ผลปกป้องของดีเฟอริโพรนต่อการเกิดออกซิเดชันของไลโปโปรตีน ชนิดความหนาแน่นต่ำและสูงที่ถูกเหนี่ยวนำด้วยฮีมิน

นางสาวอุษา สุวรรณสรวล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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PROTECTIVE EFFECT OF DEFERIPRONE ON HEMIN-INDUCED OXIDATION OF LOW AND HIGH DENSITY LIPOPROTEINS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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อุษา สุวรรณสรวล: ผลปกป้องของดีเฟอริโพรนต่อการออกซิเดชันของโปโปรตีนชนิดความหนาแน่นต่ำและสูงที่ถูกเหนี่ยวนำด้วยฮีมิน (PROTECTIVE EFFECT OF DEFERIPRONE ON HEMIN-INDUCED OXIDATION OF LOW AND HIGH DENSITY LIPOPROTEINS)
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การเปลี่ยนแปลงของไลโปโปรตีนชนิดความหนาแน่นต่ำและสูง (LDL, HDL) โดยปฏิกิริยาออกซิเดชัน มีบทบาทสำคัญต่อพยาธิกำเนิดของภาวะหลอดเลือดแข็ง ฮีมินเป็นสารประกอบเหล็กที่เหนี่ยวนำให้เกิดออกซิเดชันได้ สามารถตรวจพบในระดับสูงในผู้ป่วยธาลัสซีเมีย ซึ่งมีส่วนสำคัญทำให้เกิดภาวะหลอดเลือดแข็ง เป็นผลก่อให้เกิดภาวะแทรกซ้อนทางหลอดเลือดในผู้ป่วย deferiprone (L1) เป็นยาขับเหล็กชนิดรับประทานที่ใช้ในผู้ป่วยที่มีเหล็กเกินในพลาสมา วัตถุประสงค์ของงานวิจัยครั้งนี้เพื่อศึกษาผลของ L1 ต่อการป้องกันการเกิดปฏิกิริยาออกซิเดชันของ LDL และ HDL ที่เหนี่ยวนำด้วยฮีมินและผลของ L1 ต่อการจับกิน LDL ที่ถูกออกซิไดซ์ด้วยฮีมินโดย U937 macrophage เมื่อเติม L1 ในหลอดทดลองที่ความเข้มข้น 5, 10, 50, 100 และ 500 ไมโครโมลาร์ ใน LDL และ HDL แล้วเหนี่ยวนำให้เกิดปฏิกิริยาออกซิเดชันด้วยการเติมฮีมิน เป็นเวลา 24 ชั่วโมง ซึ่งตรวจวัดระดับการเกิดปฏิกิริยาออกซิเดชันของไขมันจากปริมาณการเกิด thiobarbituric acid reactive substances (TBARs), วัดปริมาณของ α-tocopherol และองค์ประกอบไขมันโดยใช้เครื่อง HPLC

ผลการศึกษาครั้งนี้พบว่า L1 ทุกๆความเข้มข้นที่ใช้ มีผลปกป้องการเกิดปฏิกิริยาออกซิเดชันของไขมันจากการเหนี่ยวนำด้วยฮีมินใน LDL และ HDL ได้ โดยยับยั้งการเกิด TBARs ได้ประมาณ 50-80 % และเกือบ 100 % ใน LDL และ HDL ตามลำดับ ทั้งที่เวลา 9 และ 24 ชั่วโมงของการเหนี่ยวนำให้เกิดปฏิกิริยาออกซิเดชัน อย่างไรก็ตาม L1 ไม่สามารถปกปักรักษา α-tocopherol ไว้ได้ทั้งใน LDL และ HDL ที่ถูกออกซิไดซ์ด้วยฮีมิน นอกจากนี้ L1 ที่ความเข้มข้น 500 ไมโครโมลาร์ สามารถป้องกันการลดลงของระดับ cholesteryl arachidonate และ cholesteryl linoleate อย่างไรก็ตาม L1 ไม่มีผลต่อการจับกิน LDL ที่เหนี่ยวนำด้วยฮีมินโดย macrophage จากการศึกษาครั้งนี้สรุปได้ว่า L1 มีฤทธิ์เป็นสารต้านออกซิเดชันทั้งใน LDL และ HDL ที่ถูกเหนี่ยวนำด้วยฮีมิน

สาขาวิชาเภสัชวิทยา (สหสาขาวิชา) ลายมือชื่อนิสิต.....อุษา.....
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USA SUWANNASUAL: PROTECTIVE EFFECT OF DEFERIPRONE ON HEMIN-INDUCED OXIDATION OF LOW AND HIGH DENSITY LIPOPROTEINS. THESIS ADVISOR: ASSIST. PROF. RATAYA LUECHAPUDIPORN Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. THITIMA PENGSUPARP Ph.D., 76 pp.

Oxidative modification of low density lipoproteins (LDL) and high density lipoproteins (HDL) play a major role in the pathogenesis of atherosclerosis. The hemin, iron compound is an oxidant that can be detected in thalassemic patients at high level. This is importantly a prerequisite for the development of atherosclerosis with vascular complications in the patients. Deferiprone (L1) is an oral iron chelator used to treat the patients having excessive plasma iron. The objective of this study is to investigate the effect of L1 on hemin-induced oxidation of low and high density lipoproteins and the effect of L1 on U937 derived macrophage uptake of hemin-oxidized low density lipoprotein. *In vitro* study was conducted by adding the L1 at the concentrations of 5, 10, 50, 100, and 500 μM into the LDL and HDL prior to oxidation induction using the hemin with the incubation period of 24 h. The degree of the lipoprotein oxidation was determined by thiobarbituric acid reactive substances (TBARs) formation. The levels of α-tocopherol and lipid composition were measured by HPLC.

The results showed that the L1 at all tested concentrations have the protective effect on the hemin-oxidized LDL and HDL by inhibition of TBARs formation approximately 50-80 % and almost 100% in LDL and HDL, respectively at time 9 and 24 h of incubation. However, the L1 could not preserve the α-tocopherol both in hemin-oxidized LDL and HDL. In addition, the L1 at the concentration of 500 μM could protect the decrease of cholesteryl arachidonate and cholesteryl linoleate level. However, L1 did not affect macrophage uptake of hemin oxidized LDL. This study was concluded that L1 possessed an antioxidative activity both in LDL and HDL oxidation induced by hemin.

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LIST OF ABBREVIATIONS

α-tocopherol	Alpha-tocopherol
Apo	Apoprotein
BHT	Butylated hydroxytoluene
β-thal/HbE	β-thalassemia/hemoglobin E
CA	Cholesteryl arachidonate
CE	Cholestery ester
CL	Cholesteryl linoleate
CO	Cholesteryl oleate
CP	Cholesteryl palmitate
°C	Degree celcius
FC	Free cholesterol
G	Gram
HDL	High density lipoprotein
HPLC	High perfomance liquid chromatography
h	Hour
L1	deferiprone
LDL	Low Density Lipoprotein
MDA	Malondialdehyde
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mm	Millimeter
µg	Microgram
µl	Microliter
µM	Micromolar
EDTA	Ethylenediamine tetraacetic acid disodium salt
nm	Nanometer

nmol	Nanomole
OD	Optical density
oxHDL	Oxidized high Density Lipoprotein
oxLDL	Oxidized Low Density Lipoprotein
PBS	Phosphate buffer saline
PUFAs	Polyunsaturated fatty acids
rpm	Revolution per minute
S.E.M.	Standard error of mean
TBARs	Thiobarbituric acid reactive substances

CHAPTER I

INTRODUCTION

1. Background and rationale

Thalassemia is one of the genetically heritable diseases. At present, there are approximately 16-24 millions (40%) of Thai people being carriers or traits of thalassemia, which are the highest proportion in the world, while a million of Thai people are thalassemic. The annually-increasing numbers of thalassemic patients of about 10,000 have been expected (Fucharoen *et al.*, 1997; Tanpaichitr, 1999; Tongsong *et al.*, 2000). The disease causes production of abnormal shape and premature destruction of red blood cells. This results in excessive amount of plasma hemin and iron. The hemin, which is normally found within the range of 50-280 μM in thalassemic patients, induces oxidation of low density lipoproteins (LDL), leading to abnormality of arteries i.e. pulmonary artery occlusion, cerebral thrombosis, leg ulcer, and atherosclerosis (Pascher *et al.*,1957; Sonakul *et al.*, 1988; Wong *et al.*, 1990; Miller and Shaklai, 1999).

The oxidation of both LDL and high density lipoprotein (HDL) is the initial step of the atherosclerosis since macrophages tend to engulf the oxidized LDL, subsequently formed as intracellular cholesteryl esters, and the macrophages become macrophage foam cells within the tunica intima of blood vessels, especially arteries (Jessup *et al.*, 2002). The oxidized LDL also induces smooth muscle cell proliferation. This causes thickening of arteries and plaque formation, which narrows the vascular diameter and consequently impedes blood circulation (Gurr *et al.*, 2002). In addition, oxidized HDL reduces the release of the cholesteryl esters by the macrophage foam cell while the macrophage can phagocytose the oxidized HDL. The oxidized HDL also attenuates protective effects against LDL oxidation. This, therefore, abolishes anti-atherosclerotic activity of the HDL (Throne *et al.*, 2007).

The hemin induces LDL and HDL oxidation by changing lipid components of the LDL and HDL (Chalermchoung, 2004). Moreover, the oxidized LDL is engulfed by the macrophage (Camejo *et al.*, 1998). Thus, the detected plasma hemin in those patients probably reflects the oxidation reaction causing vascular diseases.

Drugs, which can remove excessive iron, are used to treat the thalassemic patients. Until recently, the most wildly used is deferoxamine with administration by injection, but it provides low therapeutic effects. Nowadays, the drug deferiprone (L1) with its oral administration has better therapeutic effects (Eybl *et al.*, 2002). L1 prevents the hemin-induced LDL oxidation *in vitro* and it also reduces plasma very low density lipoprotein and LDL in animals (Matthews *et al.*, 1997). Nevertheless, there are no reports of protective effects of L1 on HDL oxidation, phagocytosis of oxidized LDL and HDL by the macrophage, and the formation of the macrophage foam cell.

This study was aimed to investigate the protective effects of the L1 on both LDL and HDL oxidation. The alterations of lipid compositions within lipoproteins, phagocytosis of the LDL and HDL, and the macrophage foam cell formation were observed.

2. Objectives

2.1 To study the effect of deferiprone on hemin-induced oxidation of low and high density lipoproteins.

2.2 To study the effect of deferiprone on macrophage uptake of hemin-oxidized low density lipoprotein and macrophage foam cell formation.

CHAPTER II

LITERATURE REVIEW

1. Lipoprotein (Voet *et al.*, 1999)

Lipoproteins are globular micelle-like particles, the outer parts of which are composed of polar lipids, namely phospholipids and free cholesterols. The core of the lipoprotein particles consists of the more hydrophobic lipids, cholesteryl esters and triacylglycerols. These form a central droplet which is anchored by their hydrophobic regions, surface-coating molecules of the phospholipids, cholesterols and proteins. There are four classes of the lipoproproteins: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). The major function of the chylomicrons is to transport the exogenous triacylglycerols and cholesterols from the intestine to tissues while that of the VLDL and LDL is to transport the endogenous triacylglycerols and cholesterol from the liver to tissues, but vice versa for that of the HDL (Table 2.1).

The protein components of the lipoproteins are apolipoproteins showing immense structural diversity, some of which have a largely structural role, others of which are major metabolic regulators and others of which may influence immunological and hemostatic responses apparently unconnected with lipid transport.

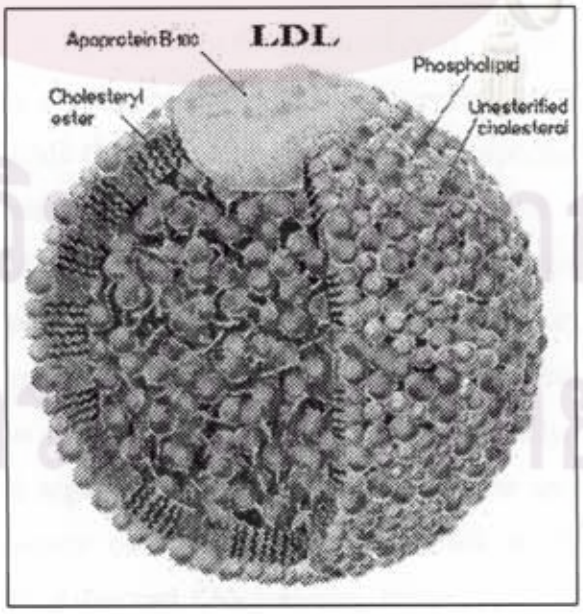


Figure 2.1 Structure of LDL

Table 2.1 Compositions and characteristics of the human plasma lipoproteins

	Chylomicrons	VLDL	LDL	HDL
Density ($g \cdot ml^{-1}$)	<0.95	<1.006	1.006-1.019	1.063-1.210
Diameter (nm)	80-1000	30-90	18-22	5-12
Mass (kD)	400,000	10,000-80,000	2300	175-360
% Protein	1.5-2.5	5-10	20.25	40-55
% Phospholipid	7-9	15-20	15-20	20-35
% Free cholesterols	1-3	5-10	7-10	3-4
% Triacylglycerols	84-89	50-65	7-10	3-5
% Cholesteryl esters	3-5	10-15	35-40	12
α-tocopherol (molecule/particle)	-	-	8-12	0.3
Major apopooroteins	A- I, A-II, B-48, C-I, C-II, C-III, E	B-100, C-I, C-II, C-III,E	B-100	A-I, A-II, C-I, C-II, C-III, D, E

2. Lipoprotein oxidation and its consequences

LDL circulates in the plasma, a portion of which traverses subendothelial spaces and can be removed from the general circulation. It is generally believed that LDL oxidation does not take place in the circulation because of its antioxidant properties. In the subendothelial spaces, the presumed site of LDL oxidation, the LDL may be exposed more frequently to cell-derived oxidants and at the same time may be less protected by antioxidants compared to the circulating LDL (Matsuura *et al.*, 2006). Moreover, endothelial cells, smooth muscle cells and macrophages have been shown to be capable of oxidizing LDL *in vitro* (Cominacini *et al.*, 1996).

The oxidation of the lipoproteins is a lipid peroxidation process in which polyunsaturated fatty acids (PUFAs) contained in the phospholipids are transformed into lipid hydroperoxides and then to some unsaturated aldehydes such as malondialdehyde (MDA), 4-hydroxynonenol (HIVE), and hexenol. These products can neutralize some positively-charged amino acids, i.e. lysine, arginine, and histidine residues, and increase apoB 100 affinity to the scavenger receptor (Cominacini *et al.*, 1996). These aldehydes can bind with the amino group of the lysine of the apoB 100 of LDL. The increased negative charge on the surface of the apoB 100 is recognized by the scavenger receptor on macrophages and leads to foam cell formation (Cominacini *et al.*, 1996; Rifici and Khachadirian, 1996).

Lipid peroxidation (LPO), which generates reactive lipid peroxides ($LOO^{\bullet}$), can be divided into three phases: initial, propagation, and terminal phase. The initial phase is the event of lipoprotein antioxidant depletion, but that of the polyunsaturated fatty acids does not take place significantly. As the PUFAs (mainly linoleic and arachidonic acid) are rapidly oxidized to lipid hydroperoxides and aldehydes, the lipoprotein antioxidants are reduced. The endogenous tocopherol in the LDL, which prevents against the lipid peroxidation, can prolong duration of the lag phase and reduce rate of the propagation phase (Francis, 2000).

Polyunsaturated lipids are essential for the entire supporting system of cells, including cell membranes, endoplasmic reticulum, and mitochondria. Disruption of their structural properties can therefore have dire consequences for cellular functions. The LPO is implicated in a diverse range of cellular insults and has traditionally been thought to be a major effect of free radicals. Because of this, many of the assay methods to establish free radical-induced injury have measured by-products of the reaction of these molecules with the lipids. However, other cellular components may be as important as, or more important than, lipids in free radical injury. Any assay method that assesses the effects of the free radicals on a particular component of cellular architecture needs to be accompanied by consideration of importance of the type of injury being determined.

The free radicals have high affinity for electron-rich unsaturated covalent bonds, such as those found in the PUFAs. The net result of this reaction is a free radical-mediated abstraction of an electron from the unsaturated covalent bonds of the PUFA, thus generating a PUFA radical ($L^{\bullet}$). Hydroxy, lipoxy, and lipid peroxy radicals have been implicated in initiating this process. Once formed, the lipid radical has several possible fates. It may arrange to a more stable conjugated diene configuration, which enters the self-propagating LPO cascade. Alternatively, it may combine with another molecule, such as another lipid radical or a so-called free radical scavenger, and form a non-reactive complex. In the latter case, the dimer is formed at the expense of a cross-linkage of the PUFAs within the membrane, causing a decrease in membrane fluidity. Because of the reactivity of PUFA radicals, the process is self-propagating. The end result is the chemical alteration of the PUFAs with the disruption of integral cellular components. Whether the LPO is the major site of free radical damage to cells has been questioned. However, even if disruption

of cellular lipids is not the final pathway to cell death, it is certainly a key route whereby additional radicals and other toxic substances are produced. Figure 2.2 shows the actual sequence of events in free radical attack of the PUFAs (Baskin and Salem, 1997).

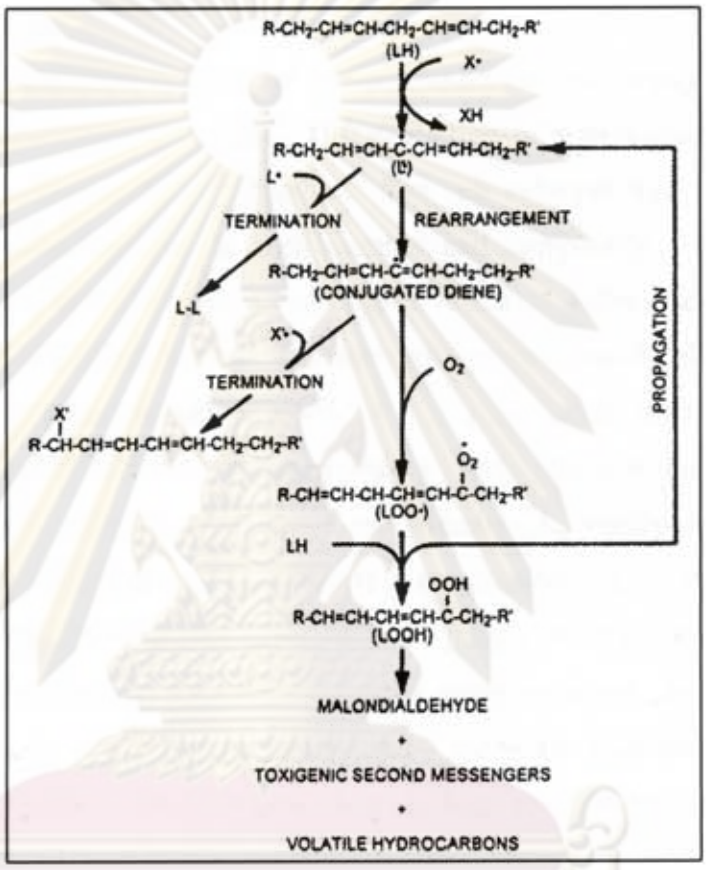


Figure 2.2 The lipid peroxidation cascade

It is known that the oxidized LDL can be taken up by the macrophages via scavenger receptor and CD 36. Accumulation of lipids in the macrophages leads to the foam cellformation which is the hallmark of atherosclerosis (Fubrman *et al.*, 2005). However, unlike the LDL counterpart, the HDL is considered as an anti-atherogenic lipoprotein (Berrougui *et al.*, 2006). *In vitro* HDL shows a key role in protecting LDL from oxidation by neutralizing the atherogenic effects of the oxLDL. This result from HDL carries the bulk of plasma paraoxonas, an arylesterase believed to cleave oxidized fatty acids from the phospholipids of oxLDL. Moreover, the HDL is able to remove cholesterol excess from peripheral cells (Rifici and Khachadiran, 1996; Fracis, 2000). The results of recent studies were shown that the oxidized HDL has a

diminished capacity of promoting cellular cholesterol efflux. This output occurs from the loss of the PUFAs and consequently a reduction of the HDL fluidity (Berrougui *et al.*, 2006).

3. Macrophage and low density lipoprotein

3.1 Macrophage

The mononuclear phagocytic system consists of monocytes circulating in blood and the macrophages in tissues. Differentiation of a monocyte into a tissue macrophage involves a number of changes. The cell enlarges five- to tenfold; its intracellular organelles increase in both number and complexity. In addition, it increases phagocytic ability by producing higher levels of hydrolytic enzymes and secreting a variety of soluble factors. The macrophages are capable of ingesting and digesting exogenous antigens such as whole microorganisms and insoluble particles. In the first step in phagocytosis, the macrophages create pseudopodia which extend around the attached materials. Fusion of the pseudopodia encloses the materials within a membrane-bounded structure called a phagosome, which then enters the endocytic processing pathway. In this pathway, the phagosome moves toward the cell interior, where it fuses with a lysosome to form a phagolysosome. In the process of the phagocytosis, the macrophages can produce cytotoxic substances to destroy phagocytosed materials. Oxygen-dependent killing mechanism is one of the phagocytosed material destruction. Activated macrophages produce a number of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates that can be toxic to the phagocytosed materials. This process is known as the respiratory burst. When the activated macrophages engulf materials or antigens in the phagocysome, they release superoxide anions. The other powerful oxidizing agent generated from superoxide anion can have extreme toxicity to the ingested antigens (Glodsby *et al.*, 2000).

3.2 Interaction between macrophage and LDL (Jessup *et al.*, 2002)

3.2.1. Macrophage as mediator of LDL oxidation

The oxidation of the LDL is thought to occur locally within the intima, and to be influenced, or even mediated by the cells that are in close proximity to the lipoprotein particles. These are predominantly endothelial cells and macrophages, also smooth muscle cells and lymphocytes as the lesion develops. It is

well-established that lesion lipoproteins are partially oxidized, but how this oxidation occurs is less clear. It is thought to occur locally within the intima, and to be influenced, or even mediated, by the cells that are in close proximity to the lipoprotein particles. By using cultured cell systems, it has been shown that all of the above, including the macrophages, can stimulate the LDL oxidation under some circumstances, while in other cases they are able to limit the oxidative events. The mechanisms by which such cells may influence lipoprotein oxidation have been the subject of intense interest as potentially therapeutic targets. The macrophages are rich potential sources of oxygen-centerd free radicals, through the activities of myeloperoxidase (MPO) and the respiratory burst oxidase (RBO). However, while their products are capable of oxidizing LDL *in vitro*, macrophage-mediated oxidation of the LDL does not appear to depend on these systems. Furthermore, *in vivo*, lesion development was normal in mice deficient in phagocyte RBO or overexpressing extracellular superoxide dismutase and was actually enhanced in MPO-deficient mice. The macrophages also generate reactive nitrogen species capable of oxidizing the lipoproteins through the activities of inducible nitric oxide synthase (iNOS) or MPO. Stimulation of macrophage iNOS activity does not enhance the LDL oxidation. On the other hand, recent studies indicate that lesion development is attenuated in iNOS-deficient mice bred onto the atherosclerosis-susceptible apoE -/- strain. Whether this is related to lipoprotein oxidation or macrophage activity remains to be shown. No studies published to date have conclusively shown a role for lipoxygenase(s) (LO) in the pro-oxidant activity of the macrophages towards the LDL, although indirect contribution of the enzyme to macrophage-accelerated LDL oxidation has been proposed. 15-LO mRNA and the enzyme and specific lipid oxidation products have been found in atherosclerotic lesions, and atherosclerosis was reduced in 15-LO-deficient mice. On the other hand, overexpression of the enzyme in the macrophages actually protected transgenic rabbits against atherosclerosis.

3.2.2 Effects of OxLDL on macrophage proliferation and death

Local proliferation of the macrophages in the intima is thought to stimulate lesion progression. OxLDL is mitogenic for the macrophages, relative to sterol loading induced by AcLDL. However, the active component(s) and mechanism(s) of action are still uncertain. It was suggested that lysophosphatidylcholine (lyso-PC) is the active component of the oxLDL, since

generation of lyso-PC in AcLDL by treatment with PLA2 increased its mitogenic activity. However, lyso-PC alone is not mitogenic, and removal of lyso-PC from OxLDL does not affect its influence on macrophage proliferation (Martens *et al.*, 1999). Modifications of apoB during the LDL oxidation have been suggested to account for much of the growth-promoting activity of OxLDL. The suggestion that the OxLDL stimulates GM-CSF secretion, inducing autocrine growth stimulation, is not supported by studies using the macrophages from op/op, GM-CSF -/- double knockout mice (deficient in both CSF-1 and GM-CSF), which responded normally to growth induction by the OxLDL. While modest levels of the OxLDL stimulate macrophage survival and proliferation, there is agreement that high doses cause death. The toxic components of OxLDL are not well defined, but may include oxysterols. However, it seems unlikely that the concentration of heavily-oxidized LDL required to induce macrophage death is ever reached *in vivo*.

3.2.3. Effect of native LDL on foam cell formation

Previously, the macrophage foam cell formation was thought to occur only through uptake of modified forms of LDL such as oxidized or aggregated LDL and native LDL (Ma *et al.*, 2006; Kruth *et al.*, 2002). The native LDL can be taken up by a receptor in dependent fashion mediated by fluid-phase macropinocytosis, ingesting large amounts of the surrounding extracellular fluid (Ma *et al.*, 2003; Janeway *et al.*, 2005). When the macrophages are differentiated from human monocytes in human serum, activatior, phorbol 12-myristate 13-acetate (PMA), stimulates macrophage uptake of Fluid in macropinosomes and any LDL contained in these human macrophages, 125II-LDL uptake is linearly related to LDL concentration, does not show saturation at high LDL concentration, and cannot be competed by unlabeled LDL.

3.2.4 Effects of OxLDL on foam cell formation

As detailed above, heavy oxidation of the LDL enhances its uptake by the macrophages and has been implicated in the foam cell formation *in vivo*. The failure to detect the LDL oxidized to this degree in lipoproteins isolated from atherosclerotic lesions argues against this. Heavily-oxidized LDL is also fairly indigestible, so that the majority of sterols remain trapped within the lysosomal system, as oxidized cholesteryl and oxysteryl esters. This contrasts with foam cells in the developing fatty streak, where the majority of cholesterols are present as

unoxidized esters in the cytoplasm. However, as lesion development progresses, an increasing proportion of lipids can be seen associated with lysosomal structures in lesion foam cells, so that the LDL oxidation might contribute to lipid accumulation in the advancing lesion. Even under conditions where cholesterol accumulation is stimulated, most cells, including macrophages, are able to export the excessive cholesterols to extracellular acceptors such as high density lipoprotein (HDL) or to its major protein, apoA-1, preventing the foam cell formation. Cholesterol removal by apoA-1 involves in a membrane transporter, ABCA1. Mutations in the ABCA1 cause Tangier disease, a condition associated with accumulation of cholesterol in tissue macrophages, and a predisposition to atherosclerosis. In macrophages loaded with OxLDL, such cholesterol export is inhibited. This inhibition appears to depend both on (i) the inaccessibility of cholesterol trapped within lysosomes for export, and (ii) a direct inhibitory effect of the major oxysterol present in the LDL, 7K, on cholesterol export. Recent studies have shown that the ability of the 7K to block apoA-1-mediated removal of cholesterol from the macrophages depends partly on its capacity to prevent interaction of apoA-1 with the plasma membrane, the site at which cholesterol export is initiated. As this can be reproduced in artificial membranes, it appears that the 7K directly affects the membrane–apoA-1 interaction. However, the possibility that it also indirectly influences the distribution or activity of ABCA1 cannot be excluded. As the 7K is present in lesion foam cells at concentrations similar to those used in the above studies, it seems likely that impaired cholesterol efflux contributes to overall cholesterol accumulation *in vivo*.

4. Atherosclerosis and lipoproteins (Sherwood, 2004)

Atherosclerosis is the process which leads to the thickening of the artery walls and gives rise to the many pathology that cause cardio vascular disease as coronary heart disease. Atherosclerosis is progressive, degenerative arterial disease that gradually blocks the affected vessels, thus reducing blood flow through them. Progressive development of the atherosclerosis is in the following:

1. Atherosclerosis is believed to start with injury of the blood vessel wall, which triggers an inflammatory response that sets the stage for the build-up of plaque. Normally, inflammation is a protective response that fights infection and promotes repair of damage tissue. However, when the cause

of the injury persists within the vessel wall, the sustained, low grade inflammatory response over a course of decades can insidiously lead to arterial plaque formation and heart disease. Suspected artery-abusing agents that may set off the vascular inflammatory response included oxidized cholesterols, free radicals, high blood pressure, homocysteines, or even bacteria and viruses that damage blood vessel walls. The most common triggering agent appears to be the oxidized cholesterols.

2. Typically, the initial stage of atherosclerosis is characterized by the accumulation beneath the endothelium of excessive amounts of low-density lipoprotein (LDL), the so-called "bad" cholesterol, in combination with a protein carrier. As the LDL accumulates within the vessel wall, this cholesterol product becomes oxidized, primarily by oxidative wastes produced by the blood vessel cells. These wastes are free radicals, very unstable electron-deficient particles that are highly reactive. Antioxidant vitamins that prevent LDL oxidation, such as vitamin E, vitamin C, and beta-carotene, have been shown to slow plaque deposition.
3. In response to the presence of the oxidized LDL and/or other irritants, the endothelial cells produce chemicals that attract monocytes to the site. These immune cells trigger a local inflammatory response.
4. Once they leave the blood and enter the vessel wall, the monocytes settle down permanently, enlarge, and become large phagocytic cells called the macrophages. They actively phagocytize the oxidized LDL until these cells become so packed with fatty droplets that they appear foamy under a microscope. Now called the foam cells, these greatly engorged macrophages accumulate beneath the vessel lining and form a visible fatty streak, the earliest form of an atherosclerotic plaque.
5. The earliest stage of plaque formation is, therefore, characterized by the accumulation beneath the endothelium of a cholesterol-rich deposit. The disease progresses as smooth muscle cells within the blood vessel wall migrate from the muscular layer of the blood vessel to a position on top of the lipid accumulation, just beneath the endothelium. The migration is triggered by chemical release at the inflammatory site. At their new location, the smooth muscle cells, continue to divide and enlarge,

producing atheromas, which are benign (non-cancerous) tumors smooth muscle cells within the blood vessel walls. Together, the lipid-rich core and overlying smooth muscle form a maturing plaque.

6. The plaque progressively bulges into the lumen of the vessel as it continues to develop. The protruding plaque narrows the opening through which blood can flow.
7. Further contributing to vessel narrowing, the oxidized LDL inhibits the release of nitric oxide from the endothelial cells. The nitric oxide is a local chemical messenger that causes relaxation of the underlying layer of normal smooth-muscle cells within the vessel wall. The relaxation of these smooth muscle cells causes the vessel to dilate. Because of reduced nitric oxide release, vessels damaged by developing plaques cannot dilate as readily as normal.
8. The thickening plaque also interferes with nutrient exchange for the cells located within the involved arterial wall, leading to degeneration of the wall in the vicinity of the plaque. The damaged area is invaded by fibroblasts (scar-forming cells), which form a connective tissue cap over the plaque.
9. In the later stages of the disease, Ca^{2+} often precipitates in the plaque. A vessel so afflicted becomes hard and poorly distensible.

5. Hemin and Lipoprotein oxidation

In models of lipoprotein oxidation, it has been found that the heavy metals were used as a mediator to induce oxidation. The lipoproteins could be oxidized not only by copper and iron, but also by hemin (figure 2.3) (Camejo *et al.*, 1998; Matthews *et al.*, 1997; Phumala *et al.*, 2003; Wang *et al.*, 2006). Under normal conditions, hemin and hemoglobin released into the plasma from red blood cells at different sites of the vascular bed are removed by binding to hemopexin and haptoglobin, respectively. However, when hemalysis and/or intramural microhemorrhage occurs and RBCs are captured at sites of vascular turbence as in the case of individuals suffering of atherosclerosis, excessive heme and hemoglobin are released into serum. The heme is oxidized and rapidly converted into hemin (Tsiftsoglou *et al.*, 2006).

In thalassemic patients, the hemin could be detected at 50-280 μM in the blood. These iron complexes may induce oxidation of several biological molecules, including the LDL and the HDL, which increases atherosclerosis risk in the patients (Livea *et al.*, 1998; Morales *et al.*, 2006). It has been shown that the atherosclerosis-related vascular complications could be found in these patients. In addition, vascular dysfunction with endothelial dysfunction has been demonstrated in these patients. Hemin-oxidized lipoproteins may generate the atherosclerosis-related vascular complication in the thalassemic patients (Cheung *et al.*, 2006; Panigrahi and Agarnial, 2007; Sonakul *et al.*, 1998; Wong *et al.*, 1990; Pascher *et al.*, 1957).

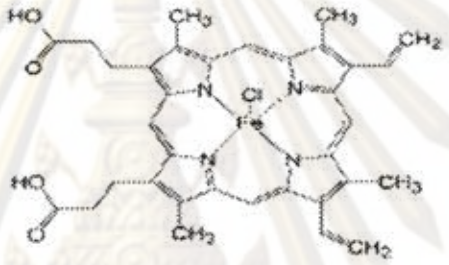


Figure 2.3 The chemical structure of hemin

6. Deferiprone

Deferiprone (1,2-dimethy-3-hydroxypyridin-4-one; L1,CP20) is the first oral iron-chelating drug used in thalassaemia and other iron-loaded patients. Progress in its development has been very slow because it was mostly undertaken through research-orientated projects and supported by non-profit establishments. Pharmaceutical companies only showed an interest on the L1 after it became clear that it would be a profitable financial venture. It is estimated that more than 6,000 patients in 40 countries have received the L1 since the initiation of clinical trials. There are groups of patients in India, Switzerland, and Cyprus who have been taking the L1 daily since 1989. India was the first country where the L1 has been registered in 1994 and now there are more patients receiving the L1 than those receiving deferoxamine (DF). The L1 has also been registered in Europe in 1999, following the recommendation by the EC CPMP that its use is restricted to patients not tolerating or using DF and also that it is closely monitored for toxicity. Registration of the L1 is pending in many other countries. The L1 is a white crystalline solid offered to

patients as capsules or as tablets. It is very stable at room temperature and in solution of physiological or acidic pH and forms red color complexes with iron, similar to the red color of the urine of patients treated with it. Its affinity for iron is higher than that for Cu, Al, Zn and other metals. Three molecules of the L1 are needed to bind one molecule of iron at physiological pH in contrast to DF which binds the iron at 1:1 molar ratio. The L1 is a neutral molecule forming a neutral complex with the iron, whereas the DF is positively charged and forms a positively-charged complex with the iron at the physiological pH. At low concentrations (10^{-6} M) the L1-iron complex is less stable than that of DF and the labile complexes of L1 with the iron or copper may promote the formation of toxic oxygen-activated species. Both L1 and DF are hydrophilic and, unlike lipophilic chelators, they do not accumulate in lipids, for example in cell membranes or in the brain. The L1 is about 10 times more lipophilic than the DF and may be easier to diffuse through the cell membranes than the DF unless other forms of membrane transport are involved.

Many centers have reported the results of clinical trials since the first report of the iron removal effects of the L1 in transfusional iron loaded patients in 1987, all confirming the original findings that the L1 is an effective, non-toxic, orally active chelator. Overall, the L1 has so far been assessed in three different therapeutic areas of medicine, mainly for iron removal from iron-loaded patients, aluminium removal from renal dialysis patients and in rheumatoid arthritic patients where it has been used for reducing infammation and for correcting the anemia of chronic diseases. Iron excretion by the L1 in humans depends on the dose and frequency of administration of the drug and the iron loading and rate of iron loading of the individual. Doses of a total of 50-120 mg/kg/day are currently being used which are administered in 2- 4 divided doses of 25-50 mg/kg. Doses as low as 10 mg/kg are sufficient at increasing urinary iron excretion in patients with relatively low serum ferritin levels (0.9-1.7 mg/L). Typical doses of 50-100 mg/kg of the L1 can result in the excretion of 10-120 mg iron in patients with serum ferritin of 3-12 mg/l. By comparison, a dose of about 50 mg/kg in normal individuals could cause much lower iron excretion (1-2 mg). Intensive chelation using escalating doses of the L1 further increases iron excretion. In one thalassaemic patient of 67 kg with serum ferritin of 8 mg/l repeated administration of 5x2 g and 2x3 g within 24 h resulted in the excretion of 325 mg, which is equivalent to a 13-day intake of iron from transfusions. In general, there is

variation among patients in iron excretion and sometimes the same patients, when given the same dose, respond differently.

Some patients can excrete more iron when given high single doses over 24 h, whereas others excrete more iron when smaller doses are administered more frequently over the same period. The factors causing such variations are unknown but the same result is observed during DF therapy. Dietary components, vitamin C status, erythropoietic activity, rate and extent of chelator biotransformation and the presence of labile low molecular weight iron could be few of many such factors influencing iron excretion by the L1 (Kontoghiorghes *et al.*, 2000).

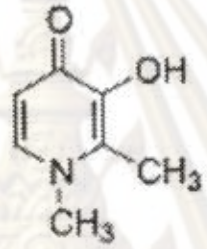


Figure 2.4 The chemical structure of deferiprone

L1 is a small neutral water-soluble molecule that forms a strong 3:1 complex with Fe^{3+}. L1 has been shown to as antioxidant in several models L1 may protect against doxorubicin-induced damage to myocytes by displacing iron bound to doxorubicin,or chelating free or loosely bound iron, thus preventing site-specific iron-based oxygen radical damage (Barnabae *et al.*, 2002). This suggests that [deferiprone:Fe^{3+}] complex is reduced by a ferrireductase present on the hepatocyte membrane to form [deferiprone:Fe^{2+}] complex, which then scavenges superoxide radicals. Therefore, the clinically used deferiprone (L1) may have therapeutic advantages over deferoxamine in having a double role therapeutically: (a) it chelates iron to alleviate iron overload pathology, and (b) L1 may have the readily formed iron complex protects hepatocytes from superoxide radical-mediated hypoxia-reoxygenation injury (Moridani, 2001).

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Chemicals

All chemicals were obtained commercially and without further purification. The following chemicals were purchased from Sigma Chemical Co., St Louis, U.S.A.: Standard free cholesterol, cholesteryl arachidonate, cholesteryl linoleate, cholesteryl oleate, cholesteryl palmitate, standard bovine albumin, α-tocopherol, ethylenediamine tetraacetic acid disodium salt (EDTA), 2-thibarbituric acid, trichloroacetic acid (TBA), sodium dodecyl sulfate (SDS), sodium hydrogenphosphate, sodium dihydrogenphosphate, folin Phenol reagent, phorbol, myristate acetate (PMA), 2, 5-diphenyltetrazolium bromide (MTT), nitro blue tetrazolium (NBT).

Potassium bromide, sodium chloride, sodium hydroxide, and sodium potassium tetrahydrate were purchased from E. Merck, Darmstadt, Germany. Deferiprone was kindly provided by Dr. Chada Phisalaphong, Government Pharmaceutical Organization.

1.2 Instruments

The following items were major equipments used in this study

1. Beckman ultracentrifuge (XL-100, USA) with fixed angle rotor (100 Ti)
2. Centrifuge (Allegra X-12R Beckman Coulter)
3. Spectrofluorometer (UV-160A SHIMASU)
4. Spectrophotometer (FP-777 JASCO)
5. High Performance Liquid Chromatography (HPLC; LC 10 SHIMASU)
6. Microplate reader (Anthos htl)
7. Laminar flow (ISSCO LAMINAR FLOW MODEL)
8. Incubator (Forma Scientific CO_2 water Jacketed incubator)

2. Methods

2.1 Plasma preparation

Blood from healthy overnight fasting volunteers were recruited, all gave their informed consent. None of the subjects were on drug therapy or were taking

upplementary antioxidants for least 2 weeks. After an overnight fast, blood was taken in heparin-containing tubes (10 I.U. /ml blood). Plasma was obtained by centrifugation at 3,250 rpm for 15 min at 4 °C, then stored at -80 °C.

2.2 Isolation of LDL and HDL

LDL and HDL were isolated from the plasma and using density gradient ultracentrifugation in fixed angle rotor (Beckman 100Ti) at 95,000 rpm (802,000g) at 10 °C. The plasma was centrifuged sequentially at densities of 1.019, 1.063, and 1.20 to separate lipoproteins. The first fraction ($d<1.019$), obtained by centrifugation for 4 h contains chylomicrons and VLDL, the second fraction ($1.019<d<1.063$) obtained by centrifugation for 4 h 40 min contained LDL, and the third fraction ($1.063<d<1.20$) obtained by centrifugation for 8 h contained HDL (Havel *et al.*, 1995). Isolated LDL and HDL was dialyzed against 10 mM PBS pH 7.4 for 12 h to remove salts and used immediately.

2.3 Protein determination

Protein concentration was determined by the modification of Lowry method using bovine albumin serum as standard (BSA) (Markwell *et al.*, 1978). The reagents were used to determine LDL and HDL protein concentrations, where 1 part Reagent B (4 % (w/v) $CuSO_4$) was mixed to 100 parts of Reagent A (containing Na_2CO_3, KNa tartrate, SDS, sodium tartrate in 4% NaOH). Folin Phenol reagent is added to the mixer and the protein concentration, in µg/ml, was determined by comparison to BSA protein standard curve.

2.4 Hemin-induced lipoproteins oxidation

Oxidation of lipoprotein was carried out at 37 °C under air in a shaking water bath. LDL and HDL 300 µg/ml was incubated with or without deferiprone (L1) (0-500 µM) for 0, 9, and 24 h in the presence of 5 µM hemin. Ten mM Phosphate buffer saline (PBS) pH 7.4 was presented as solvent control. Oxidation was terminated by the addition of EDTA and BHT with final concentration 100 µM and 5 mM, respectively.

2.5 Measurement of oxidation parameters of modified lipoproteins

Thiobarbituric reactive substances (TBARs)

Lipid peroxidation, the oxidative breakdown of polyunsaturated fatty acids, is widely accepted as a general mechanism for cellular injury and death. Lipid peroxidation has been implicated in diverse pathological conditions, including atherosclerosis, aging and cancer associated with certain solvents, drugs and metals. Lipid peroxidation is known to generate reactive aldehydes such as 4-hydroxy-2-nonenal and malondialdehyde (MDA). In this test, the chromogen is formed by the reaction of one molecule of MDA with two molecules of TBA. The method involves heating the sample in the presence of TBA under acidic conditions; the MDA-TBA adduct formed.

Lipid peroxidation was determined by quantification of TBARs. TBARs, mainly malondialdehyde (MDA) were assayed by adding modified LDL and HDL with 100 mM BHT, 10% TCA, 5 mM EDTA, 8% SDS and 0.6% TBA in test tube. The reaction tubes were incubated at 100 °C for 1 h. The absorbencies of the mixer solution were measured in a spectrofluorometer at an excitation wavelength of 515 nm and an emission wavelength of 553 nm. TBARs were expressed as nmol malondialdehyde (MDA) by using tetramethoxypropane as a standard (Asakawa and Matsushita, 1980). Percentage of inhibition of TBARs formation was calculated by the following equation.

$$\% \text{ inhibition} = \frac{[MDA_{oxidized} - MDA_{native}] - [MDA_{oxidized\text{-}L1} - MDA_{native}]}{[MDA_{oxidized} - MDA_{native}]} \times 100$$

Level of α-tocopherol and lipid conpositions of modified LDL and HDL

Modified LDL and HDL were extracted with cold methanol followed by hexane. Samples were vortexed, then centrifuged at 1,700 rpm at 4 °C for 5 min. Part of hexane layer was removed, dried under N_2, and re-dissolved in mobile phase, acetonitrile/isopropanol (75:25, v/v) and transferred to HPLC vials. High Liquid Chromatography (HPLC) was nmol to measure level of α-tocopherol and lipid compositions.

Lipoprotein endogenous vitamin E was assayed as α-tocopherol by reversed-phase-HPLC, and UV at wavelength of 292 nm. α-Tocopherol was assayed on hypersil BDS C18 column with isocratic acetonitrile/isopropanol (75:25, v/v) at a

flow rate of 1.2ml/min. The column temperature was controlled at 50 °C. (Seta *et al.*, 1990). The retention time of α-Tocopherol is 5.154 min.

Lipid compositions of LDL and HDL were such as free cholesterol (FC), cholesteryl arachidonate (CA), cholesteryl linoleate (CL), cholesteryl oleate (CO), and cholesteryl palmitate(CP) were determined. Level of lipid compositions were assayed by reversed-phase- HPLC with UV monitor at 210 nm for free cholesterol and cholesteryl esters on hypersil BDS C18 column with isocratic acetonitrile/isopropanol (75:25, v/v) at a flow rate of 1.2ml/min. The column temperature was controlled at 50 °C (Seta *et al.*, 1990). The retention time of each lipid is 7.268, 18.847, 23.623, 30.415, and 32.189 min at for FC, CA, CL, CO, and CP, respectively.

2.6 Cell culture

The human monocyte U937 were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin. Cells were cultured at 1×10^4 cell/ml in T25 and T75 tissue culture flask. Cell cultures were maintained and incubated in at 37 C°, 97 % humidity, and 5 % CO_2.

2.7 U937 monocyte-derived macrophage

U937 cells (2×10^5 cell/ml) in RPMI 1640 medium containing 10% FBS and 1% penicillin and streptomycin were differentiated to macrophage by addition of 100 ng/ml PMA in 24 and 12 well plate for 72 h at 37 C°, 97 % humidity, and 5 % CO_2. When U937 cells were induced to differentiation into macrophage, the cells were washed three times with PBS.

2.8 Cytotoxicity assay (Manosroi *et al.*, 2003)

The cytotoxicity effect of modified LDL on U937 monocyte-derived macrophage was determined by tetrazolium salt reduction, as an indicator of cell growth. The yellow tetrazolium salts MTT is reduced by metabolically active cell, in part by the action of dehydrogenase enzyme, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan in nonsoluble in water and can be solubilized with DMSO.

U937 monocyte-derived macrophage in 24 well plates was added with LDL (30 μg/ml) at time 24 h of incubation. The cells were maintained in serum-free medium and then incubated 24 hr at 37 C°, 97 % humidity, and 5 % CO_2.The medium were removed off and added 10 μl/well of MTT solution (400 μg/ml) in the plate then incubate the cells at 37 C°, 97 % humidity, 5% CO_2 for 4 h. The MTT solution was removed and added 1 ml of DMSO. Of this 200 μl was placed in 96 well plates and measured the optical density (OD) of the plate at 570 and 650 nm in a microplate reader. Cytotoxicity of modified LDL was determined by calculate as % cytotoxicity of modified LDL which compared with negative control.

$$\%\text{cytotoxicity} = \frac{\left[OD_{\text{negative control}} - OD_{\text{sample}}\right]}{OD_{\text{negative control}}} \times 100$$

2.9 Phagocytotic assay (Manosroi *et al.*, 2003)

Phagocytosis is a key defence mechanism which, in multicellar animals, allows the entrapment and ingestion of invading potentially pathogenic foreign particles. It involves three consecutive steps, namely recognition, engulfment, and digestion, and is associated with a series of biochemical events such as the increased oxidative metabolism, nitric oxide synthase activation, and excretion of hydrolytic enzymes, leading to the production and release into the extracellular medium of microbicidal products (Ballarin *et al.* 1997).

Nitroblue tetrazolium reduction assay is an indirect measurement of the respiratory burst by the macrophage. Superoxide anions produced by macrophage have the ability to reduce NBT to dark blue formazan (Brekalo *et al.* 2006). U937 monocyte–derived macrophage was added with 80 μl of LDL at time 24 h of incubation (30 μg/ml) in 24 well plate, containing serum-free medium. Afterwards, it was added 80 μl/well of NBT solution (400 μg/ml) and incubated for 1 h at 37 C°, 97 % humidity, and 5 % CO_2. The supernatant was pipetted off and washed four times with 800 μl methanol. The cell pellets were air dried. Amount of 240 μl of 2 M KOH and 280 μl of DMSO were added respectively. Of this 200 μl was pipetted in 96 well-plate and measurd at 570 nm.

The absorbance was used in the following equation to determine percentage of phagocytosis.

$$\%\text{phagocytosis} = \frac{\left[OD_{\text{negative control}} - OD_{\text{sample}}\right]}{OD_{\text{negative control}}} \times 100$$

2.10 Measurement of macrophage foam cell formation

Measurement of free and total cholesteryl esters (Bolton *el al.*, 1997; Seta *et al.*, 1990)

The LDL (30 µg/ml) at time 24 h of incubation was added to U937 monocyte-derived macrophage in serum-free medium in 12 well plates, incubated for 24 h at 37 °C, 97 % humidity, and 5 % CO_2. The cells were washed three times in PBS. Free cholesterol, cholesterol esters were extracted from individual cell cultures. Cells were lysed with 0.6 ml 0.2 M NaOH on ice, then mixed with 0.2 ml PBS, then 0.5 ml cold methanol follow by 2.5 ml of hexane. Samples were vortexed after each extraction, then centrifuged at 1,700 rpm at 4 °C for 4 min two ml of hexane layer was removed, evaporated under vacuum, and then re-dissolved in 200 µl of HPLC mobile phase. Of this, 50 µl was injected onto a reverse-phase C-18 column. Analysis of cholesterol and cholesteryl esters was detected at 210 nm with mobile phase containing 75% (v/v) acetonitrile and 25% (v/v) isopropanol. Free cholesterol and cholesteryl ester chromatographic peaks were changed to µg lipid /mg cell protein (using total cholesterol = free cholesterol + cholesteryl esters) and total cell protein was determined by modified Lowry method. Results were expressed in percent of total cholesterol.

Foam cell assay (Deng *et al.*, 2005)

U937-derived macrophages were incubated in serum-free medium in the presence of The LDL (30 µg/ml) at time 24 h of incubation for 24 h. The cells were washed three times with PBS, fixed by 10% formalin in PBS for 1 h. at room temperature, and then stained with 1% Oil Red O in 60% isopropanol for 3 h and washed three with water, and vaporized all water. The cells were examined at ×4 magnification on microscope.

CHAPTER IV

RESULTS

1. The effect of L1 on lipid peroxidation in hemin induced oxidation of LDL and HDL

To evaluate the oxidation stage of LDL induced by hemin, LDL 300 μg/ml were incubated with hemin 5 μM for 9 and 24 h. The MDA level of treated LDL was measured compared with non-treated LDL. From table 4.1 and figure 4.1 MDA level of oxidized LDL (Hemin-oxLDL) significantly increased ($P<0.05$) from native LDL at time 9 and 24 hr of incubation. A similar pattern was observed in oxidized HDL (HDL 300 μg/ml treated with hemin 5 μM) as shown in table 4.3 and figure 4.2. However, the MDA level of oxidized HDL was less increased than that of oxidized LDL and occurred in later time of incubation. After treated HDL with L1, the lipid peroxidation determined as the level of MDA was reduced at time 9 and 24 h of incubation. However, L1 could not completely inhibit lipid peroxidation of LDL induced by hemin.

Table 4.1 MDA level of hemin induced oxidation of LDL in the presence of L1 at time 0, 9, and 24 h of incubation

LDL	MDA (nmol/mg protein)		
	0 h	9 h	24 h
Native LDL	2.4±0.2	2.3±0.2 [a]	2.6±0.4 [a]
Hemin-oxLDL	3.3±1.0	18.8±0.8 [c]	18.6±0.2 [c]
Hemin-oxLDL + L1 5 μM	2.6±.0.1	11.0±4.4 [b]	8.5±1.8 [b]
Hemin-oxLDL + L1 10 μM	2.4±0.2	7.0±0.9 [ab]	8.8±1.3 [b]
Hemin-oxLDL + L1 50 μM	2.4±0.2	6.5±0.7 [ab]	6.9±0.2 [b]
Hemin-oxLDL + L1 100 μM	3.2±.0.4	5.2±0.6 [ab]	6.7±0.5 [b]
Hemin-oxLDL + L1 500 μM	2.7±0.2	5.9±0.4 [ab]	6.9±0.4 [b]

Results are expressed as mean±S.E.M. (n=3).

a, b and c showed different within the same time statistically at $P<0.05$.

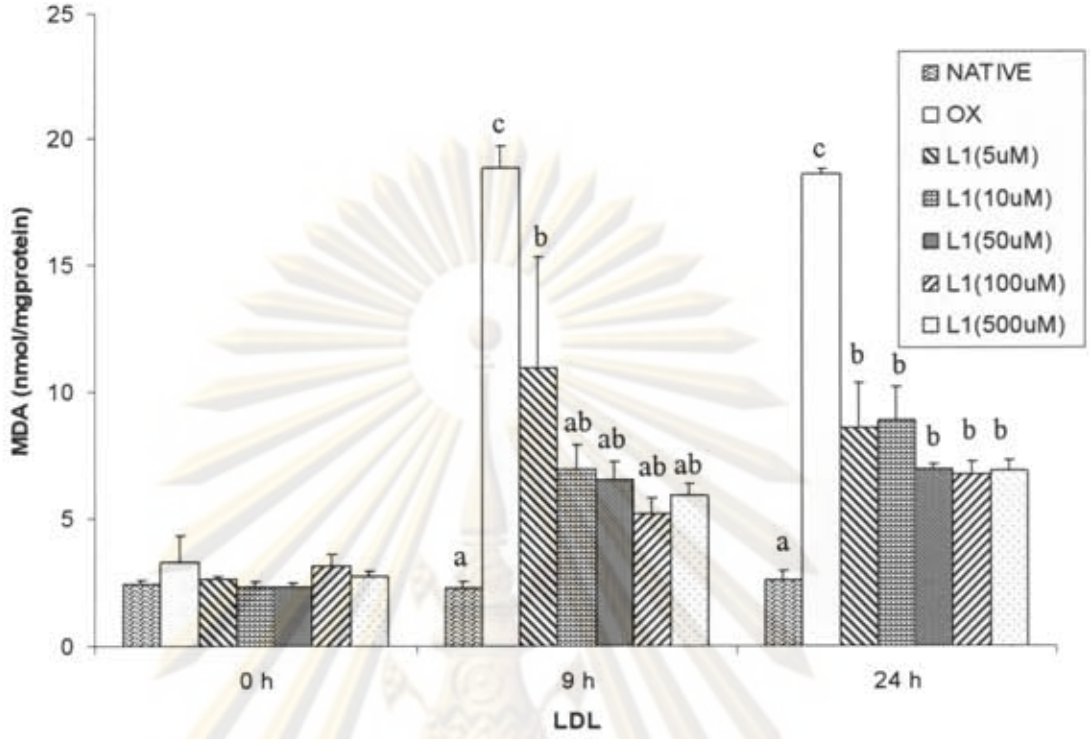


Figure 4.1 Effect of L1 (5-500 μM) on MDA formation during hemin induced oxidation of LDL at time 0, 9, and 24 h of incubation (n=3)

a, b and c showed different within the same time statistically at $P<0.05$.

Table 4.2 %inhibition of MDA level in hemin-oxidized LDL at the presence of L1 at time 9, and 24 h of incubation

	%inhibiton	
LDL	9 h	24 h
Hemin-oxLDL + L1 5 μM	49.4±25.0	62.8±10.0
Hemin-oxLDL + L1 10 μM	73.4±3.7	62.6±6.9
Hemin-oxLDL + L1 50 μM	75.9±1.6	73.0±1.8
Hemin-oxLDL + L1 100 μM	83.9±2.6	74.3±2.7
Hemin-oxLDL + L1 500 μM	79.4±1.4	73.4±3.6

Results are expressed as mean±S.E.M. (n=3).

Table 4.3 MDA level of hemin induced oxidation of HDL in the presence of L1 at time 0, 9, and 24 h of incubation

HDL	MDA (nmol/mg protein)								
	0 h			9 h			24 h		
	1	2	mean	1	2	mean	1	2	mean
Native HDL	2.5	2.3	2.4	2.9	2.0	2.4	2.7	2.3	2.5
Hemin-oxHDL	3.1	2.3	2.7	6.9	2.8	4.9	7.9	3.1	5.5
Hemin-oxHDL + L1 5 μM	3.5	2.3	2.9	2.8	4.1	3.5	2.9	2.8	2.9
Hemin-oxHDL + L1 10 μM	2.8	2.2	2.5	3.2	2.6	2.9	2.8	2.8	2.8
Hemin-oxHDL + L1 50 μM	2.4	2.2	2.3	3.2	2.6	2.9	3.2	3.0	3.1
Hemin-oxHDL + L1 100 μM	3.4	2.4	2.9	3.5	2.4	3.0	3.2	2.8	3.0
Hemin-oxHDL + L1 500 μM	3.9	2.6	3.3	2.9	2.6	2.7	3.4	2.7	3.1

Results are expressed as mean (n=2).

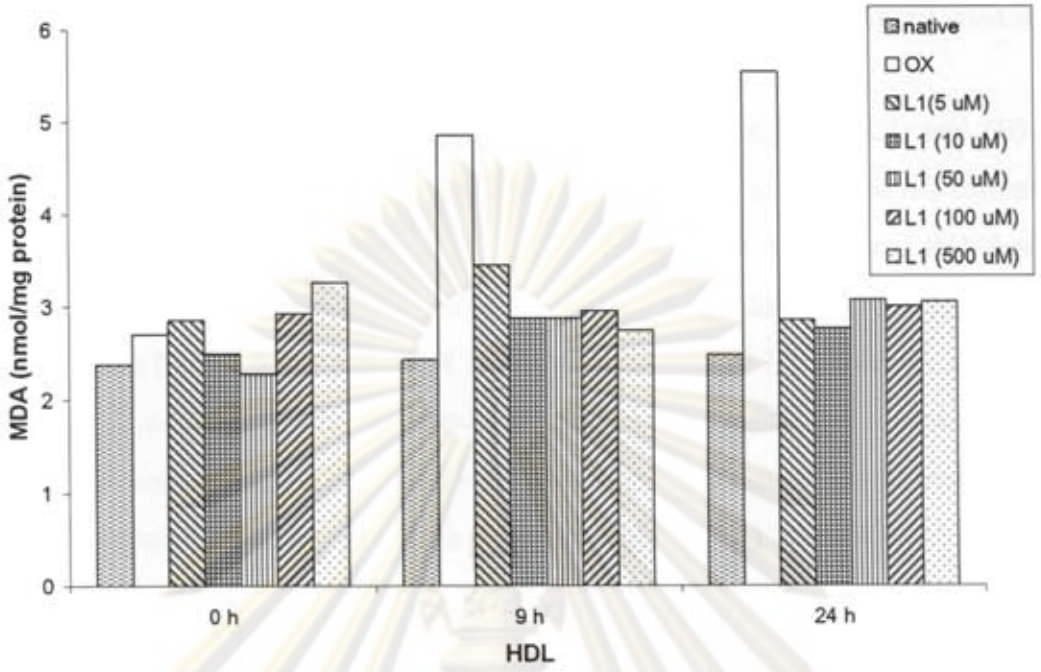


Figure 4.2 Effect of L1 (5-500 μM) on MDA formation during hemin induced oxidation of HDL at time 0, 9, and 24 h of incubation (n=2)

Table 4.4 %inhibition of MDA level in hemin-oxidized HDL at the presence of L1 at time 9, and 24 h of incubation

	%inhibition					
	9 h			24 h		
HDL	1	2	mean	1	2	mean
Hemin-oxHDL + L1 5 μM	110.2	97.0	103.6	92.8	97.64	95.2
Hemin-oxHDL + L1 10 μM	94.3	91.8	93.0	96.9	97.62	97.3
Hemin-oxHDL + L1 50 μM	94.3	91.5	92.9	89.9	90.41	90.2
Hemin-oxHDL + L1 100 μM	84.5	85.9	85.2	88.9	90.11	89.5
Hemin-oxHDL + L1 500 μM	97.1	99.2	98.1	88.4	84.27	86.4

Results are expressed as mean (n=2).

2. The effect of L1 on α-tocopherol preservation in hemin induced oxidation of LDL and HDL

Since α-tocopherol is the major antioxidant found in both LDL and HDL, the determination of α-tocopherol level should be measured to evaluate the oxidation of LDL and HDL induced by hemin. Table 4.5 and figure 4.3 showed that there was significant disappearance of α-tocopherol in hemin-oxidized LDL when compared with native LDL at time 9 and 24 h of incubation. The L1 could not preserve the amount of α-tocopherol in hemin-induced LDL oxidation. The α-tocopherol level in HDL was not significantly reduced by 5 μM of hemin at time 9 and 24 h of incubation when compared with native HDL as shown in table 4.6 and figure 4.4.

Table 4.5 α-tocopherol level of hemin induced oxidation of LDL in the presence of L1 at time 0, 9, and 24 h of incubation

LDL	α-tocopherol (nmol/mg protien)		
	0 h	9 h	24 h
Native LDL	11.5±0.6	8.8±0.3 [a]	10.2±1.6 [a]
Hemin-oxLDL	9.4±2.0	2.2±1.3 [b]	1.1±1.1 [b]
Hemin-ox LDL + L1 5 μM	11.0±1.1	0.7±0.7 [c]	0.7±0.7 [b]
Hemin-ox LDL + L1 10 μM	11.0±1.8	2.0±0.1 [b]	0.7±0.7 [b]
Hemin-ox LDL + L1 50 μM	9.3±2.1	2.1±0.1 [b]	1.1±1.1 [b]
Hemin-ox LDL + L1 100 μM	7.3±2.3	3.2±0.1 [b]	1.8±1.0 [b]
Hemin-ox LDL + L1 500 μM	10.6±1.4	2.8±0.3 [b]	1.9±1.1 [b]

Results are expressed as mean±S.E.M. (n=3).

a, b and c showed different within the same time statistically at $P<0.05$.

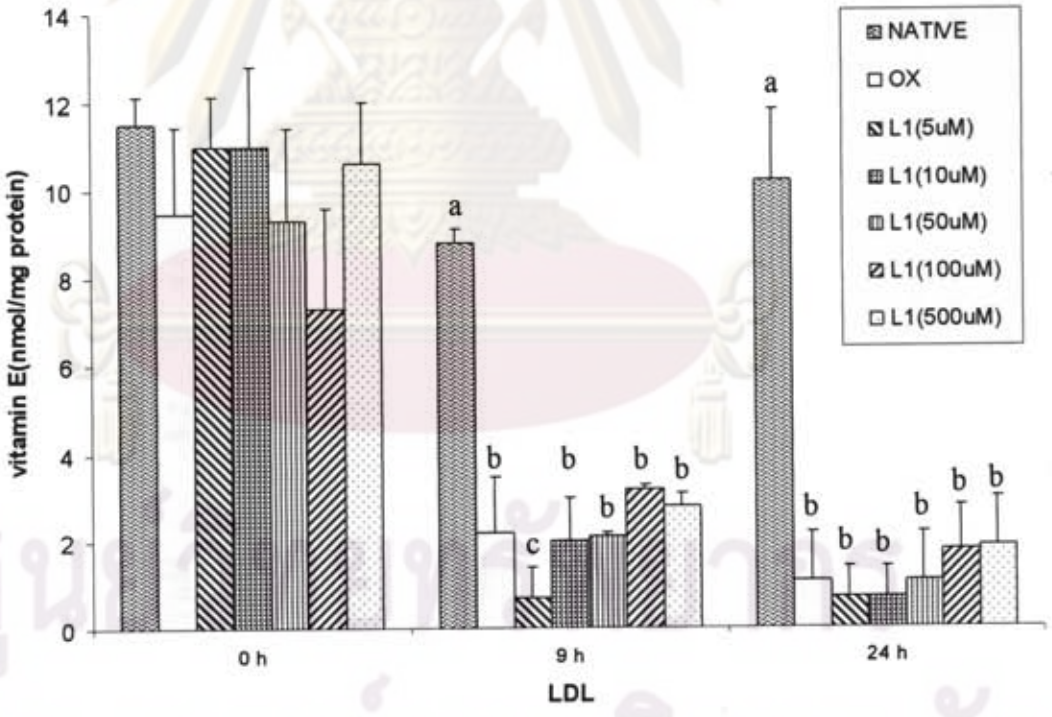


Figure 4.3 Effect of L1 (5-500 μM) on depletion of α-tocopherol during hemin induced oxidation of LDL at time 0, 9, and 24 h of incubation (n=3)

a, b and c showed different within the same time statistically at $P<0.05$.

Table 4.6 α-tocopherol level of hemin induced oxidation of HDL in the presence of L1 at time 0, 9, and 24 h of incubation

HDL	α-tocopherol (nmol/mg protien)								
	0 h			9 h			24 h		
	1	2	mean	1	2	mean	1	2	mean
Native HDL	9.0	4.1	6.6	6.7	4.4	5.6	5.3	4.8	5.0
Hemin-oxHDL	9.0	4.9	6.9	7.5	0.0	3.7	5.2	0.8	3.0
Hemin-oxHDL + L1 5 μM	8.2	4.6	6.4	4.3	2.3	3.3	3.6	1.8	2.7
Hemin-oxHDL + L1 10 μM	7.0	3.6	5.3	4.3	3.1	3.7	3.6	1.9	2.8
Hemin-oxHDL + L1 50 μM	6.3	3.3	4.8	4.4	2.9	3.6	3.8	1.9	2.8
Hemin-oxHDL + L1 100 μM	7.3	5.3	6.3	5.3	2.7	4.0	3.7	1.8	2.7
Hemin-oxHDL + L1 500 μM	7.2	4.2	5.7	11.4	2.1	6.8	3.4	2.1	2.7

Results are expressed as mean. (n=2).

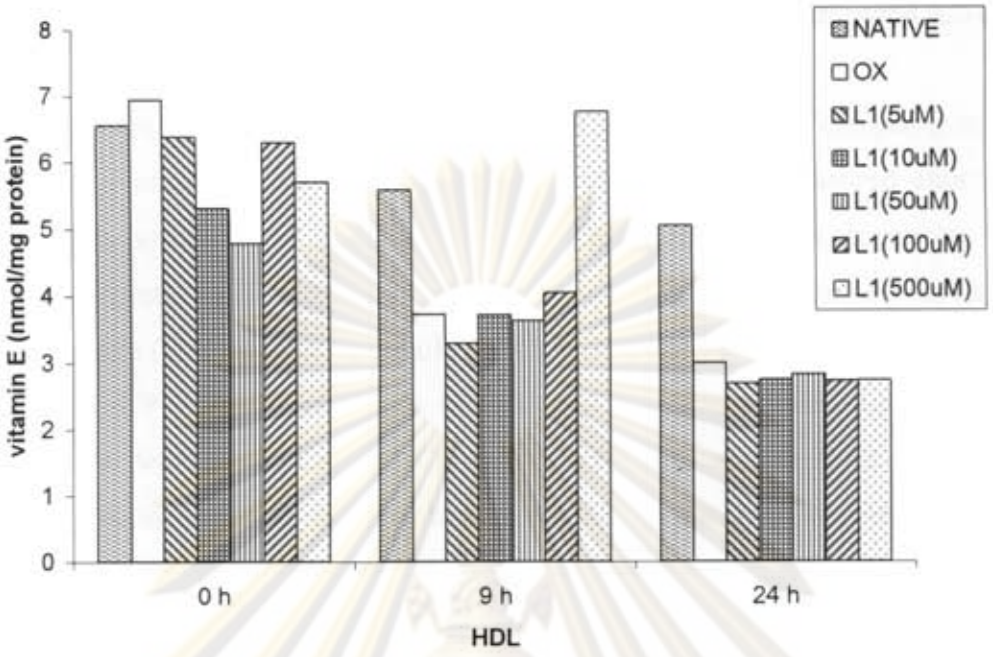


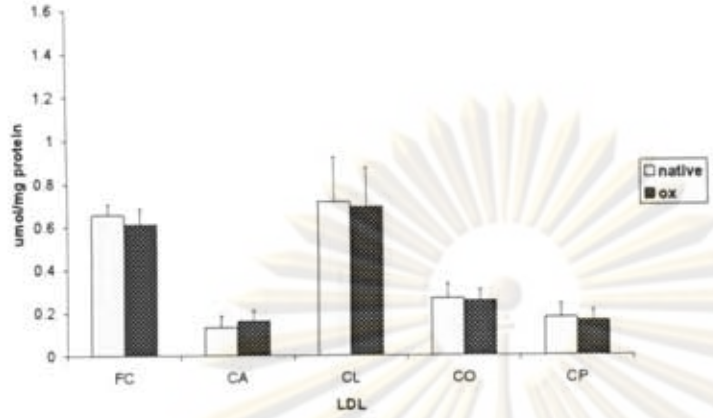
Figure 4.4 Effect of L1 (5-500 μM) on depletion of α-tocopherol during hemin induced oxidation of HDL at time 0, 9, and 24 h of incubation (n=2)

3. The effect of L1 on lipid composition change of hemin induced LDL and HDL oxidation

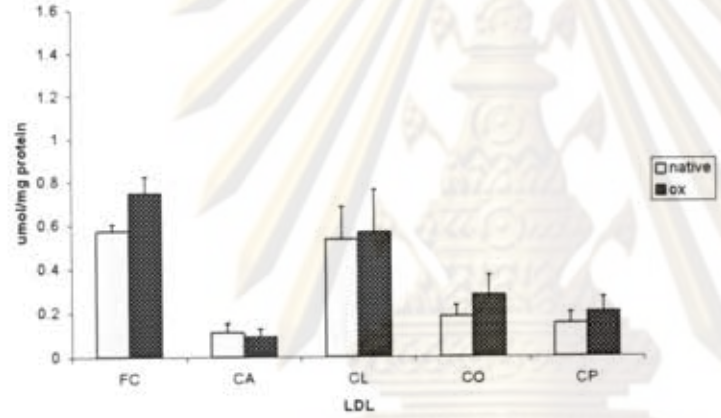
Oxidation of lipoproteins could cause the change of lipid composition (mainly cholesteryl esters). The change of cholesteryl esters was measured after incubate LDL with hemin 5 μM for 9 and 24 h. This interest lipid composition of LDL cholesteryl esters were free cholesterol (FC), and cholesteryl esters such as cholesteryl arachidonate (CA), cholesteryl linoleate (CL), cholesteryl oleate (CO), and cholesteryl palmitate (CP). The result shown in figure 4.5 (C) demonstrated that there were the significant decrease of CA and CL in oxidized LDL at time 24 h compared with native LDL measured at the same time ($P<0.05$). Exposure of various concentrations of L1 to oxidized LDL did not protect the decrease level of CA and CL, except the highest concentration of L1 (figure 4.7 (C)). The 500 μM of L1 could significantly preserve the level of CA and CL at time 24 h of incubation ($P<0.05$).

However, the cholesteryl esters were completely disappeared at time 24 h in both oxidized-and native HDL, while the level of FC was increased as shown in figure 4.8 (C). Oxidized HDL treated with 100 and 500 μM of L1 was able to protect the decrease of CA and CL from oxidation at time 24 h of incubation.

(A) 0 h



(B) 9 h



(C) 24 h

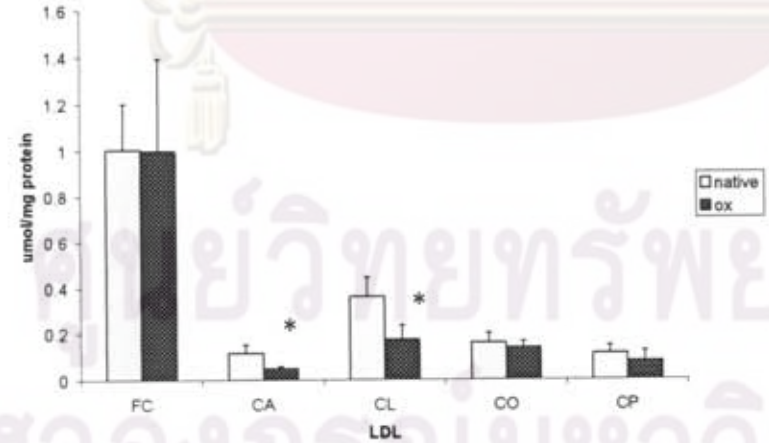
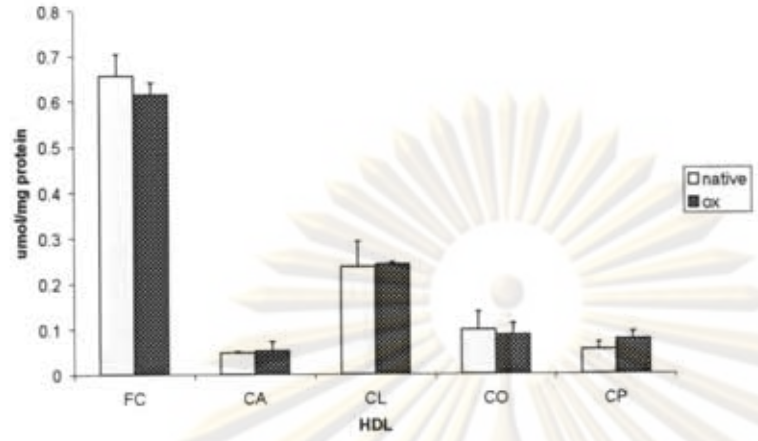


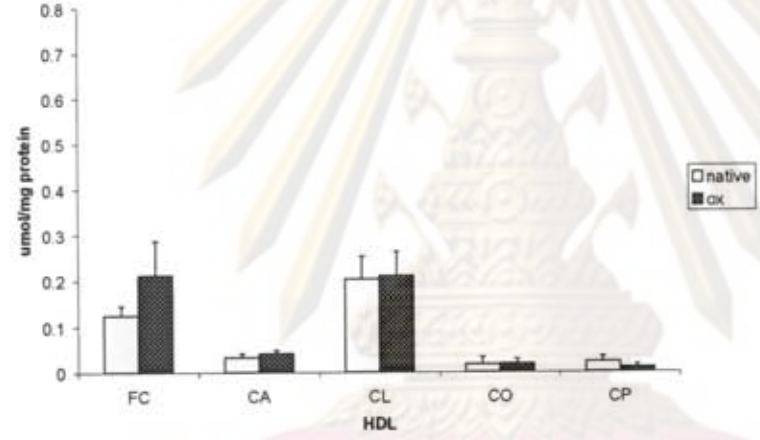
Figure 4.5 Lipid composition of hemin induced oxidation of LDL at time 0 h (A), 9 h (B), and 24 h (C) of incubation (n=3)

* It was significantly different at $P<0.05$ (compare with native LDL)

(A) 0 h



(B) 9 h



(C) 24 h

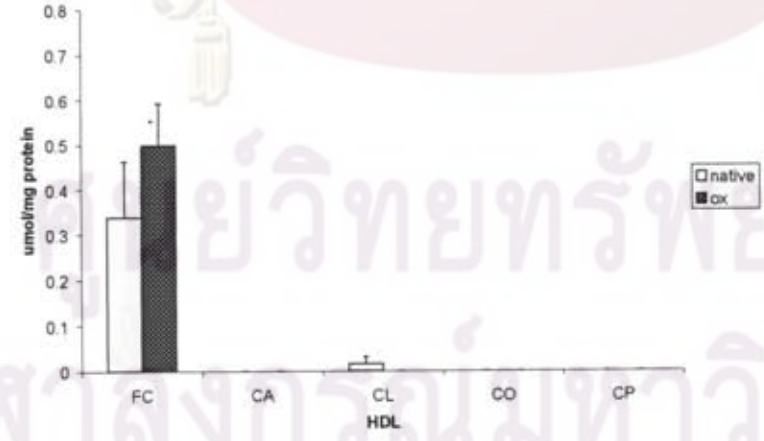
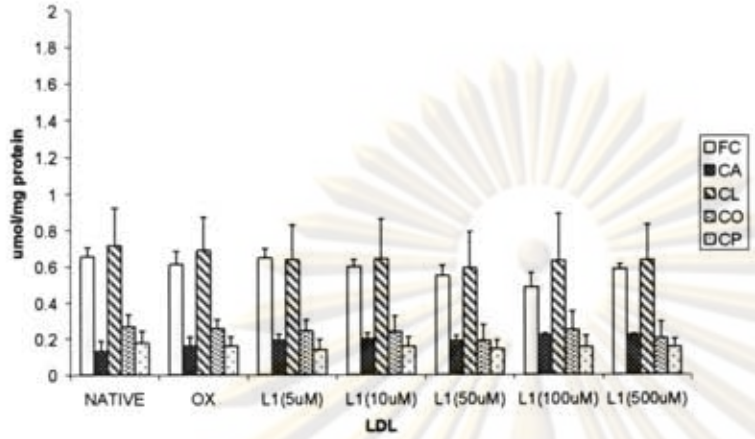
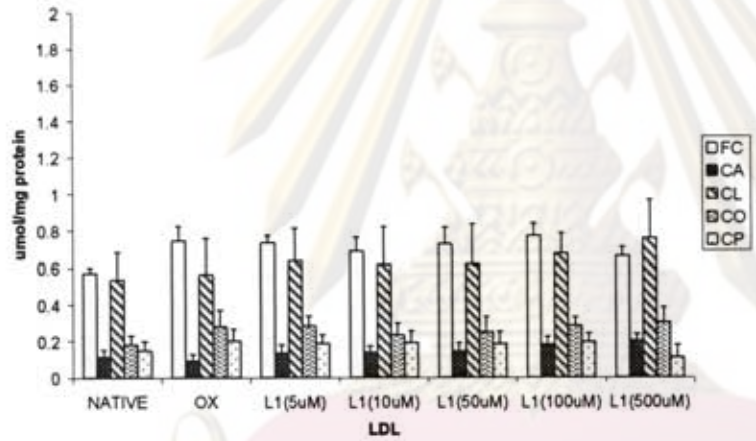


Figure 4.6 Lipid composition of hemin induced oxidation of HDL at time 0 h (A), 9 h (B), and 24 h (C) of incubation (n=3)

(A) 0 h



(B) 9 h



(C) 24 h

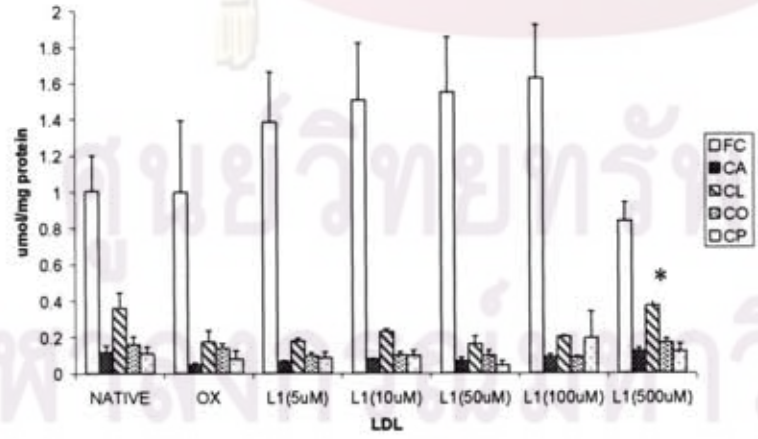
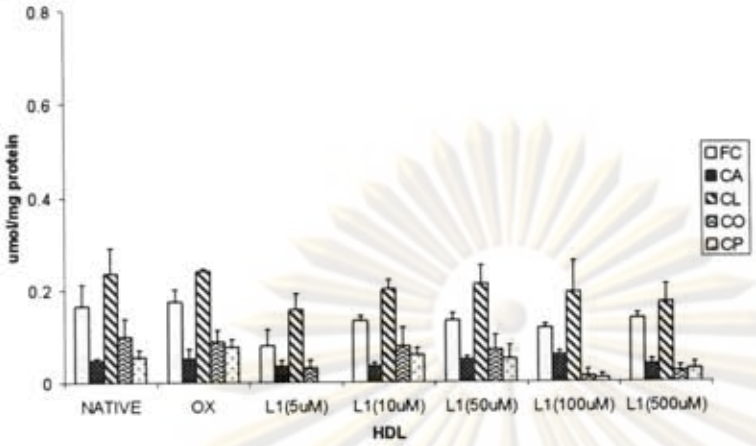


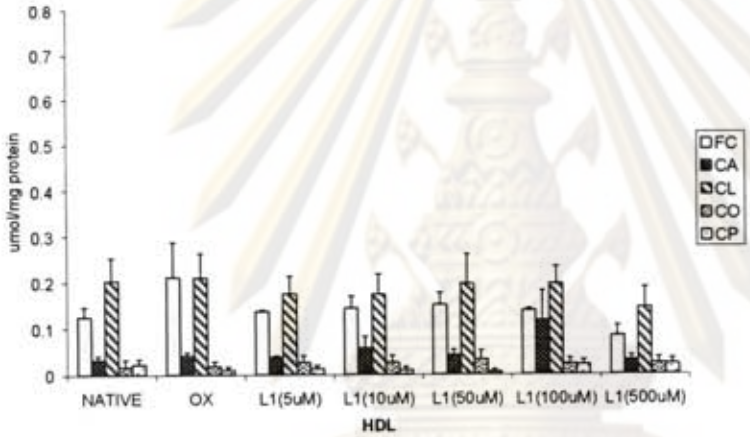
Figure 4.7 Lipid composition in the presence of L1 on hemin induced oxidation of LDL at time 0 h (A), 9 h (B), and 24 h (C) of incubation (n=3)

* It was significantly different at $P<0.05$ (compare with native LDL).

(A) 0 h



(B) 9 h



(C) 24 h

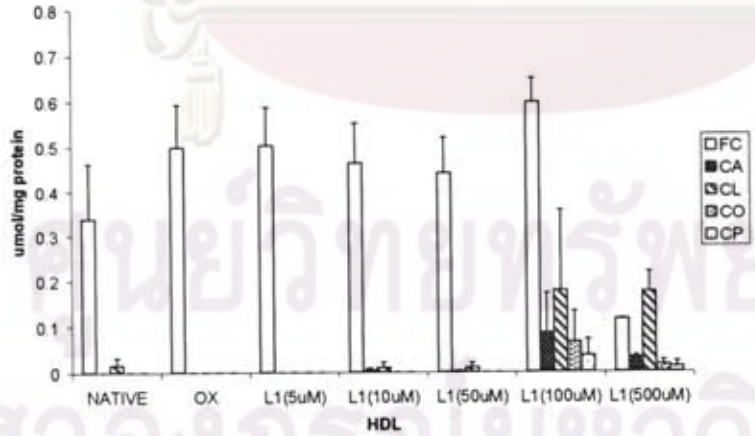


Figure 4.8 Lipid composition in the presence of L1 on hemin induced oxidation of HDL at time 0 h (A), 9 h (B), and 24 h (C) of incubation (n=3)

4. Macrophage formation induced by PMA

Activation induced by PMA of monocytes with the protein kinase C (PKC) activator, phorbol myristate acetate, can cause the differentiation of these cells to macrophages. This experiment determined phorbol (PMA) concentration for activation of U937 human monocytes to form macrophages and tested for the function of U937-derived macrophages induced by PMA. The differentiation was measured by the adherence capacity of the macrophages. The functional property of macrophage was determined as zymosan phagocytosis using on nitroblue tetrazolium (NBT) reduction assay. From table 4.7, and figure 4.9 both 100 and 1000 ng/ml of PMA could induce the differentiation of U937-monocytes into macrophages higher than 85% at time 72 h of incubation. These U937-derived macrophages induced by 100 ng/ml PMA can phagocyte zymosan (600 µg/ml) at approximately 20% as shown in table 4.8.

Table 4.7 The macrophage formation induced by PMA

PMA	% macrophage formation					
	1	2	3	4	5	mean±S.E.M.
10 ng/ml	87.7	72.4	67.2	81.0	61.0	73.9±4.8 [a]
100 ng/ml	90.2	83.3	82.0	87.1	85.6	85.6±1.4 [b]
1000 ng/ml	97.0	93.9	79.8	85.5	92.6	89.8±3.1 [b]

Results are expressed as mean±S.E.M. (n=3).

a, and b show different statistically at $P<0.05$.

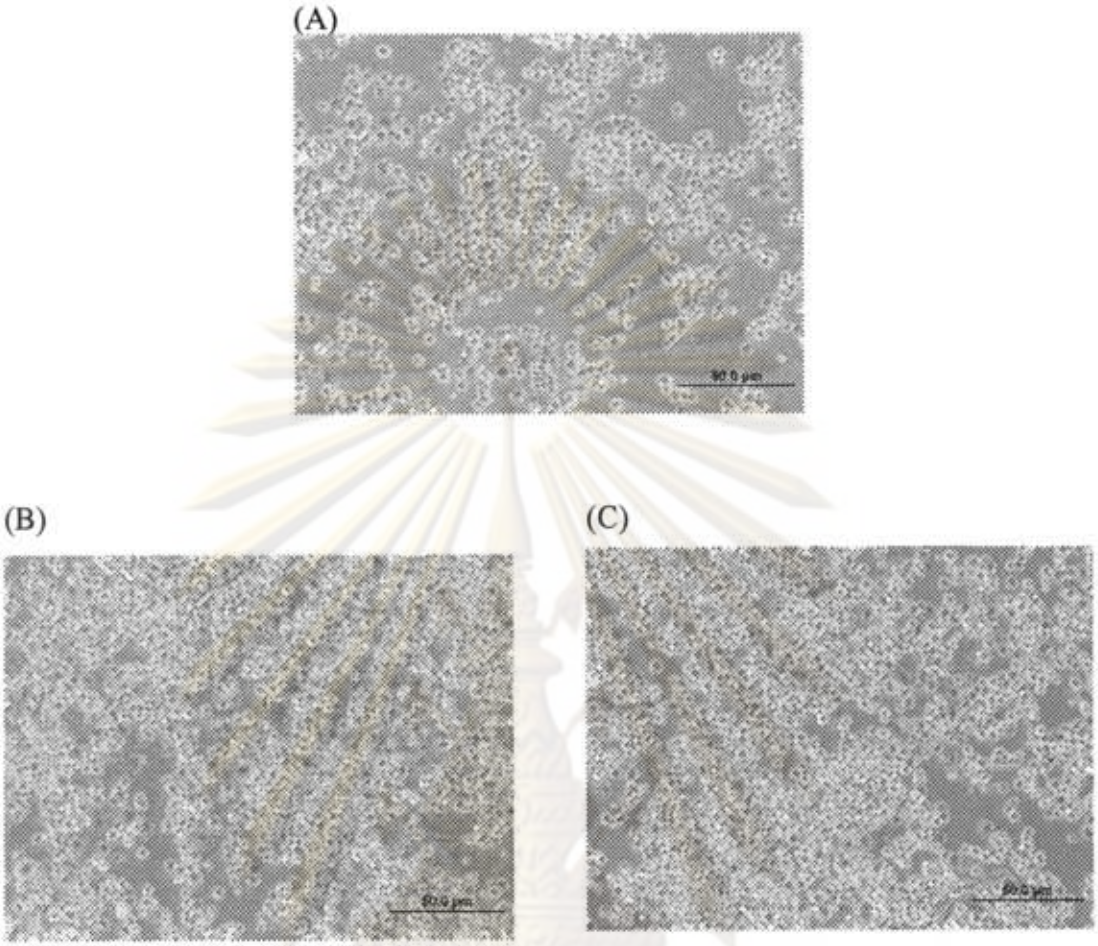


Figure 4.9 The macrophage formation induced by the 10 (A), 100 (B), and 1000 (C) ng/ml of PMA

Table 4.8 %phagocytosis of zymosan 600 µg/ml by monocyte derived macrophage

replication	%phagocytosis (mean±S.E.M.)
1	13.8±3.3
2	25.2±1.0
3	21.2±10.3
4	18.6±2.2

5. The effect of hemin-oxidized LDL on PMA-induced macrophage

5.1 Cytotoxicity

The cytotoxicity of LDL, hemin and L1 on the macrophage were determined using MTT assay, as an indicator of cell viability. Hemin and L1 at all tested concentrations were not toxic to macrophages as seen in table 4.9 and 4.10. In the macrophages, all concentration of treated hemin and L1 were not affect on viability except at 1,000 μM of L1 (table 4.9 and 4.10). Exposure of the macrophage to both native LDL and hemin-oxidized LDL result in increase in cytotoxicity (table 4.11). The cytotoxicity values for cells exposed to oxidized LDL at concentration 30 μg/mg protein was not significantly different from native LDL treated with the cells at value of 78 %. The cytotoxicity of oxidized LDL in the presence of L1 also was not significantly different from untreated oxidized LDL.

Table 4.9 Effect of hemin on cell viability of PMA-induced macrophage at time 24 h of incubation

Hemin (μM)	%cell viability
2.1	114.2±1.6
4.2	121.27±6.5
8.4	121.8±12.6

Results are expressed as mean±S.E.M. (n=3).

Table 4.10 Effect of L1 on cell viability of PMA-induced macrophage at time 24 h of incubation

	%cell viability		
L1(μM)	1	2	mean
1	111.9	107.8	109.8
10	97.5	108.8	103.1
100	100.6	96.1	98.3
1000	71.8	85.8	78.8

Results are expressed as mean. (n=2).

Table 4.11 The cell viability and cytotoxicity of oxidized LDL on PMA-induced macrophage at time 24 h of incubation

LDL	%cell viability	%Cytotoxicity
Native LDL	21.8±2.3	78.1±2.4
Hemin-oxLDL	22.8±3.4	77.2±3.4
Hemin-oxLDL + L1 5 μM	23.0±4.6	77.0±4.6
Hemin-oxLDL + L1 10 μM	23.7±2.8	76.3±2.8
Hemin-oxLDL + L1 50 μM	20.1±3.6	80.0±3.6
Hemin-oxLDL + L1 100 μM	22.6±2.8	77.4±2.8
Hemin-oxLDL + L1 500 μM	23.6±2.3	76.4±2.3

Results are expressed as mean±S.E.M. (n=3).

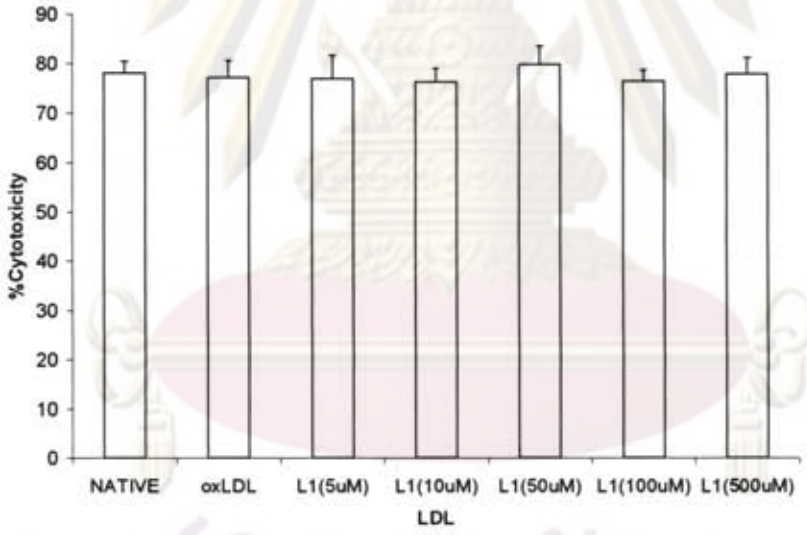


Figure 4.10 The cytotoxicity of oxidized LDL on PMA-induced macrophage at time 24 h of incubation (n=3)

5.2 The phagocytosis assay

The LDL phagocytosis of the macrophage was measured by using nitroblue tetrazolium (NBT) reduction assay. This assay is based on the superoxide anions produced by macrophage after phagocytosis can reduce NBT to blue formazan. From table 4.12, the macrophages had ability to phagocytose oxidized LDL as well as native LDL with value of 131.5%. The phagocytosis of oxidized LDL at all concentrations of L1 was not different except for that of L1 at 500 μM.

Table 4.12 The phagocytosis of oxidized LDL on PMA-induced macrophage at time 24 h of incubation

LDL	%Phagocytosis
Native LDL	131.5±12.7
Hemin-oxLDL	137.6±12.6
Hemin-oxLDL + L1 5 μM	126.2±5.6
Hemin-oxLDL + L1 10 μM	133.0±8.3
Hemin-oxLDL + L1 50 μM	130.0±5.7
Hemin-oxLDL + L1 100 μM	132.0±15.0
Hemin-oxLDL + L1 500 μM	86.7±23.0

Results are expressed as mean±S.E.M. (n=3).

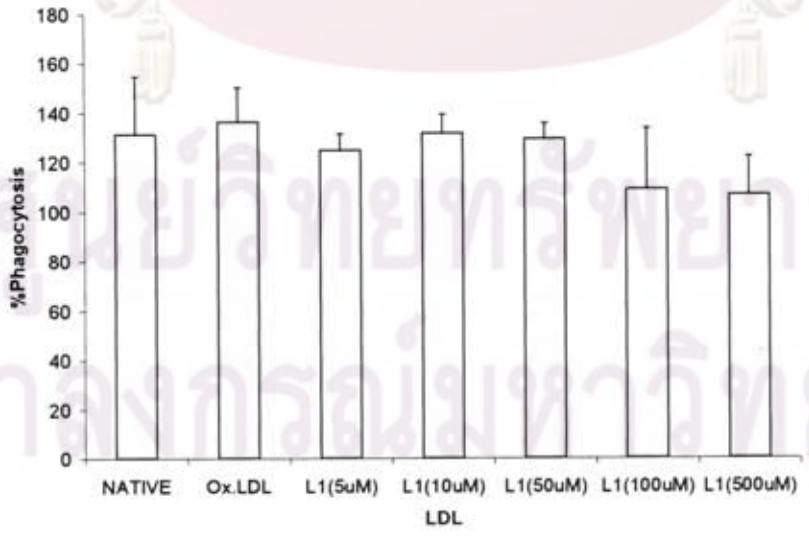


Figure 4.11 The phagocytosis of oxidized LDL (30 μg/mg protein) on PMA-induced macrophage at time 24 h of incubation (n=3)

5.3 The macrophage foam cell formation

The macrophage foam cell formation was determined by measuring the accumulation of cholesteryl esters in cell and confirmed with Oil red O staining (Deng *et al.*, 2005). This preliminary study found only CA and CL accumulated in macrophage foam cell. The percent of cholesteryl esters in the macrophage after exposure to oxidized LDL at 30 μg protein/ml was increased significantly similar to those of the native LDL-treated macrophages at time 24 h of incubation as shown in figure 4.12. The L1 at 5, 10, and 100 μM -treated oxidized LDL could inhibit the accumulation of cholesteryl esters in macrophages. The macrophage loaded with oxidized LDL accumulated less amount of cholesteryl esters compared with native LDL loading, and the distribution of cellular cholesterol between free and the cholesteryl esters was different. For example, CL represented 20.8% of total cholesterol content after native LDL loading, but less than 12.9% after oxidized LDL loading (Table 4.13).

The macrophage foam cell formation was confirmed by staining cell with Oil red O. All cells treated with any forms of LDL could be stained with Oil red O (figure 4.13).

Table 4.13 Free cholesterol and cholesteryl esters content in macrophage foam cell

Macrophage	%of total cholesterol							
	FC		CA		CL		CE (CA+CL)	
	1	2	1	2	1	2	1	2
PBS (normal cell)	100.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0
Native LDL	54.7	100.0	3.8	0.0	41.5	0.0	45.3	0.0
Hemin-oxLDL	80.0	92.3	1.8	0.0	18.2	7.7	20.0	7.7
Hemin-ox LDL + L1 5 μM	100.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0
Hemin-ox LDL + L1 10 μM	100.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0
Hemin-ox LDL + L1 50 μM	82.9	100.0	0.0	0.0	17.1	0.0	17.1	0.0
Hemin-ox LDL + L1 100 μM	100.0	100.0	0.0	0.0	0	0.0	0.0	0.0
Hemin-ox LDL + L1 500 μM	60.0	75.4	4.3	1.7	35.7	22.9	40.0	24.6

Total cholesterol = FC+CA+CL

= FC+CE

(A) (B)

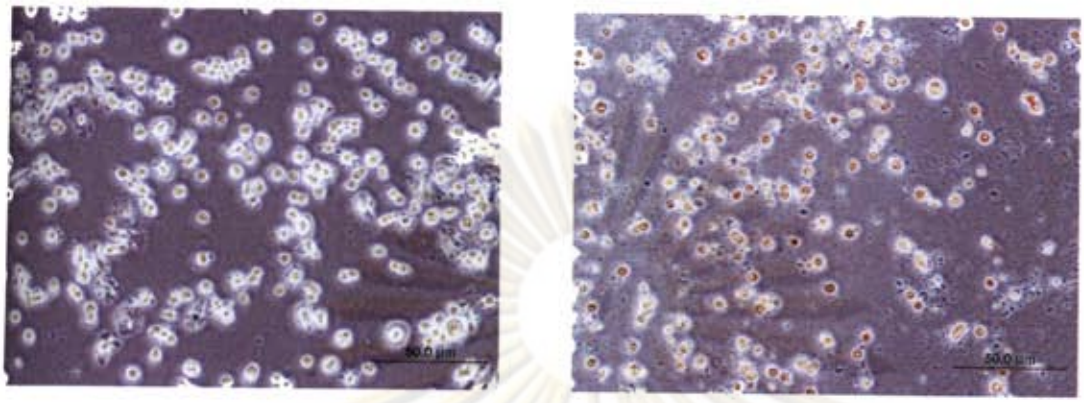


Figure 4.12 The foam cell formation detected by staining with Oil red O of PMA-induced macrophage (B) and control cell (A) at time 24 h of incubation

CHAPTER IV

DISCUSSION AND CONCLUSION

In this study, the change of chemical properties of lipoprotein induced by hemin was determined by lipid peroxidation, the level of endogenous α-tocopherol, and the change of lipid composition. Oxidation of lipoproteins can lead to this alteration of lipoprotein properties. Lipid peroxidation, the marker of oxidation stage, can be monitored as the MDA product by measuring the thiobarbitutic acid reactive substances. In addition, lipoprotein oxidation also can be determined by the depletion of endogenous antioxidant (Berrogui *et al.*, 2006). These results demonstrated that LDL 300 μg protein/ml treated with 5 μM of hemin could generate the lipid peroxidation as increasing of TBARs, and decreasing of α-tocopherol level at time 24 h of incubation. Alpha-tocopherol is the major endogenous antioxidant in lipoprotein. Normally, during the lag phage of lipid peroxidation, the α-tocopherol was consumed and no significant oxidation of PUFAs had taken place. However, when LDL was depleted of its antioxidants, PUFAs are rapidly oxidized to lipid hydroperoxides, which are then converted to a variety of other products including reactive aldehydes (Cominacini *et al.*, 1996). These results have demonstrated that the oxidation of PUFAs occurred as shown by the decrease level of cholesteryl arachidonate (CA) and cholesteryl linoleate (CL) in oxidation of LDL at time 24 h of incubation. These results complement very well with the results obtained by Luechapudiporn who demonstrated that the oxidized LDL by hemin was characterized by the reduction of cholesteryl esters especially CL and CA with the increased in TBARs level (Luechapudiporn, 2003). In addition, hemin could decrease the α-tocopherol level in LDL and HDL (Chalermchoung, 2004). Therefore, these results demonstrated that *in vitro* oxidation of LDL can be induced by hemin.

Hemin, protoporphyrin IX with a coordinated Fe^{3+} is one of the oxidant substrates. Several studies reported that, hemin could induce oxidation of lipoproteins. LDL that was oxidized by hemin could produce lipid peroxidation via free radical generated by Fenton-type reaction. Hemin had also a higher potential to induce oxidation than iron (Luechapudiporn, 2003). The hydrophobic ring might insert deep enough to reach nonpolar regions of the surface monolayer. The rapid

destruction of the porphyrin ring followed very closely diene formation and oxidation of core residing probes liberated Fe^{3+} that became the core oxidant (Tribble *et al.*, 1996).

A similar pattern was observed with HDL, however, there were the slightly increase of TBARs level, and the depletion of α-tocopherol level after treated HDL with 5 μM of hemin. Compared the oxidation of HDL with LDL, the level of HDL oxidation was less than in LDL since HDL itself possesses the antioxidant property. HDL carries the bulk of the paraoxonase (PON), a calcium-dependent enzyme associated with HDL, which plays a role in inhibiting HDL oxidation. Therefore, the susceptibility to oxidation of HDL is related to PON activity, suggesting that individuals with low PON activity are exposed to increased oxidative stress. Moreover, PON activity is reduced during cell-mediated and copper-mediated HDL oxidation (Norata *et al.*, 2006).

This study showed that while cholesteryl esters in native LDL and HDL were decreased, TBARs formation did not occur, indicating that the decrease of cholesteryl esters was not due to lipid peroxidation. As the same time, FC level was increased forward to the duration of incubation time. Perhaps the increase of FC and the decrease of cholesteryl esters may be come from the hydrolysis on ester bond due to the procedure of lipoprotein separation using very high rotor speed of ultracentrifugation (95,000 rpm). In previous studies, lipid level in native LDL was unchanged through the duration of incubation time (Luechapudiporn, 2003; Chalermchoung, 2004). Their procedure of lipoprotein separation was different from our procedure such as ultracentrifugation time, rotor speed, volume of sample required and temperature at which the lipoproptein separation occurs, all which may influence the composition of lipid result (Mackness and Durrington, 1992).

When treated oxidized LDL with L1 at various concentrations (5, 10, 50, 100 or 500 μM), all concentrations of L1 can inhibit lipid peroxidation as seen by 50-80% decreasing of TBARs level at time 9 and 24 h of incubation (table 4.2). However, the cholesteryl esters in oxidized LDL still can not be protected by L1. Interestingly, only L1 at concentration of 500 μM was able to protect the decrease of cholesteryl arachidonate (CA) and cholesteryl linoleate (CL) at time 24 h of incubation. It seemed that L1 at concentration of 500 μM might inhibit the hydrolysis of cholesteryl esters.

Although L1 was able to inhibit the lipid peroxidation of oxidized LDL, it could not preserve the amount of α-tocopherol in oxidized LDL in our study. Since the incubation time at 9 and 24 h were in log phase of lipid peroxidaton at which α-tocopherol was not found. This study did not measure α-tocopherol level in lag phase, however the decrease of TBARs level by L1 may imply that L1 could prolong the lag phase. Indeed, Matthews *et al.* (1996) have shown that L1 could exert the protective effect of lipid peroxidation and prolonged the lag time to oxidation of LDL in a concentration-dependent manner. For the protective effect of L1 on HDL oxidation, all concentration of L1 can inhibit lipid peroxidation almost 100% since hemin can slightly induce the oxidation of HDL. One hundred and 500 μM of L1 were able to protect the decrease of cholesteryl esters level at time 24 h of incubation. Taking these results together, it can be postulated that L1 acts as a potential antioxidant.

In cell culture experiment, the monocyte-derived macrophages were able to taken up either native LDL or oxidized LDL at 130 % phagocytosis. Macrophage could also accumulate cholesterol via fluid phase endocytosis (Kruth *et al.*, 2002). This model was suggested that foam cell formation could be mediated not only by modified LDL, but also by native LDL taken up by activated macrophages (Kruth *et al.*, 2002). In this study, U937 human monocytes were induced to the activated macrophage by phorbol myristate acetate (PMA). The native LDL uptake by macrophage might be due to activating macrophage by PMA. Ma *et al.* (2006) reported the macrophages are differentiated from human monocytes in human serum, activation of these macrophages with the protein kinese C (PKC) activator, phorbol 12-myristate 13-acetate (PMA) can stimulate macrophages. The macrophage foam cell formation can occur through uptake of native LDL in a receptor-independent fashion mediated by fluid-phase macropinocytosis. LDL uptake by macrophages is linearly related to LDL concentration, and does not show saturation at high LDL concentration, and cannot be competed by unlabeled LDL. Moreover, LDL can be visualized during entering macrophage within the fluid-phase of macropinosomes. From this experiment, L1 could not protect uptake of oxidized LDL by PMA-induce macrophage. Perhaps, native LDL at time 24 h of incubation, which was taken up by macrophage, may be the modified LDL as a result of changing of lipid composition by hydrolysis.

Cytotoxicity study found that the hemin and L1 were not cytotoxicity on the macrophages, while the native LDL and oxidized LDL were toxic on the macrophages. The cytotoxicity of native LDL might occur from the oxidation in macrophage after incubation. Since macrophages were crucial sources of oxygen-centerd free radical, which induce the oxidation of LDL (Jessup *et al.*, 2002).

From preliminary study (4.13), exposure of macrophages to all conditions of LDL cause the accumulation the lipid in cells at time 24 h of incubation. In addition, the uptake of macrophages was not different. The macrophages can accumulate FC but not cholesteryl esters in the cells treated with L1 at concentration of 5, 10, and 100 μM treated LDL. However, cholesteryl esters were found in macrophage treated with native, oxidized LDL and L1 at 500 μM with treated LDL. The accumulated lipid compositions in the cells correlated with individual cholesteryl esters in oxidized LDL prior to incubation with cells.

In conclusion, this study demonstrated that L1 possesses an antioxidant activity in *in vitro* oxidation of LDL and HDL. LDL is susceptible to lipid peroxidation induced by hemin more than HDL. L1 can inhibit TBARs formation both in LDL and HDL. The concentration of L1 at 500 μM can protect the decrease of CA and CL level in LDL. However in U937 derived macrophage model, the uptake of native and oxidized LDL can not be distinguished. This may be due to the concentration of LDL used in the experiment and the property of LDL which altered by the separation procedure. The effect of L1 on macrophage uptake can not be observed. Further study should be performed to solve the problem of this model such as lipoprotein separation procedure which influenced on lipid composition of which LDL and HDL and property of macrophage.

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APPENDICES

The TBARs levels in hemin-oxidized LDL at time various of incubation

1. Time 0 h

	TBARs (nmol/mg protein)		
N / Condition	1	2	3
Native LDL	2.76	2.09	2.44
Hemin-oxLDL	5.32	2.08	2.60
Hemin-oxLDL + L1 5 μM	2.81	2.55	2.55
Hemin-oxLDL + L1 10 μM	3.01	2.45	2.43
Hemin-oxLDL + L1 50 μM	2.68	2.08	2.28
Hemin-oxLDL + L1 100 μM	3.58	3.62	2.25
Hemin-oxLDL + L1 500 μM	3.16	2.49	3.12

2. Time 9 h

	TBARs (nmol/mg protein)		
N / Condition	1	2	3
Native LDL	2.77	2.07	2.04
Hemin-oxLDL	19.56	19.78	17.17
Hemin-oxLDL + L1 5 μM	19.64	7.72	5.56
Hemin-oxLDL + L1 10 μM	8.42	7.16	5.26
Hemin-oxLDL + L1 50 μM	7.09	7.33	5.20
Hemin-oxLDL + L1 100 μM	6.33	4.95	4.27
Hemin-oxLDL + L1 500 μM	6.67	6.01	5.11

3. Time 24 h

	TBARs (nmol/mg protein)		
Condition \ N	1	2	3
Native LDL	3.27	2.03	2.54
Hemin-oxLDL	18.91	18.64	18.20
Hemin-oxLDL + L1 5 μM	12.01	7.68	5.94
Hemin-oxLDL + L1 5 μM	11.25	7.27	7.16
Hemin-oxLDL + L1 5 μM	7.13	7.12	6.57
Hemin-oxLDL + L1 5 μM	7.47	6.98	5.75
Hemin-oxLDL + L1 5 μM	6.71	7.64	6.32

Inhibition percent of MDA level in hemin-oxidized LDL at the presence of L1 at time various of incubation

1. Time 9 h

	%inhibition		
Condition \ N	1	2	3
Hemin-oxLDL + L1 5 μM	-0.5	72.1	76.7
Hemin-oxLDL + L1 10 μM	66.3	75.2	78.7
Hemin-oxLDL + L1 50 μM	74.3	74.2	79.1
Hemin-oxLDL + L1 100 μM	78.8	87.7	85.3
Hemin-oxLDL + L1 500 μM	76.8	81.7	79.7

2. Time 24 h

	%inhibition		
Condition \ **N**	**1**	**2**	**3**
Hemin-oxLDL + L1 5 μM	44.1	66.0	78.3
Hemin-oxLDL + L1 10 μM	48.9	68.4	70.5
Hemin-oxLDL + L1 50 μM	75.3	69.4	74.3
Hemin-oxLDL + L1 100 μM	73.1	70.2	79.5
Hemin-oxLDL + L1 500 μM	78.0	66.2	75.9

The TBARs levels in hemin-oxidized HDL at time various of incubation

1. Time 0 h

	TBARs (nmol/mg protein)	
Condition \ **N**	**1**	**2**
Native HDL	2.47	2.29
Hemin-oxHDL	3.11	2.31
Hemin-oxHDL + L1 5 μM	3.46	2.25
Hemin-oxHDL + L1 10 μM	2.78	2.21
Hemin-oxHDL + L1 50 μM	2.38	2.18
Hemin-oxHDL + L1 100 μM	3.41	2.44
Hemin-oxHDL + L1 500 μM	3.91	2.62

2. Time 9 h

TBARs (nmol/mg protein)		
N / Condition	1	2
Native HDL	2.87	2.00
Hemin-oxHDL	6.93	2.79
Hemin-oxHDL + L1 5 μM	2.76	4.14
Hemin-oxHDL + L1 10 μM	3.16	2.60
Hemin-oxHDL + L1 50 μM	3.17	2.59
Hemin-oxHDL + L1 100 μM	3.47	2.45
Hemin-oxHDL + L1 500 μM	2.94	2.56

3. Time 24 h

TBARs (nmol/mg protein)		
N / Condition	1	2
Native HDL	2.70	2.26
Hemin-oxHDL	7.95	3.14
Hemin-oxHDL + L1 5 μM	2.90	2.82
Hemin-oxHDL + L1 10 μM	2.79	2.75
Hemin-oxHDL + L1 50 μM	3.17	2.98
Hemin-oxHDL + L1 100 μM	3.20	2.82
Hemin-oxHDL + L1 500 μM	3.37	2.74

The α-tocopherol levels in hemin-oxidized LDL at time various of incubation

1. Time o h

	α-tocopherol (nmol/mg protein)		
N / Condition	1	2	3
Native LDL	14.41	10.26	11.61
Hemin-oxLDL	14.34	5.89	9.76
Hemin-oxLDL + L1 5 μM	14.00	8.94	11.25
Hemin-oxLDL + L1 10 μM	14.55	6.51	11.01
Hemin-oxLDL + L1 50 μM	15.27	5.07	10.77
Hemin-oxLDL + L1 100 μM	14.60	7.41	11.26
Hemin-oxLDL + L1 500 μM	15.89	8.20	10.41

2. Time 9 h

	α-tocopherol (nmol/mg protein)		
N / Condition	1	2	3
Native LDL	9.97	9.40	8.37
Hemin-oxLDL	7.23	0.00	1.78
Hemin-oxLDL + L1 5 μM	8.29	0.00	0.00
Hemin-oxLDL + L1 10 μM	3.79	2.12	1.85
Hemin-oxLDL + L1 50 μM	3.61	2.16	1.89
Hemin-oxLDL + L1 100 μM	5.18	2.13	2.20
Hemin-oxLDL + L1 500 μM	4.70	2.31	2.84

3. Time 24 h

Condition \ N	α-tocopherol (nmol/mg protein)		
	1	2	3
Native LDL	3.89	8.89	8.41
Hemin-oxLDL	3.48	0.00	0.00
Hemin-oxLDL + L1 5 μM	3.49	0.00	0.00
Hemin-oxLDL + L1 10 μM	3.60	0.00	0.00
Hemin-oxLDL + L1 50 μM	3.60	0.00	0.00
Hemin-oxLDL + L1 100 μM	3.89	0.00	1.96
Hemin-oxLDL + L1 500 μM	3.80	0.00	1.97

The α-tocopherol levels in hemin-oxidized LDL at time various of incubation

1. Time 0 h

Condition \ N	α-tocopherol (nmol/mg protein)	
	1	2
Native HDL	8.97	4.14
Hemin-oxHDL	8.97	4.91
Hemin-oxHDL + L1 5 μM	8.16	4.60
Hemin-oxHDL + L1 10 μM	7.00	3.63
Hemin-oxHDL + L1 50 μM	6.30	3.28
Hemin-oxHDL + L1 100 μM	7.28	5.31
Hemin-oxHDL + L1 500 μM	7.25	4.16

2. Time 9 h

α-tocopherol (nmol/mg protein)		
N / Condition	1	2
Native HDL	6.73	4.44
Hemin-oxHDL	7.47	0.00
Hemin-oxHDL + L1 5 μM	4.30	2.28
Hemin-oxHDL + L1 10 μM	4.29	3.15
Hemin-oxHDL + L1 50 μM	4.38	2.88
Hemin-oxHDL + L1 100 μM	5.35	2.75
Hemin-oxHDL + L1 500 μM	11.42	2.10

3. time 24 h

α-tocopherol (nmol/mg protein)		
N / Condition	1	2
Native HDL	5.29	4.81
Hemin-oxHDL	5.23	0.77
Hemin-oxHDL + L1 5 μM	3.57	1.81
Hemin-oxHDL + L1 10 μM	3.63	1.87
Hemin-oxHDL + L1 50 μM	3.81	1.85
Hemin-oxHDL + L1 100 μM	3.70	1.76
Hemin-oxHDL + L1 500 μM	3.41	2.07

Free cholesterol and cholesteryl esters levels in oxidized LDL after incubate with hemin at various incubation time.

Free cholesterol (FC) in LDL

1. Time 0 h

	FC (µmol/mg protein)		
N / Condition	1	2	3
Native LDL	0.56	0.68	0.73
Hemin-oxLDL	0.51	0.75	0.58
Hemin-oxLDL + L1 5 µM	0.55	0.67	0.73
Hemin-oxLDL + L1 10 µM	0.54	0.60	0.67
Hemin-oxLDL + L1 50 µM	0.55	0.45	0.65
Hemin-oxLDL + L1 100 µM	0.54	0.33	0.59
Hemin-oxLDL + L1 500 µM	0.55	0.57	0.64

2. Time 9 h

	FC (µmol/mg protein)		
N / Condition	1	2	3
Native LDL	0.52	0.62	0.58
Hemin-oxLDL	0.59	0.85	0.79
Hemin-oxLDL + L1 5 µM	0.66	0.78	0.77
Hemin-oxLDL + L1 10 µM	0.54	0.76	0.77
Hemin-oxLDL + L1 50 µM	0.55	0.79	0.84
Hemin-oxLDL + L1 100 µM	0.71	0.91	0.70
Hemin-oxLDL + L1 500 µM	0.57	0.67	0.74

3. Time 24 h

FC (µmol/mg protein)			
N / Condition	1	2	3
Native LDL	0.72	0.91	1.38
Hemin-oxLDL	0.30	1.03	1.66
Hemin-oxLDL + L1 5 µM	0.85	1.51	1.79
Hemin-oxLDL + L1 10 µM	1.00	1.43	2.09
Hemin-oxLDL + L1 50 µM	1.00	1.59	2.05
Hemin-oxLDL + L1 100 µM	1.18	1.52	2.18
Hemin-oxLDL + L1 500 µM	0.64	0.88	0.99

Cholesteryl arachidonate (CA) in LDL

1. Time 0 h

CA(µmol/mg protein)			
N / Condition	1	2	3
Native LDL	0.04	0.13	0.23
Hemin-oxLDL	0.20	0.07	0.22
Hemin-oxLDL + L1 5 µM	0.22	0.13	0.24
Hemin-oxLDL + L1 10 µM	0.22	0.14	0.24
Hemin-oxLDL + L1 50 µM	0.23	0.14	0.20
Hemin-oxLDL + L1 100 µM	0.20	0.22	0.24
Hemin-oxLDL + L1 500 µM	0.21	0.23	0.23

2. Time 9 h

Condition \ N	CA(µmol/mg protein) 1	2	3
Native LDL	0.13	0.06	0.17
Hemin-oxLDL	0.06	0.06	0.17
Hemin-oxLDL + L1 5 µM	0.13	0.06	0.22
Hemin-oxLDL + L1 10 µM	0.14	0.08	0.20
Hemin-oxLDL + L1 50 µM	0.14	0.07	0.23
Hemin-oxLDL + L1 100 µM	0.24	0.10	0.21
Hemin-oxLDL + L1 500 µM	0.24	0.14	0.24

3. Time 24 h

Condition \ N	CA(µmol/mg protein) 1	2	3
Native LDL	0.05	0.17	0.14
Hemin-oxLDL	0.03	0.06	0.06
Hemin-oxLDL + L1 5 µM	0.06	0.08	0.06
Hemin-oxLDL + L1 10 µM	0.07	0.09	0.07
Hemin-oxLDL + L1 50 µM	0.07	0.10	0.04
Hemin-oxLDL + L1 100 µM	0.10	0.11	0.06
Hemin-oxLDL + L1 500 µM	0.14	0.14	0.09

Cholesteryl linoleate(CL) in LDL

1. Time 0 h

	CL(µmol/mg protein)		
Condition \ **N**	**1**	**2**	**3**
Native LDL	0.48	0.49	1.12
Hemin-oxLDL	0.48	0.54	1.06
Hemin-oxLDL + L1 5 µM	0.48	0.42	1.02
Hemin-oxLDL + L1 10 µM	0.49	0.36	1.08
Hemin-oxLDL + L1 50 µM	0.54	0.28	0.96
Hemin-oxLDL + L1 100 µM	0.48	0.28	1.14
Hemin-oxLDL + L1 500 µM	0.51	0.37	1.02

2. Time 9 h

	CL(µmol/mg protein)		
Condition \ **N**	**1**	**2**	**3**
Native LDL	0.30	0.51	0.81
Hemin-oxLDL	0.24	0.55	0.91
Hemin-oxLDL + L1 5 µM	0.41	0.51	0.98
Hemin-oxLDL + L1 10 µM	0.31	0.52	1.01
Hemin-oxLDL + L1 50 µM	0.30	0.52	1.03
Hemin-oxLDL + L1 100 µM	0.52	0.61	0.89
Hemin-oxLDL + L1 500 µM	0.53	0.57	1.18

3. Time 24 h

CL (µmol/mg protein)			
N / Condition	1	2	3
Native LDL	0.19	0.46	0.43
Hemin-oxLDL	0.06	0.26	0.21
Hemin-oxLDL + L1 5 µM	0.19	0.19	0.16
Hemin-oxLDL + L1 10 µM	0.22	0.21	0.26
Hemin-oxLDL + L1 50 µM	0.11	0.24	0.13
Hemin-oxLDL + L1 100 µM	0.20	0.20	0.21
Hemin-oxLDL + L1 500 µM	0.31	0.41	0.38

Cholesteryl oleate(CO) in LDL

1. Time 0 h

CO (µmol/mg protein)			
N / Condition	1	2	3
Native LDL	0.18	0.22	0.40
Hemin-oxLDL	0.17	0.26	0.34
Hemin-oxLDL + L1 5 µM	0.18	0.19	0.37
Hemin-oxLDL + L1 10 µM	0.17	0.13	0.41
Hemin-oxLDL + L1 50 µM	0.09	0.11	0.37
Hemin-oxLDL + L1 100 µM	0.18	0.11	0.45
Hemin-oxLDL + L1 500 µM	0.11	0.12	0.39

2. Time 9 h

Condition \ N	CO (μmol/mg protein) 1	2	3
Native LDL	0.09	0.20	0.26
Hemin-oxLDL	0.11	0.32	0.41
Hemin-oxLDL + L1 5 μM	0.20	0.28	0.38
Hemin-oxLDL + L1 10 μM	0.13	0.22	0.35
Hemin-oxLDL + L1 50 μM	0.11	0.25	0.39
Hemin-oxLDL + L1 100 μM	0.20	0.29	0.36
Hemin-oxLDL + L1 500 μM	0.19	0.27	0.46

3. Time 24 h

Condition \ N	CO (μmol/mg protein) 1	2	3
Native LDL	0.08	0.18	0.22
Hemin-oxLDL	0.08	0.17	0.16
Hemin-oxLDL + L1 5 μM	0.08	0.07	0.13
Hemin-oxLDL + L1 10 μM	0.08	0.08	0.14
Hemin-oxLDL + L1 50 μM	0.04	0.13	0.12
Hemin-oxLDL + L1 100 μM	0.07	0.10	0.08
Hemin-oxLDL + L1 500 μM	0.12	0.20	0.18

Cholesteryl palmitate(CP) in LDL

1. Time 0 h

	CP (µmol/mg protein)		
N / Condition	1	2	3
Native LDL	0.06	0.19	0.28
Hemin-oxLDL	0.07	0.17	0.24
Hemin-oxLDL + L1 5 µM	0.04	0.14	0.24
Hemin-oxLDL + L1 10 µM	0.08	0.14	0.26
Hemin-oxLDL + L1 50 µM	0.07	0.12	0.24
Hemin-oxLDL + L1 100 µM	0.09	0.09	0.28
Hemin-oxLDL + L1 500 µM	0.08	0.14	0.24

2. Time 9 h

	CP (µmol/mg protein)		
N / Condition	1	2	3
Native LDL	0.05	0.19	0.21
Hemin-oxLDL	0.08	0.26	0.27
Hemin-oxLDL + L1 5 µM	0.10	0.21	0.26
Hemin-oxLDL + L1 10 µM	0.06	0.26	0.25
Hemin-oxLDL + L1 50 µM	0.06	0.23	0.27
Hemin-oxLDL + L1 100 µM	0.11	0.27	0.22
Hemin-oxLDL + L1 500 µM	0.10	0.23	0.00

3. Time 24 h

CP (µmol/mg protein)			
N / Condition	1	2	3
Native LDL	0.05	0.16	0.12
Hemin-oxLDL	0.00	0.15	0.09
Hemin-oxLDL + L1 5 µM	0.05	0.07	0.15
Hemin-oxLDL + L1 10 µM	0.05	0.10	0.15
Hemin-oxLDL + L1 50 µM	0.04	0.09	0.00
Hemin-oxLDL + L1 100 µM	0.48	0.10	0.00
Hemin-oxLDL + L1 500 µM	0.05	0.19	0.12

Free cholesterol and cholesteryl esters levels in oxidized HDL after incubate with hemin at various incubation time.

Free cholesterol (FC) in HDL

1. Time 0 h

FC (µmol/mg protein)			
N / Condition	1	2	3
Native HDL	0.11	0.26	0.13
Hemin-oxHDL	0.22	0.18	0.13
Hemin-oxHDL + L1 5 µM	0.11	0.01	0.12
Hemin-oxHDL + L1 10 µM	0.12	0.15	0.13
Hemin-oxHDL + L1 50 µM	0.11	0.16	0.13
Hemin-oxHDL + L1 100 µM	0.10	0.13	0.12
Hemin-oxHDL + L1 500 µM	0.12	0.16	0.13

2. Time 9 h

Condition \ N	FC (µmol/mg protein) 1	2	3
Native HDL	0.10	0.11	0.17
Hemin-oxHDL	0.13	0.36	0.14
Hemin-oxHDL + L1 5 µM	0.14	0.14	0.13
Hemin-oxHDL + L1 10 µM	0.11	0.19	0.13
Hemin-oxHDL + L1 50 µM	0.10	0.16	0.19
Hemin-oxHDL + L1 100 µM	0.13	0.14	0.14
Hemin-oxHDL + L1 500 µM	0.09	0.12	0.04

3. Time 24 h

Condition \ N	FC (µmol/mg protein) 1	2	3
Native HDL	0.28	0.15	0.58
Hemin-oxHDL	0.32	0.62	0.56
Hemin-oxHDL + L1 5 µM	0.34	0.62	0.54
Hemin-oxHDL + L1 10 µM	0.29	0.58	0.52
Hemin-oxHDL + L1 50 µM	0.28	0.52	0.52
Hemin-oxHDL + L1 100 µM	0.64	0.66	0.49
Hemin-oxHDL + L1 500 µM	0.11	0.12	0.11

Cholesteryl arachidonate (CA) in HDL

1. Time 0 h

	CA (µmol/mg protein)		
N / Condition	1	2	3
Native HDL	0.05	0.06	0.04
Hemin-oxHDL	0.09	0.04	0.02
Hemin-oxHDL + L1 5 µM	0.04	0.05	0.01
Hemin-oxHDL + L1 10 µM	0.05	0.03	0.02
Hemin-oxHDL + L1 50 µM	0.06	0.06	0.03
Hemin-oxHDL + L1 100 µM	0.05	0.08	0.05
Hemin-oxHDL + L1 500 µM	0.04	0.05	0.01

2. Time 9 h

	CA (µmol/mg protein)		
N / Condition	1	2	3
Native HDL	0.05	0.03	0.03
Hemin-oxHDL	0.04	0.06	0.03
Hemin-oxHDL + L1 5 µM	0.04	0.04	0.03
Hemin-oxHDL + L1 10 µM	0.03	0.05	0.10
Hemin-oxHDL + L1 50 µM	0.05	0.06	0.02
Hemin-oxHDL + L1 100 µM	0.07	0.24	0.03
Hemin-oxHDL + L1 500 µM	0.05	0.04	0.01

3. Time 24 h

CA (µmol/mg protein)			
N / Condition	1	2	3
Native HDL	0.00	0.00	0.00
Hemin-oxHDL	0.00	0.00	0.00
Hemin-oxHDL + L1 5 µM	0.00	0.00	0.00
Hemin-oxHDL + L1 10 µM	0.00	0.01	0.00
Hemin-oxHDL + L1 50 µM	0.00	0.00	0.00
Hemin-oxHDL + L1 100 µM	0.26	0.00	0.00
Hemin-oxHDL + L1 500 µM	0.03	0.04	0.03

Cholesteryl linoleate(CL) in LDL

1. Time 0 h

CL (µmol/mg protein)			
N / Condition	1	2	3
Native HDL	0.14	0.09	0.24
Hemin-oxHDL	0.25	0.15	0.23
Hemin-oxHDL + L1 5 µM	0.12	0.11	0.12
Hemin-oxHDL + L1 10 µM	0.16	0.13	0.24
Hemin-oxHDL + L1 50 µM	0.13	0.17	0.25
Hemin-oxHDL + L1 100 µM	0.13	0.14	0.12
Hemin-oxHDL + L1 500 µM	0.12	0.12	0.14

2. Time 9 h

Condition \ N	CL (μmol/mg protein) 1	2	3
Native HDL	0.14	0.09	0.30
Hemin-oxHDL	0.11	0.15	0.25
Hemin-oxHDL + L1 5 μM	0.10	0.11	0.22
Hemin-oxHDL + L1 10 μM	0.09	0.13	0.19
Hemin-oxHDL + L1 50 μM	0.11	0.17	0.15
Hemin-oxHDL + L1 100 μM	0.14	0.14	0.18
Hemin-oxHDL + L1 500 μM	0.11	0.12	0.09

3. Time 24 h

Condition \ N	CL (μmol/mg protein) 1	2	3
Native HDL	0.00	0.03	0.00
Hemin-oxHDL	0.00	0.00	0.00
Hemin-oxHDL + L1 5 μM	0.00	0.00	0.00
Hemin-oxHDL + L1 10 μM	0.00	0.02	0.00
Hemin-oxHDL + L1 50 μM	0.00	0.02	0.00
Hemin-oxHDL + L1 100 μM	0.53	0.00	0.00
Hemin-oxHDL + L1 500 μM	0.10	0.14	0.17

Cholesteryl oleate(CO) in LDL

1. Time 0 h

	CO (µmol/mg protein)		
N / Condition	1	2	3
Native HDL	0.03	0.11	0.16
Hemin-oxHDL	0.08	0.05	0.13
Hemin-oxHDL + L1 5 µM	0.03	0.06	0.00
Hemin-oxHDL + L1 10 µM	0.04	0.04	0.16
Hemin-oxHDL + L1 50 µM	0.03	0.04	0.13
Hemin-oxHDL + L1 100 µM	0.04	0.00	0.00
Hemin-oxHDL + L1 500 µM	0.03	0.04	0.00

2. Time 9 h

	CO (µmol/mg protein)		
N / Condition	1	2	3
Native HDL	0.05	0.00	0.00
Hemin-oxHDL	0.03	0.02	0.00
Hemin-oxHDL + L1 5 µM	0.03	0.05	0.00
Hemin-oxHDL + L1 10 µM	0.03	0.05	0.00
Hemin-oxHDL + L1 50 µM	0.03	0.07	0.00
Hemin-oxHDL + L1 100 µM	0.04	0.03	0.00
Hemin-oxHDL + L1 500 µM	0.04	0.04	0.00

3. Time 24 h

	CO (µmol/mg protein)		
Condition \ N	1	2	3
Native HDL	0.00	0.00	0.00
Hemin-oxHDL	0.00	0.00	0.00
Hemin-oxHDL + L1 5 µM	0.00	0.00	0.00
Hemin-oxHDL + L1 10 µM	0.00	0.00	0.00
Hemin-oxHDL + L1 50 µM	0.00	0.00	0.00
Hemin-oxHDL + L1 100 µM	0.20	0.00	0.00
Hemin-oxHDL + L1 500 µM	0.03	0.01	0.00

Cholesteryl palmitate(CP) in HDL

1. Time 0 h

	CP (µmol/mg protein)		
Condition \ N	1	2	3
Native HDL	0.02	0.07	0.07
Hemin-oxHDL	0.05	0.07	0.11
Hemin-oxHDL + L1 5 µM	0.00	0.00	0.00
Hemin-oxHDL + L1 10 µM	0.08	0.03	0.07
Hemin-oxHDL + L1 50 µM	0.03	0.01	0.11
Hemin-oxHDL + L1 100 µM	0.03	0.00	0.00
Hemin-oxHDL + L1 500 µM	0.04	0.05	0.00

2. Time 9 h

Condition \ N	CP (µmol/mg protein) 1	2	3
Native HDL	0.04	0.03	0.00
Hemin-oxHDL	0.02	0.01	0.00
Hemin-oxHDL + L1 5 µM	0.02	0.02	0.00
Hemin-oxHDL + L1 10 µM	0.02	0.01	0.00
Hemin-oxHDL + L1 50 µM	0.02	0.00	0.00
Hemin-oxHDL + L1 100 µM	0.02	0.04	0.00
Hemin-oxHDL + L1 500 µM	0.03	0.04	0.00

3. Time 24 h

Condition \ N	CP (µmol/mg protein) 1	2	3
Native HDL	0.00	0.00	0.00
Hemin-oxHDL	0.00	0.00	0.00
Hemin-oxHDL + L1 5 µM	0.00	0.00	0.00
Hemin-oxHDL + L1 10 µM	0.00	0.00	0.00
Hemin-oxHDL + L1 50 µM	0.00	0.00	0.00
Hemin-oxHDL + L1 100 µM	0.11	0.00	0.00
Hemin-oxHDL + L1 500 µM	0.04	0.00	0.00

Effect of hemin on cell viability of PMA-induced macrophage at time 24 h of incubation

Hemin (µM) \ N	%cell viability 1	2	3
2.1	114.7	111.2	116.7
4.2	133.2	110.7	119.9
8.4	144.5	101.0	119.9

Effect of L1 on cell viability of PMA-induced macrophage at time 24 h of incubation

N / L1(μM)	%cell viability	
	1	2
1	111.9	107.8
10	97.5	108.8
100	100.6	96.1
1000	71.8	85.8

The cytotoxicity of hemin oxidized LDL on PMA-induced macrophage at time 24 h of incubation

	%cytotoxicity		
N / Condition	1	2	3
Native LDL	78.2	74.1	82.2
Ox LDL	81.3	70.4	79.9
L1 5 μM	83.7	68.1	79.2
L1 10 μM	80.3	70.9	77.6
L1 50 μM	85.0	72.8	81.8
L1 100 μM	79.7	71.9	80.7
L1 500 μM	73.7	74.5	81.1

The phagocytosis of hemin oxidized LDL by PMA-induced macrophage at time 24 h of incubation

	%Phagocytosis		
N / Condition	1	2	3
Native LDL	171.7	131.6	90.6
Ox LDL	156.5	142.7	110.3
L1 5 μM	137.3	121.5	116.1
L1 10 μM	140.2	116.4	138.3
L1 50 μM	142.0	123.7	122.2
L1 100 μM	160.5	126.3	105.8
L1 500 μM	43.5	122.0	91.4

BIOGRAPHY

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