factor (Grundy SM, 1995). A large number of epidemiologic studies demonstrated a strong positive correlation between elevated LDL cholesterol levels and the development of coronary artery disease (CAD). Genetic studies also documented that inheritable hypercholesterolemias (familial hypercholesterolemia, familial defective apo B-100), mainly with elevated levels of LDL cholesterol, are the primary cause of premature CAD (Goldstein JL and Brown MS, 1973). In addition, apo B and LDL particles were identified in atherosclerotic plaques and *in vitro* studies showed that elevated LDL levels can damage endothelial cell (EC) layer and penetrate into the arterial intima. The accumulation of

LDL in the arterial wall initiates monocyte and smooth muscle cell migration and transforms macrophages and smooth muscle cells into cholesterol-loaded foam cells, which are the major cell components found in the plaque (Goldstein JL, *et al.*, 1979). Furthermore, pathological studies have demonstrated that the lowering of LDL-cholesterol is associated with reduced severity of atherosclerotic lesion and improvement of cardiac functional parameters. For example, reduction in cholesterol levels may reduce the susceptibility of LDL to oxidation which is a causal factor for the initiation and progression of atherosclerosis. Protection of LDL from oxidation could increase nitric oxide bioavailability and improve endothelium-dependent vasomotor, anti-inflammatory, and anticoagulant properties of the endothelium (Guetta V and Cannon RO, 1996).

There are many evidences indicating that oxidized LDL is present in atherosclerotic lesions *in vivo*. First of all, LDL isolated from atherosclerotic lesions is in part oxidatively modified. Second, immunological techniques demonstrated that atherosclerotic lesions contain materials reactive with antibodies generated against oxidized LDL. Third, serum contains autoantibodies against oxidized LDL. Fourth, treatment with antioxidants prevent the development or slow the progression of atherosclerosis (Carew TE, *et al.*, 1987). In principle, any modified LDL in plasma could be rapidly removed by hepatic sinusoidal cells (Kupffer cells), which contain abundant scavenger receptors. Moreover, a variety of antioxidants remain in plasma. Therefore, it was presumed that oxidized LDL mainly occurred locally in the arterial wall after entrance of native LDL, whereby it was sequestered from antioxidants in plasma (Steinberg D, *et al.*, 1989). However, recent studies suggested that very small amounts of oxidized LDL are also present in plasma. These changes could be occurred elsewhere, or during a previous transient passage through the artery wall. Such minimally modified LDL might then be "primed" for more rapid oxidative modification on a subsequent entry into the intima. Therefore, oxidized LDL in the arterial wall can be derived both from native LDL oxidized locally in the arterial intima and from oxidized LDL in plasma (Nielsen LB, 1999).

1.2.5 Small dense LDL and atherogenesis

Kinetic studies have shown that particle of VLDL1 (VLDL subfractions) are large TG-rich VLDL particles and the precursor of small dense LDL, and the long residence time of VLDL1 particles favours the lipid exchange (Packard CJ, *et al.*, 2000). In the metabolic process,

CE remains in the core of LDL particles, but part of CE is transferred by CETP to VLDL in exchange for TG. Correspondingly, LDL particles become enriched in TG that is a better substrate for hepatic lipase (HL). Thereafter, HL remodels the large LDL particles by hydrolysis of TG in the core and phospholipids on the surface to convert them to smaller, denser particles. In addition, small dense LDL have been shown to be affected by age, gender, diet, hypertriglyceridemia, obesity, insulin resistance, hormone status, and drugs.

Small dense LDL is bound less avidly by the LDL receptor than large LDL resulting in decreased hepatic clearance and longer residence time in plasma. The rate of LDL particles into the intima is inversely related to particle size. Small dense LDL particles penetrate more easily into the subendothelial space of artery wall from the circulation. Small dense LDL particles contain less phospholipids and free cholesterol in their surface monolayer than do large LDL particles. This difference in lipid content appears to induce changes in conformation of apo B-100, leading to exposure of proteoglycan-binding regions. This may give small dense LDL particles increased binding affinity to arterial proteoglycans. The end-result is trapping of these particles in the arterial extracellular matrix. The retention of LDL in the subendothelial space has been recently suggested to be the initiating event in the early stage of atherosclerosis. Small dense LDL particles are more susceptible to *in vitro* oxidation than large LDL. This increased LDL susceptibility to oxidation may be due to either reduced content of antioxidants or increased content of PUFA in small dense LDL. In addition, small dense LDL particles may also promote the thromboxane synthesis and further influence platelet aggregation (Chapman MJ. *et al.*, 1998).

1.2.6 Risk factors and mediators of atherosclerosis

1.2.6.1 LDL modification

Although long term increased blood cholesterol and LDL levels will increase risk of atherosclerosis. It is difficult in cell culture models as monocytes to ingest cholesterol or LDL to form foam cells, due to monocyte cholesterol uptake is saturatable. The biologically important modification of LDL is oxidation forming oxidized LDL, which has a fundamental role in atherosclerosis. Increased level of oxidized LDL was also found in atherosclerotic plaques. Animals predisposed to atherosclerosis are protected by antioxidants which prevent oxidation of LDL. Patients with atherosclerosis have increased levels of antibodies to LDL. Finally,

preliminary results from human trials are now showing protection from atherosclerosis by antioxidants. Oxidized LDL uptake by monocytes and macrophages is not saturatable. LDL oxidation also induces monocyte chemotaxis, promotes cytokine secretion by the endothelial cells and depresses release of nitric oxide, a potent vasodilator. Lastly, oxidized LDL promotes a hypercoagulable state of platelet by preventing activation of protein C and upregulation of the procoagulant, tissue factor (Yla-Herttuala S, 1989).

Derivatives of LDL modification

Acetylated LDL

The resultant of *in vitro* LDL acetylation is a particle, which is taken up by macrophage's receptors at a higher speed than native LDL (Steinberg D, *et al.*,1989).

Oxidized LDL (ox-LDL)

LDL may undergo numerous changes by peroxidation of its polyunsaturated fatty acids (PUFA) and by degradation of its apo B. The ox-LDL has great affinity for macrophage receptors, while its interaction with physiologic receptors is decreased. This confers on ox-LDL a high atherogenic potential.

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

In culture, all the vascular cells can initiate oxidation of LDL, but the relative contributions of EC, monocytes and macrophages, or smooth muscle cells (SMC) to such modification *in vivo* are unknown. *In vitro*, LDL can bind to copper which can promote rapid lipid peroxidation. Free copper and iron, or complexes of these metals, exist *in vivo* can promote LDL peroxidation, although intact ceruloplasmin can act as a pro-oxidant. In macrophages,

enhanced 15-lipoxygenase activity could generate increased cellular lipid hydroperoxides, which could be transferred to extracellular LDL, providing the "seed" that would lead to enhanced lipid peroxidation (Heinecke JW, 1998).

Glycosylated LDL

Glycosylated LDL constitutes the non-enzymatic union of glucose with the amino group of N-terminal amino acid of the LDL apolipoprotein forming of a ketoaminic union. This type of modified LDL, probably plays a main role in the development of diabetic atherosclerosis.

Carbamylated LDL

In patients having high level of urea in their blood with chronic renal disease, the LDL particles have qualitative alterations with atherogenic potential for these individuals. These modifications take place by carbamylation of lysine residuals from apo B with cyanide derived from urea.

Triglyceride-riched LDL

The formation of LDL, involves enzymatic activity and interchange of lipids with other lipoproteins. In this process, the formation of triglyceride-riched LDL, is favoured by an increase in the half-life of the particles as well as a low enzymatic activity (Deckelbaum RJ, *et al.*, 1982). Triglyceride-riched LDL has larger size, lower density and is more susceptible to oxidation than native LDL. Recent works, showed that greater concentrations of triglycerides in LDL produce a lesser exposure to apo B-100 epitopes to be identified by B/E receptors (Aviram M, *et al.*, 1988). This modification of LDL also has atherogenic implications.

1.2.6.2 Diabetes mellitus

Hyperglycemia contributes to interactions between endothelial functions producing abnormal responses to acetylcholine, increased production of thromboxane and prostaglandins, raised intracellular Ca^{2+}. All of which stimulate the release of endothelial vasoconstricting agents such as acetylcholine and endothelin 1. The shunt in glucose to sorbitol *via* aldose reductase also produces fructose. Sorbitol enhances cell damage by augmenting cell swelling. Endothelium derived aldose reductase contributes to highly abnormal cellular functioning and oxidative stress. Hyperglycemia also accelerates the generation of free radical mediated LDL oxidation. Furthermore, available glucose can bind covalently to proteins by a process called glycation. This

process increases the production of free radicals causing glycoxidation, and glycative stress within the cell, raises the quantity of glycated LDL and the atherogenic potential of LDL. Fat metabolism is also disturbed in diabetes, with high blood levels of cholesterol and triglycerides (Sato Y, *et al.*, 1979).

1.2.6.3 Cigarette Smoking

Metabolites of cigarette smoke, allylamine and the end product acrolein and reactive oxygen species are the cause of oxidative stress and antioxidants reduction causing reduced ability to inhibit lipid peroxidation, endothelial dysfunction in particular subendothelial edema and mitochondial swelling. Fatty streaks may developed before endothelial cell denudation during severe cytotoxic changes to oxidise LDL which then develops to a complicated lesion. This adaptive change also attracks circulating monocytes to penetrate into the intimal layers and act trap oxidized LDL by scavenger receptor forming foam cell or a so called cholesterol clefts which accelerate the formation of the fatty streak. Smoking also increases platelet aggregation and the plasma concentrations of fibrinogen which both contribute to the occlusion of arteries.

1.2.6.4 Hypercholesterolemia

For most people, a cholesterol level more than 200 mg/dL is considered too high. One explanation for this disparity in risk for a given total cholesterol level is due to the fact that not all cholesterol is created equal. There are actually two main types of cholesterol, "LDL-cholesterol" and "HDL-cholesterol". LDL cholesterol is the so called "bad" cholesterol because this type of cholesterol deposits inside the arteries and plugs them up. The HDL-cholesterol is the so called "good" cholesterol because this cholesterol actually removes the cholesterol that is clogging your arteries. Therefore, it is important to know what proportion of total cholesterol is found as LDL or HDL-cholesterol. In addition, blood cholesterol is only one risk factor contributing to the development of atherosclerosis.

Small, dense LDL is more atherogenic than larger, buoyant forms. Accompanying by raised triglycerides, reduced HDL, LDL binding to apo A-I decreased. Insulin resistance increases the HDL susceptibility to oxidation. liver, circulation or peripheral tissues. As lipoprotein lipase positively correlate with HDL, low concentrations increase apo A-I and AII,

catabolism reduces levels of available lipoprotein lipase and increases hepatic lipase leading to hypertriglyceridemia.

1.2.6.5 Hypertension

Hypertension induces endothelial dysfunction by reducing nitric oxide mediated vasodilation and increased vascular resistance. This may relate to increased Ca^{2+} by either reduced NO synthetase or excess production of oxygen derived free radicals which inhibit NO production.

1.2.6.6 Lipoprotein(a)

Lipoprotein(a) is a LDL-like lipoprotein that greatly increases the risk of atherosclerosis. Individuals with elevated levels of this lipoprotein suffer from premature stroke, myocardial infarction, and peripheral vascular disease. These patients may develop atherosclerotic heart disease in their thirties despite the absence of other risk factors. Lipoprotein(a) has been shown to block fibrinolysis *in vitro* and may interact with the endothelium to stimulate the growth of atherosclerotic plaques or promote thrombotic complications of atherosclerosis. Recently one complicated mechanism for lipoprotein(a) pro-atherogenic mechanism has been elucidated. Transforming growth factor (TGF) is secreted in the latent formed and is activated by plasmin. Lipoprotein(a) blocks plasmin generation thus blocking TGF and promoting smooth muscle growth. The treatment of patients with elevated levels of lipoprotein(a) is problematic. Traditional cholesterol lowering maneuvers are known to be ineffective. High dose niacin has been reported to reduce lipoprotein(a) levels, but the long term benefits and toxicities of this approach are unknown. Aggressive control of other risk factors such as smoking, diabetes, and elevated levels of LDL cholesterol are clearly warranted (Maranhao RC, 1995 and Dahlen G, *et al.*, 1976).

1.2.6.7 Homocysteine

Homocysteine is recognized as an independent risk factor for atherosclerotic vascular disease due to its damaging effects to vascular endothelial cells, resulting in lipid deposition and plaque formation. Damaged endothelial cells attract blood platelets which form clots, in part, as a result of the production of thromboxanes. Genetic, dietary and lifestyle factors accelerate

conversion of methionine to homocysteine, and homocysteine to homocysteine thiolactone. Homocysteine thiolactone reacts with the free amino group of LDL, causing aggregation and increased uptake of LDL by macrophages, which results in lipid deposition. Homocysteine thiolactone released within the vascular wall from this homocysteine-LDL complex promotes oxidation of cholesterol and unsaturated lipids, platelet aggregation, thrombogenic activity, glycosaminoglycan disruption, fibrosis, intimal injury, and calcification of atherosclerotic plaques. Homocysteine also stimulates proliferation of smooth-muscle cells, a key component in atherosclerosis. Recent studies have shown that even moderately-elevated homocysteine levels are correlated with an increasing risk of myocardial infarction, cardiovascular disease, and stroke (Glueck CJ, *et al.*, 1995).

1.2.7 Oxidized LDL receptor and metabolism

Oxidized LDL (ox-LDL) particles are taken up by so-called scavenger receptors. In the liver, Kupffer cells are the main site for mediating the *in vivo* uptake of ox-LDL from the circulation and might thus protect against circulating ox-LDL. Increased LDL levels in plasma lead to an increase entry of LDL into the intima through the injured endothelium, resulting in accumulation of LDL in the intima as shown in figure at Page 18. LDL in the arterial wall can be oxidatively modified by all the major cells of the arterial wall, such as EC, SMC, monocytes, macrophages, and in cell-free system by transition metals, lipoxygenase, myeloperoxidase, and nitric oxide. Ox-LDL is taken up by a family of scavenger-receptors (SR) on the surfaces of cells such as macrophages, platelets, and EC. However, which part of the ox-LDL particle is being recognized by scavenger receptors is not fully understood (Greaves DR, *et al.*, 1998). Certainly, both the lipids and the protein are oxidized under the oxidative condition. It was shown that the modified apolipoproteins extracted from ox-LDL particles were efficiently internalized and degraded by macrophage scavenger receptors, while the oxidized lipids extracted from ox-LDL were also recognized by scavengers. During the oxidation of LDL, the PUFA are broken down to smaller fragments and become conjugated with the amino group of lysine residues. Therefore, that the recognition of ox-LDL by scavenger receptors appears to be due to the masking of lysine-amino group and subsequent changes in protein charge and configuration. The SRs mediate the endocytosis of the ox-LDL, where the process is not down regulated forming foam cell which is

the hallmark of fatty streaks and atherosclerotic plaques. The SR activity on the macrophages exhibits a remarkable broad binding specificity, they not only recognize ox-LDL but also other chemically modified proteins such as acetylated LDL (Ac-LDL), methylated LDL, suggesting they are multiligand receptors. So far, there are more than six classes of SR (10 types) that have been shown to be responsible for endocytosis of ox-LDL (Krieger M, 1997).

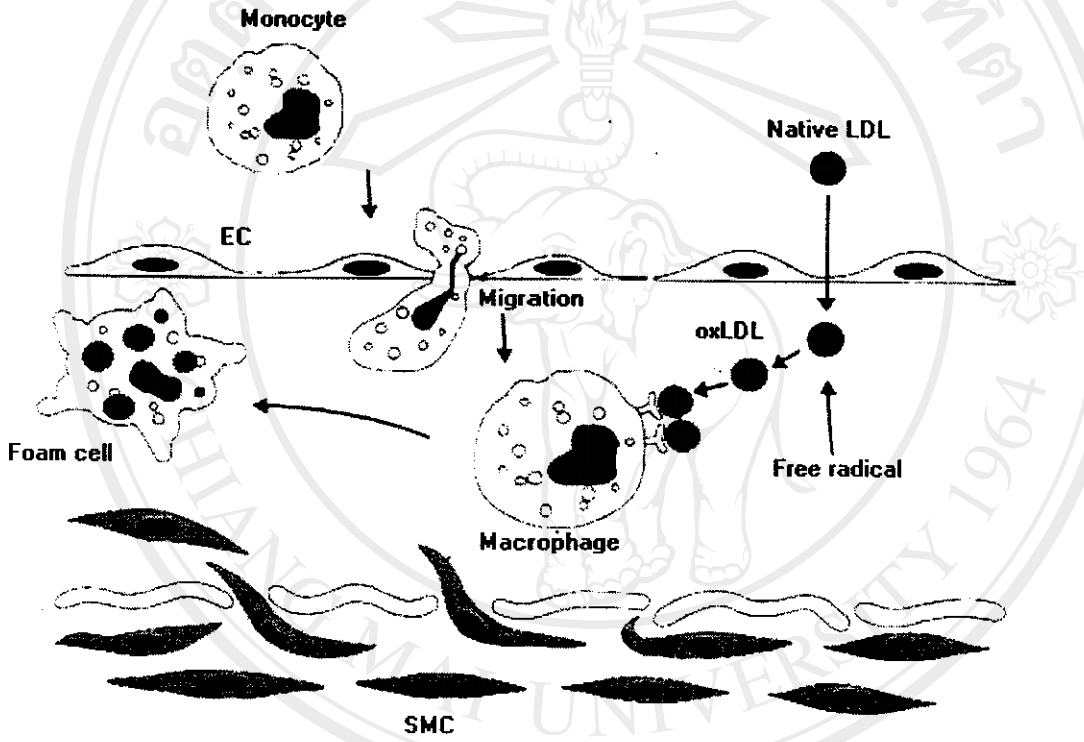

Figure. Pathway for ox-LDL initiated atherosclerotic lesion formation

EC:endothelial cells, M: monocytes, SMC: smooth muscle cells

(http://www.gik.gr.jp/~skj/arteriosclerosis/ images/plaque-LDL.jpg)

1.2.8 Possible mechanisms of atherogenesis

Although there are multiple evidences supporting the relationship between hyperlipidemia and atherosclerosis, the physiopathologic mechanism is not well understood. The most important clues in this association are:

1. Endothelial damage: This appears to be the first step that triggers a chain of events leading to plaque formation. Platelet aggregation occurs over a surface of endothelial damage with subsequent release of growth factors that in turn, stimulate the proliferation of smooth muscle cells. In vitro models suggest that high concentrations of LDL may be toxic to endothelial surface. The earliest endothelial damage consists of functional alterations of the endothelium without substantial morphologic changes, and it is characterized by a loss of the relaxation of the vessel after being stimulated by nitric oxide agonists such as acetylcholine. On the other hand, ox-LDL stimulates the release of endothelin, the most important vasoconstrictor expressed by endothelial cells (Ross R, 1986).

2. Penetration of LDL into the subendothelial space: LDL particles are small enough to cross the endothelial barrier at a speed related to their plasma concentration (Stender S. and Zilbersmit DB, 1981).

3. Chemotaxis of monocytes: ox-LDL is a ligand for the scavenger receptor that is expressed when monocytes differentiate into tissue macrophages. It also recruits these cells avoiding their return to the blood stream (Berliner JA. *et al.*, 1990).

4. Transformation of macrophages into foam cells: The recruited monocytes express specific receptors for modified lipoproteins becoming macrophages. This shift in receptor recognition leads to cellular uptake of ox-LDL by receptors that are not regulated by the cholesterol content of the cell. The result is a massive accumulation of cholesterol. Such cholesterol-loaded cells are called foam cells and eventually die by an apoptotic process releasing substances that produce further endothelial damage. This phenomenon is not observed with the non-oxidized LDL (Yla-Hertuala S, *et al.*, 1989).

5. Migration and proliferation of smooth muscle cells and matrix synthesis: These mechanisms are produced by cytokines and growth factors such as: IL-1b, platelet derived growth factor (PDGF), fibroblast growth factor (FGF), and tumor necrosis factor alpha (TNF-alpha). The ox-LDL, and its subproducts, stimulate the synthesis of the above factors by macrophages The smooth muscle cell determines the amount of matrix at the atheromatous plaque, which are in turn activated by these factors.

6. Genesis of the necrotic core and calcification: The apoptotic foam cells death leads to calcification of the plaque, or its rupture, or thrombus formation according with the magnitude of

necrosis. It constitutes the so-called unstable plaque diffuse calcification predominates making vascular stiffens and cardiac overload increases (Berliner JA, and Heinecke JW, 1996).

1.2.9 Determination of LDL oxidation

Since ox-LDL in plasma is rapidly removed from the circulation by macrophages, lipid peroxidation of LDL is evaluated measuring this process *in vitro*. Hence, plasma concentration of ox-LDL does not reflect neither the real dimension of LDL oxidation nor the events evolving in the arterial wall. Resistance of LDL to oxidation depends upon the equilibrium between pro-oxidants and antioxidants characteristics of the lipoprotein, *e.g.* the degree of unsaturation of PUFA and the vitamin E content. One of the agents most extensively used for in vitro oxidation, is the cupric cation. It can be assumed that LDL oxidized by copper has similar structural and functional characteristics as LDL oxidized in the arterial wall. It is important to establish the oxidative conditions in vitro, and select the most adequate markers of modified LDL. These markers are applied both to basal status and after oxidation was induced (Esterbauer H and Jungens G, 1993).

In LDL particle, cholesteryl linoleate represents quantitatively the single most important PUFA which is the substrate for peroxidation. If PUFA becomes oxidized to lipid hydroperoxides, their isolated carbon-carbon double bonds are converted to conjugated double bonds showing a strong UV-absorption at 234 nm, designated as conjugated diene (CD) formation (Esterbauer H, and Jurgens G, 1993). Therefore, its content in LDL may influence the determination of oxidation resistance measured by CD or TBARs formation. Reaven *et al.* (Reaven PD, *et al.*, 1993) reported that LDL particles riched in PUFA are more readily oxidized than LDL particles enriched in saturated fatty acids or monounsaturated fatty acids. In addition, elevated levels of performed lipid hydroperoxides and cholesterol in LDL were associated with increased oxidation susceptibility. Studies have shown that monoenic fatty acids enriched LDL, for example oleic acid was remarkably resistant to oxidative modification as measured by decreased formation of CD and TBARS. The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase inhibitors reduced the susceptibility of LDL to oxidation by altering the LDL particle composition containing less lipid relative to protein (Lavy A, *et al.*,1991) or preserve endogenous antioxidants. In deed, most of these HMG-CoA reductase inhibitors themselves are

antioxidants and could become bound to lipoprotein in the circulation to protect them against oxidation. LDL particle size could also influence its oxidation susceptibility. For example, small dense LDL particles display diminished resistance to oxidative stress in vitro. Since LDL is the major extracellular transport vehicle for lipid-soluble antioxidants, it contains relatively large amounts of α-tocopherol, β-carotene, and ascorbic acid and among them α-tocopherol is the most important antioxidant known with 6 molecules per LDL particle (Esterbauer H and Ramos P, 1995). As the oxidation goes on, α-tocopherol is the first and β-carotene is the last endogenous antioxidant in LDL particles to be depleted. The endogenous antioxidants contained in LDL particles might influence oxidation resistance. Dietary antioxidant supplementation could increase their content in LDL and therefore, increase oxidation resistance of lipoproteins (Nyyssö nen K, *et al.*, 1994).

1.2.10 LDL cholesterol and U937 cells

Neoplastic, histiocytic cell line (U937 cells) derived from a patient with generalized histiocytic lymphoma. The morphology of the cell line was identical to that of the tumor cells in the pleural effusion from which the line was derived. In all these respects U937 differed from prototype lymphoblastoid cell lines. The histiocytic origin of the cell line was shown by its capacity for lysozyme production and the strong esterase activity of the cells.

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

Furthermore, the activation of endocytosis by LDL is not inhibited by the inclusion of heparin or acetylation of the LDL indicating that binding of LDL to the LDL receptor is not required for these effects. Phorbol myristate acetate (PMA) and 12-tetradecanoyl-phorbol-13-acetate (TPA) were induced for differentiation of U-937 cells into monocyte-macrophage-like cells, and up-regulation of scavenger receptors (Hayashi K, *et al.*, 1998 and Hass R, *et al.*, 1989).

1.2.11 Free radicals

Free radicals are chemical species that possess an unpaired electron in the outer (valence) shell of the molecule making them highly reactive, means that they have low chemical specificity, so they can react with most molecules in its vicinity including proteins, lipids, carbohydrates and DNA. It also means that in trying to gain stability by capturing the needed electron they have very short survive and quickly react with their surroundings. Hence, free radicals attack the nearest stable molecule, "stealing" its electron. When the "attacked" molecule loses its electron, it becomes a free radical itself initiating chain reaction. Once the process is started, it can cascade, finally resulting in the disruption of a living cell. Free radicals are produced continuously in cells either as by-products of metabolism or deliberately as in phagocytosis (Del Mastero RF, 1980).

Sources of Free Radicals

Endogenous sources

Autoxidation

Autoxidation is a by product of the aerobic internal milieu of the molecules that undergo autoxidation, such as catecholamines, hemoglobin, myoglobin, reduced cytochrome C and thiol. Autoxidation of any of the above molecules in a reaction results in the reduction of the oxygen diradical and the formation of reactive oxygen species. Superoxide is the primary radical formed. Ferrous ion (Fe^{2-}) can also have its electron stolen by oxygen to produce superoxide and Fe^{3-}, by the process of autoxidation.

Enzymatic oxidation

A variety of enzyme systems is capable of generating significant amounts of free radicals, including xanthine oxidase (activated in ischemia-reperfusion), prostaglandin synthase, lipoxygenase, aldehyde oxidase, and amino acid oxidase. The enzyme myeloperoxidase produced in activated neutrophils, utilizes hydrogen peroxide to oxidize chloride ions into the powerful oxidant hypochlorous acid (HOCl).

Respiratory burst

Respiratory burst is a term used to describe the process by which phagocytic cells consume large amounts of oxygen during phagocytosis. Between 70 and 90% of this oxygen consumption can be accounted for in terms of superoxide production. These phagocytic cells

possess a membrane bound flavoprotein cytochrome-b-245 NADPH oxidase system. Cell membrane enzymes such as the NADPH-oxidase exist in an inactive form. It is the exposures to immunoglobulin-coated bacteria, immune complexes, complement 5a, or leukotriene, however activate the enzyme NADPH-oxidase. This activation initiates a respiratory burst at the cell membrane to produce superoxide. H_2O_2 is then formed from superoxide by dismutation with subsequent generation of $OH^{\bullet}$ and HOCl by bacteria.

Subcellular organelles

Organelles such as mitochondria, chloroplasts, microsomes, peroxisomes and nuclei have been shown to generate $O_2^{\bullet-}$. Mitochondria are the main cellular organelle for cellular oxidation reactions and the main source of reduced oxygen species in the cell. The leaks in mitochondrial electron transport system allow O_2 to accept a single electron forming $O_2^{\bullet-}$. It has been shown that superoxide production by the mitochondria. Microsomes are responsible for 80% of the H_2O_2 produced *in vivo* at 100% hyperoxia sites. Peroxisomes are known to produce H_2O_2, but not $O_2^{\bullet-}$, under physiologic conditions. Peroxisomal oxidation of fatty acids has recently been recognized as a potentially important source of H_2O_2 production with prolonged starvation.

Transition metals ions

Iron and copper play a major role in the generation of free radicals injury and the facilitation of lipid peroxidation. Transition metal ions participate in the Haber-Weiss reaction that generates $OH^{\bullet}$ from $O_2^{\bullet-}$ and H_2O_2.

Ischemia reperfusion injury

Ischemia confers a number of effects all contributing to the production of free radicals. Normally, xanthine oxidase is known to catalyse the reaction of hypoxanthine to xanthine and subsequently xanthine to uric acid. This reaction requires an electron acceptor as a cofactor. During ischemia two factors occur, first the production of xanthine and xanthine oxidase are greatly enhanced. Second, there is a loss of both antioxidants superoxide dismutase and glutathione peroxidase. The molecular oxygen supplied on reperfusion serves as an electron acceptor and cofactor for xanthine oxidase causing the generation of the $O_2^{\bullet-}$ and H_2O_2. Strenuous exercise has been proposed to activate xanthine oxidase-catalysed reactions and generate free radicals in skeletal muscle and myocardium.

Exogenous sources

Drugs

A number of drugs can increase the production of free radicals in the presence of increased oxygen tensions. The agents appear to act additively with hyperoxia to accelerate the rate of damage. These drugs include antibiotics that depend on quinoid group or bound metals for activity (nitrofurantoin), antineoplastic agents as bleomycin, anthracyclines (adriamycin) and methotrexate, which possess pro-oxidant activity. In addition radicals derived from penicillamine, phenylbutazone, some fenamic acids and the aminosalicylate component of sulphasalazine might inactivate protease and deplete ascorbic acid accelerating lipid peroxidation.

Radiation

Radiotherapy may cause tissue injury that is caused by free radicals. Electromagnetic radiation (X rays, gamma rays) and particulate radiation (electrons, photons, neutrons, alpha and beta particles) generate primary radicals by transferring their energy to cellular components such as water. These primary radicals can undergo secondary reactions with dissolved oxygen or with cellular solutes.

Tobacco smoking

Oxidants in tobacco exist in sufficient amounts to suggest that they play a major role in injuring the respiratory tract. It has been shown that tobacco smoke oxidants severely deplete intracellular antioxidants in the lung cells *in vivo* by a mechanism that is related to oxidative stress. It has been estimated that each puff of smoke has an enormous amount of oxidant materials. These include aldehydes, epoxides, peroxides, and other free radicals that may be sufficiently long lived as to survive till they cause damage to the alveoli. In addition nitric oxide, peroxyl radicals and carbon centred radicals are present in the gas phase. Again micro-hemorrhages are most probably the cause of iron deposition found in smokers' lung tissue. Iron in this form leads to the formation of the lethal hydroxyl radical from hydrogen peroxide. It was also found that smokers have elevated amounts of neutrophils in the lower respiratory tract that could contribute to a further elevation of the concentration of free radicals.

Inorganic particles

Inhalation of inorganic particles also known as mineral dust (*e.g.* asbestos, quartz, silica) can lead to lung injury that seems at least in part to be mediated by free radical production.

Asbestos inhalation has been linked to an increased risk of developing pulmonary fibrosis (asbestosis), mesothelioma and bronchogenic carcinoma. Silica particles as well as asbestos are phagocytosed by pulmonary macrophages. These cells then rupture, releasing proteolytic enzymes and chemotactic mediators causing infiltration by other cells such as neutrophils, thus initiating an inflammatory process (Kehrer JP, *et al.*, 1993), that leads to increased production of free radicals and other reactive oxygen species. Furthermore, asbestos fibres contain iron, which may be derived from hemoglobin during micro-hemorrhages. This iron can stimulate the formation of hydroxyl radicals.

Gases

Ozone is not a free radical but is a very powerful oxidising agent. Ozone (O_3) contains two unpaired electrons and degrades under physiological conditions to $OH^{\bullet}$, suggesting that free radicals are formed when ozone reacts with biological substrates. In support of this hypothesis, ozone can generate lipid peroxidation *in vitro*, although similar findings *in vivo* have not been demonstrated.

1.2.12 Antioxidants

In the aerobic environment, the most dangerous by product are the species of reactive oxygen. The role of antioxidants is to detoxify reactive oxygen intermediates (ROI) in the body. Over the past several years, nutritional antioxidants have attracted considerable interest as potential treatment for a wide variety of disease states, including cancer and other causes, *e.g.* atherosclerosis, chronic inflammatory diseases and aging. An antioxidant is a substance that when present in low concentrations relative to the oxidizable substrate significantly delays or reduces oxidation of the substrate. Antioxidants get their names because they combat oxidation. They are substances that protect other chemicals of the body from damaging oxidation reactions by reacting with free radicals and other reactive oxygen species within the body, hence hindering the process of oxidation. During this reaction the antioxidant sacrifices itself by becoming oxidized. However, antioxidant supply is not unlimited as one antioxidant molecule can only react with a single free radical. Therefore, there is a constant need to replenish antioxidant resources, whether endogenously or through supplementation (Gokce N and Frei B, 1996).

Non-Enzymatic antioxidants

Antioxidants from our diet appear to be of great importance in controlling damage by free radicals. Each nutrient is unique in terms of its structure and antioxidant function. Vitamin E is actually a generic term that refers to all entities (eight found so far) that exhibit biological activity of the isomer tocopherol. Alpha-tocopherol, the most widely available isomer, has the highest biopotency, or strongest effect in the body. Because it is fat-soluble alpha-tocopherol is in a unique position to safeguard cell membranes largely composed of fatty acids. Vitamin E prevents the perioxidation of polyunsaturated fatty acid in membranes from damage by free radicals. Vitamin E is found in vegetable and seed oils, in wheat germ and, in smaller quantities, in meats, fish, fruits and vegetables. Vitamin C is the predominant plasma antioxidant. This water-soluble vitamin scavenges plasma free radicals and prevents their entry into LDL particles. Vitamin C regenerates active vitamin E and increases cholesterol excretion. Vitamin C works synergistically with vitamin E to quench free radicals. Vitamin C also regenerates the reduced (stable) form of vitamin E. Dietary sources of vitamin C include citrus fruits, strawberries, cantaloupe, tomatoes, cabbage and leafy green vegetables. Beta-carotene is a vitamin A precursor carried in plasma and LDL. It is thought to be the best quencher of singlet oxygen (an energized but uncharged form of oxygen that is toxic to cells). Beta-carotene is also especially excellent at scavenging free radicals in low oxygen concentration. Sources of dietary carotenoids include fruits, yellow-orange vegetables (*e.g.*, carrots, squash and sweet potatoes) and deep-green vegetables (*e.g.*, spinach and broccoli) (Jialal I and Grundy SM, 1993).

Antioxidant Enzymes

The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) serve as primary line of defense in destroying free radicals. SOD first reduces (adds an electron to) the radical superoxide ($O_2^{\cdot}$) to form hydrogen peroxide (H_2O_2) and oxygen (O_2). Catalase and GPx then work simultaneously with the protein glutathione to reduce hydrogen peroxide and ultimately produce water (H_2O). Together, they repair oxidized DNA, degrade oxidized protein, and destroy oxidized lipids. Various other enzymes act as a secondary antioxidant defense mechanism to protect the body from further damage (Diaz MN, *et al.*, 1997).

Other Antioxidants

In addition to enzymes, vitamins, and many minerals have antioxidant properties. Among them is coenzyme Q10 (CoQ10, or ubiquinone), which is essential to energy production and can also protect the body from destructive free radicals. Also, uric acid, a product of DNA metabolism, has become increasingly recognized as an important antioxidant. Additionally, substances in plants called phytochemicals are being investigated for their antioxidant activity and health-promoting potential (Odeh RM and Cornish LA, 1991).

There are at least 4 ways expected to be antioxidant actions against free radicals (Gutteridge JM, and Halliwell B, 1994).

1. Chain breaking reactions, *e.g.* α-tocopherol which acts in lipid phase to trap "ROS" (Reactive oxygen species) radical.

2. Reducing the concentration of reactive oxygen species *e.g.* oxidized glutathione.

3. Scavenging initiating radicals such as superoxide dismutase which acts in aqueous phase to trap superoxide free radicals.

4. Chelating the transition metal catalysts: A group of compounds serves an antioxidant function by sequestration of transition metals that are well-established pro-oxidants. In this way, transferrin, lactoferrin, and ferritin function to keep iron-induced oxidant stress while ceruloplasmin and albumin as copper sequestrants.

The antioxidant defenses that prevent oxidation of LDL need to be defined. The antioxidant content of the LDL particle is critical for its protection (Esterbauer H, *et al.*, 1992) and, theoretically, if sufficient lipophilic antioxidants were present, the LDL particles would be protected from even profound oxidant challenge. *In vivo*, whether or not LDL becomes oxidized is a question of the balance between the extent of the pro-oxidant challenge and the capacity of the antioxidant defenses. Although ox-LDL is found in man, there are no conclusive intervention studies in man to support a quantitatively important role for this process. The ongoing antioxidant trials will no doubt add more beneficial evidence in the role of atherosclerosis prevention.

1.2.13 Antioxidant vitamins and diabetics

Diabetes is associated with higher levels of oxidized blood lipids, which increase the risk of diabetic complications such as cardiovascular disease. Oxidative stress may be increased because of glucose attachment to proteins. Levels of antioxidants in diabetic are also lower in circulation, and lower content in LDL, this lower level of antioxidant protection is likely to contribute to the development of diabetic complications (Packer L, 1993). Vitamin C metabolism is altered in diabetic. The cellular uptake of vitamin C is promoted by insulin and inhibited by high blood sugar; and as diabetic especially who have low insulin levels, they are at greater risk of vitamin C deficiency. Most studies have found people with diabetes to have at least 30 per cent lower vitamin C concentrations than people without the disease. Levels seem to be lower in diabetic people as a result of the disease rather than as a result of poor dietary intake. This deficiency can lead to increased capillary permeability, poor wound-healing, increased cholesterol levels, and immune suppression; which all contribute to diabetic complications (Losonczy KG, *et al.*, 1996).

1.2.14 Antioxidant vitamins and the prevention of coronary heart disease

Clinical use of antioxidant vitamin supplementation may help to prevent coronary heart disease (CHD). Epidemiologic studies found lower CHD morbidity and mortality in persons who consume larger quantities of antioxidants in foods or supplements. Clinical trials indicate that supplementation with certain nutrients is beneficial in reducing the incidence of CHD events. Recent studies show that supplementation with antioxidant vitamins E and C have benefits in CHD prevention; however, supplementation with β-carotene may have deleterious effects and is not recommended. Current evidence suggests that patients with CHD would probably benefit from taking vitamin E in a dosage of 400 IU per day and vitamin C in a dosage of 500 to 1,000 mg per day. Clinicians may also want to consider vitamin supplementation for CHD prevention in high-risk patients. Folate lowers elevated homocysteine levels, but evidence for routine supplemental use does not yet exist. Other nutritional supplements are currently under investigation. Recent experimental and epidemiologic evidence suggests that some antioxidant vitamins appear to be important in reducing the risk of coronary heart disease (CHD). These

antioxidants include ascorbic acid (vitamin C), α-tocopherol (vitamin E), folate, β-carotene, ubiquinone (CoQ10), bioflavonoids and selenium (Jha P, *et al.*, 1995).

LDL oxidation is a key factor in the development of atherosclerosis (Schwartz CJ, *et al.*, 1993 and Jialal I and Grundy SM, 1992). Excess free radicals in plasma and the arterial intima increase LDL oxidation. Oxidized LDL is cytotoxic and is taken up by arterial macrophages, which is a primary factor in plaque formation and progression. Antioxidants in plasma, the LDL particle and the cell wall reduce LDL oxidation. The major fat-soluble antioxidants are α-tocopherol and β-carotene (a vitamin A precursor). The major water-soluble antioxidant is vitamin C. These vitamins reduce LDL oxidation and preserve vasoreactivity by increasing endothelial nitric oxide release and reducing thrombogenicity. Antioxidant vitamins may also reduce the risk of plaque progression and rupture (Diaz MN, *et al.*, 1997).

1.2.15 Turmeric

Curcuma longa Linn. is a tropical plant native of south and southeast tropical Asia as shown in Figure at Page 30. It is a member of the ginger or *Zingiberaceae* family. Turmeric is widely consumed in the countries of origin for a variety of uses, including as a dietary spice, as a dietary pigment and as an Indian folk medicine for the treatment of various illnesses. It is also used in Hindu religious ceremonies in one form or another as part of the religious rites. Curcuminoids are responsible for the yellow color of turmeric, as well as the yellow color of curry (Srinivasan KR, 1953).

Figure. ***Curcuma longa*** **Linn. plant, with young rhizomes and roots**

(http://www.afuegolento.com/imgnot/ 34/foto_parnot378459.jpg)

Curcuminoids

Curcuminoids are polyphenolic pigments found in turmeric. The term turmeric is used both for the plant *Curcuma longa* Linn. and the spice derived from the rhizomes of the plant. The major curcuminoids are curcumin, demethoxycurcumin and bisdemethoxycurcumin as chemical structure shown in Figure at Page 31. These substances comprise 3 to 6% of *Curcuma longa* Linn. Curcumin makes up 70 to 75% of the curcuminoids, demethoxycurcumin 15 to 20% and bisdemethoxycurcumin about 3% (Ammon, HPT and Wahl MA, 1991).

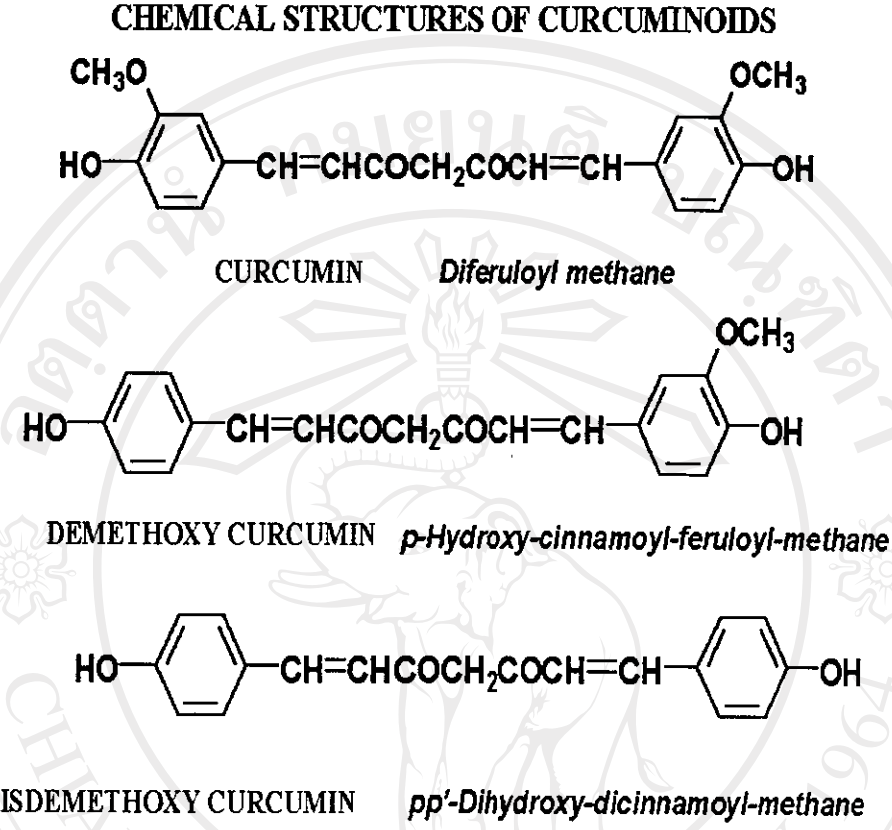

Figure. Chemical structures of curcumin, demethoxycurcumin and bisdemethoxycurcumin

(http://www.medicine.cmu.ac.th/secret/edserv/journal/41(4)/Fig1-songyot.jpg)

Actions and pharmacology

The primary pharmacological actions of curcuminoids which have been researched extensively include: antioxidant, anti-inflammation, anti-carcinogen, anti-mutagen, anti-thrombotic action, hepato-protectective action, antimicrobial action, antiviral action, antiviral, hypocholesterolemic activities and antiparasitic action (Srimal RC and Dhawan BN, 1985). These actions are attributed the unique molecular structure of the curcuminoids as shown in Figure at Page 32.

Molecular structures important for biological activity of curcuminoids:

1. Para hydroxyl group - antioxidant activity

2. Keto group - anti-inflammatory activity, anti-cancer, anti-mutagen.

3. Double bonds - anti-inflammatory activity, anti-cancer, anti-mutagen

In the three well recognized phenolics of turmeric, bisdemethoxycurcumin is often regarded as the most potent antioxidant followed by demethoxycurcumin and curcumin. This data is, however, inconsistent with other experiments, which indicate that all three curcuminoids posses almost similar antioxidant activity .

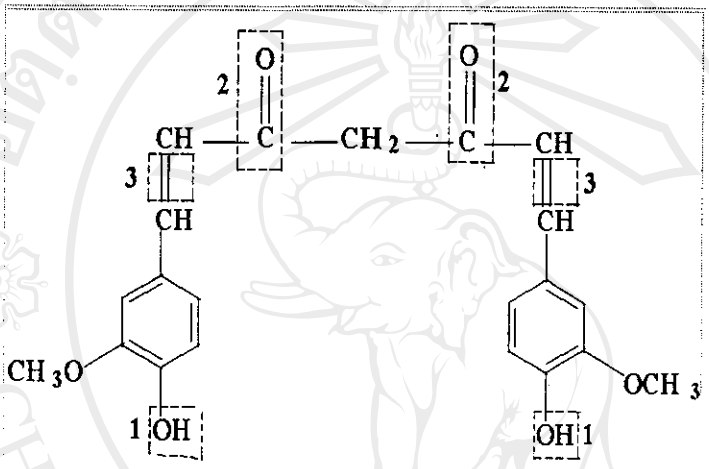

Figure. Molecular structures and biological active groups of curcuminoids

(http://www.sabinsa.com/images/literature/curcum_paper/Image37.gif)

Antioxidant mechanism of curcuminoids

The antioxidant mechanism of curcuminoids may include one or more of the following interactions:

1. Scavenging or neutralizing of free radicals

2. Interacting with oxidative cascade, and preventing its outcome

3. Oxygen quenching, and making it less available for oxidative reactions

4. Inhibition of oxidative enzymes like cytochromeP-450,

5. Chelating or disarming oxidative properties of metal ions like iron, (Fe)

Turmeric and its active curcuminoids and the water soluble peptide turmerin, have antioxidant properties and effectively inhibit the free radical damage to biomolecules both *in vitro* and *in vivo* conditions. The fact that curcuminoids act as antioxidants by prevention and intervention processes, makes them very unique natural antioxidants.

The curcuminoids have been found to have a number of antioxidant activities, including scavenging of such reactive oxygen species as superoxide anions and hydrogen peroxide, inhibition of lipid peroxidation and inhibition of the oxidation of low-density lipoprotein (LDL). The reduced derivative of curcumin, tetrahydrocurcumin, has been found to have even stronger antioxidant activity. Tetrahydrocurcumin may be formed from curcumin following ingestion; however, this is unclear (Reddy ACP, and Lokesh BR, 1992).

Anticarcinogenic mechanism

The possible anticarcinogenic activity of curcumin and the other curcuminoids may be accounted for by a few mechanisms. These include inhibition of angiogenesis, upregulation of apoptosis, interference with certain signal transduction pathways that are critical for cell growth and proliferation, inhibition of colonic mucosa cyclo-oxygenase (COX) and lipoxygenase (LOX) activities and inhibition of farnesyl protein transferase. In addition to its possible activity in preventing malignant transformation and inhibiting tumor growth, curcumin may have antimetastatic potential, as well. In this regard, curcumin has been found to inhibit matrix metalloproteinase-9 in a human hepatocellular carcinoma cell line. The possible anticarcinogenic activity of the curcuminoids may be attributed, at least in part, to their ability to inhibit activation of the transcription factors NF-Kappa B and AP-1. Curcuminoids have also been found to target the fibroblast growth factor-2 (FGF-2) angiogenic signaling pathway and inhibit expression of gelatinase B in the angiogenic process (Nagabhushan M and Bhide SV, 1987).

Anti-inflammatory action mechanism

The possible anti-inflammatory activity of the curcuminoids may also be accounted for several mechanisms, including inhibition of COX and LOX, reduction of the release of ROS by stimulated neutrophils, inhibition of AP-1 and NF-Kappa B, and inhibition of the activation of the pro-inflammatory cytokines TNF (tumor necrosis factor) -alpha and IL (interleukin)-1 beta (Ghatak N and Basu N, 1972).

Anti-HIV activity

Curcuminoids has modest anti HIV-1 activity. It has been found to inhibit HIV-1 and HIV-2 proteases, HIV-1 long terminal repeat (LTR)-directed gene expression, *Tat*-mediated transactivation of HIV-1-LTR and HIV-1 integrase. All of these actions have been demonstrated *in vitro*. There is no evidence that curcumin or the other curcuminoids significantly inhibit the replication of HIV-1 *in vivo*. The mechanism of the possible hypocholesterolemic effect of the curcuminoids is unclear (Mazumder A, *et al.*, 1995).

Contraindications and precautions

Curcuminoids may stimulate bile production. The volatile oil of turmeric is thought to be responsible for the bile-stimulating activity of turmeric, but this has not been conclusively established. Therefore, curcuminoids are contraindicated in those with bile duct obstructions and those with gallstones.

Pregnant women and nursing mothers should avoid curcuminoid supplemention. Those with gastroesophogeal reflux disease (GERD) and those with a history of peptic ulcer disease should exercise caution in the use of curcuminoid supplements. Curcuminoids may have antithrombotic activity. Therefore, those on warfarin or anti-platelet drugs should exercise caution in their use. Cancer patients should only use curcuminoid supplements under medical supervision. Curcuminoid supplements must be taken with food. Curcuminoids may cause gastric irritation and ulceration if taken on an empty stomach.

Curcuminoids and diabetics

Administration of turmeric or curcuminoids to diabetic rats reduced the blood sugar and glycosylated hemoglobin levels significantly. Oxidative stress was also reduced by turmeric and curcuminoids, as determined by the standard TBARs test. It was postulated that this could be due to decreased influx of glucose pathway, leading to an increased NADPH/NADP ratio and elevated activity of the antioxidant enzyme glutathione peroxidase. The activity of sorbitol dehydrogenase, an enzyme that catalyzes the conversion of sorbitol to fructose, was also lowered significantly on treatment with turmeric or curcuminoids. Curcuminoids was found to be more effective than turmeric in all these case (Pan MH, *et al.*, 1999).

1.3 Objectives

1. To study the inhibitory effect of curcuminoids on oxidative modification of LDL in healthy with normolipidemic compared to diabetes with hyperlipidemic groups.

2. To study the inhibitory effect of curcuminoids compare to α-tocopherol and ascorbic acid on oxidative modification of LDL.