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Effects of Oxidized Low Density Lipoprotein on Nitric Oxide Production in Macrophages

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EFFECTS OF OXIDIZED LOW DENSITY LIPOPROTEIN ON NITRIC OXIDE PRODUCTION IN MACROPHAGES

A Dissertation

Presented to

the Faculty of the Department of Physiology

James H. Quillen College of Medicine

East Tennessee State University

In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy in Biomedical Sciences

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by

Annong Huang

December 1997

APPROVAL

This is to certify that the Graduate Committee of

Annong Huang

met on the

5th day of November, 1997.

The committee read and examined her dissertation, supervised her defense of it in an oral examination, and decided to recommend that her study be submitted to the Graduate Council, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Science.

Chair, Graduate Committee

Signed on behalf of
the Graduate Council

Interim Dean, School of Graduate Studies

ABSTRACT

EFFECTS OF OXIDIZED LOW DENSITY LIPOPROTEIN

ON NITRIC OXIDE PRODUCTION IN MACROPHAGES

By

Annong Huang

The effects of oxidatively modified low density lipoprotein (oxLDL) on atherogenesis may be partly mediated by alterations in nitric oxide (NO) production by macrophages. A major goal of this study was to identify the lipid components in oxLDL modulating NO production. The effect of a water soluble antioxidants (N-acetylcysteine) and lipid soluble antioxidant (alpha-tocopherol) on NO production in macrophages was also determined. A second goal was to determine if the effects of oxLDL occurred at the transcriptional level.

Human LDL was oxidized using an azo-initiator 2,2'-azobis (2-amidinopropane) HCl (ABAP). OxLDL markedly decreased the production of NO in LPS stimulated RAW264.7 macrophages. This inhibition depended on the levels of LOOH formed in oxLDL and was not due to oxLDL cytotoxicity. In contrast, acetylated LDL (AcLDL) and native LDL showed only minor inhibition. Lipid hydroperoxides (LOOH) and lysophosphatidylcholine (lysoPC) are the primary products formed during LDL oxidation. 13-Hydroperoxyl octadecadienoic acid (13-HPODE) markedly inhibited NO production, whereas lysoPC showed only slight inhibition. Furthermore, the effects of 13-HPODE and lysoPC did not require their uptake in an AcLDL carrier. Pre-treatment of macrophages with alpha-tocopherol attenuated the inhibition due to oxLDL. Similarly, pre-treatment with N-acetylcysteine attenuated the inhibition caused by oxLDL or 13-HPODE.

OxLDL was found to decrease iNOS protein and mRNA levels in RAW264.7 macrophages induced by LPS. The activation of NF-κB was slightly suppressed after 45 minutes of treatment. 13-HPODE showed much stronger reduction of iNOS protein levels than lysoPC. These results suggest that oxLDL may inhibit NO production in macrophages at transcriptional level. 13-HPODE is likely to be the most important lipid component in oxLDL for the inhibitory effect. Antioxidants were found to preserve NO production in macrophages treated with either oxLDL or 13-HPODE. The physiological consequences of decreased NO production in macrophages caused by oxLDL are discussed with respect to atherosclerosis.

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ABBREVIATIONS

ABAP	2,2'-azobis-2-admidinopropane HCL
AcLDL	acetylated LDL
ANOVA	analysis of variance
apo B-100	apoprotein B-100
BHT	butylated hydroxy-toluene
DPA	diphenylamine
ecNOS	endothelial constitutive nitric oxide synthase
EDTA	ethylenediamine-tetraacetic acid
EDRF	endothelial derived relaxing factor
ELAM-1	endothelial leukocyte adhesion molecule 1
EMSA	electrophoretic mobility shift assay
GAPDH	glyceraldehyde-3-phosphodehydrogenase
GSH	reduced glutathione
GSSG	oxidized glutathione
GM-CSF	granulocyte-monocyte stimulating factor
HDL	high density lipoprotein
HODE	13-hydroxyl octadecadienoic acid
HPODE	13-hydroperoxyl octadecadienoic acid
HPLC	high performance liquid chromatography
HSP	heat shock protein
ICAM-1	intercellular adhesion molecule 1

IL-1α	interleukin 1α
IL-1β	interleukin 1β
IPI_2	phosphatidylinositol 4,5-bisphosphate
iNOS	inducible nitric oxide synthase
LDL	low density lipoprotein
LH	lipid fragment
13-HODE	13-hydroxyl octadecadienoic acid
LOH	lipid hydroxides
LOO*	lipid hydroxyl radicals
LOOH	lipid hydroperoxides
13-HPODE	13-hydroperoxyl octadecadienoic acid
lysoPC	lysophosphatidylcholine
LPS	lipopolysaccharide
MAD	malondialdehyde
MCP-1	monocyte chemoattratant protein 1
M-CSF	monocyte stimulating factor
MM-LDL	minimally oxidized low density lipoprotein
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NAC	N-acetylcysteine
NF-kB	nuclear factor kappa B
NO	nitric oxide
NO_2^-	nitrite

NO_3^-	nitrate
$O^{\bullet}_2^-$	superoxide radical
ONOO-	peroxynitrite
oxLDL	oxidatively modified low density lipoprotein
PBS	phosphate buffer saline
PKC	protein kinase C
PLA_2	phospholipase A_2
PUFA	polyunsaturated fatty acid
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulphate
SMC	smooth muscle cells
TLC	thin layer chromatography
TNF-α	tumor necrosis factor-α
VCAM-1	vascular cell adhesion molecule
VLDL	very low density lipoprotein

CHAPTER 1

INTRODUCTION

Oxidized LDL, Macrophages and Atherosclerosis

Oxidation of Low Density Lipoprotein

Oxidatively modified low density lipoprotein (oxLDL) is generally believed to play an important role in the initiation of atherosclerosis (Berliner and Heinecke 1996; Parthasarathy and others 1992; Steinbrecher and others 1990). Immunological evidence suggests that oxidatively modified forms of low density lipoprotein (LDL) exist in human and rabbit atherosclerotic plaques (Yla-Herttuala and others 1989). The alterations in LDL resulting from *in vitro* lipid peroxidation have been well characterized (Esterbauer and others 1992; Noguchi and others 1993; Steinberg 1997). LDL oxidation involves lipid peroxidation, epitope alterations of apoprotein B-100 (apo B-100), hydrolysis of phospholipids and an increase in the negative charge of LDL particles.

LDL is a spherical molecule, consisting of triglycerides and cholesteryl esters in a hydrophobic core surrounded by a monolayer of phospholipids and cholesterol. One large polypeptide is present termed apo B-100. LDL contains large amounts of polyunsaturated fatty acids (PUFA) such as linoleic acid (or octadecadienoic acid), which are very susceptible to peroxidation forming lipid hydroperoxides (LOOH). As shown in Figure 1-1, lipid peroxidation is initiated when a radical abstracts a hydrogen atom from a PUFA fragment (LH) present in

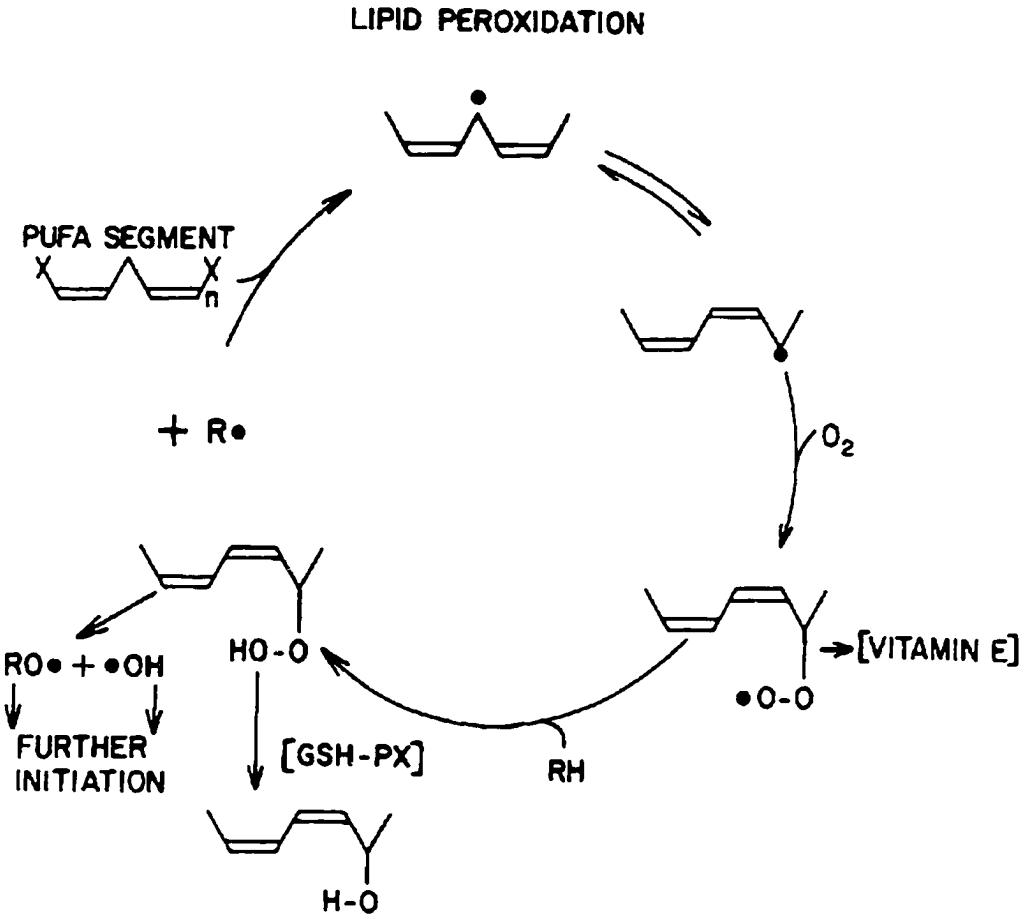


Figure 1-1 Mechanism of Lipid Peroxidation

LDL. The resulting carbon-center lipid radical undergoes double bond rearrangement to form a conjugated diene. The conjugated diene reacts rapidly with oxygen to form a lipid peroxyl radical ($LOO^{\bullet}$). In the absence of antioxidants, the lipid peroxyl radical will abstract a hydrogen atom from an adjacent LH to yield a LOOH and another carbon-center lipid radical that can then continue the chain reactions.

Even though the *in vivo* mechanism for LDL oxidation is uncertain, LOOH is likely to be the most abundant primary lipid oxidation product formed (Noguchi and others 1993). In extensively oxidized LDL, nearly 50% of the phospholipids are hydrolyzed to lysophospholipids that are the second most abundant lipid products (Steinbrecher and others 1990) (reaction 1-1).

$$\text{phospholipid} \xrightarrow{PLA_2} \text{lysophospholipid} + \text{free fatty acid} \qquad (1\text{-}1)$$

Hydrolysis of phospholipids is catalyzed by a PLA_2-like activity that is intrinsic to oxLDL but not native LDL (Parthasarathy and Barnett 1990; Reisfeld and others 1993). LDL oxidation *in vitro* with either Cu^{++} or peroxyl radicals generated from azo-initiators yields high levels of octadecadienoic acid hydroperoxides (9-HPODE and 13-HPODE) and octadecadienoic acid hydroxides (9-HODE and 13-HODE) (Thomas and Jackson 1991). 13-HPODE has been found to be a predominant form of lipid oxidation product over 13-HODE in oxLDL modified by human monocytes (Folcik and Cathcart 1994). Increasing evidence suggests

that lipoxygenase contributes to LDL oxidation during atherosclerosis (Folcik and others 1995; Kuhn and others 1994; Scheidegger and others 1997). Lipoxygenase catalyzes the peroxidation of octadecadienoic acid to form predominantly the S stereoisomer over the R stereoisomer of 13-HPODE, whereas oxidation of LDL by Cu^{++} forms equal amount of the S and R stereoisomers (Folcik and others 1995). This property of lipoxygenase activity can be distinguished from nonenzymatic lipid peroxidation. Analysis of lipid extracts from human atherosclerotic lesions indicates that 13-HPODE is produced from 15-lipoxygenase mediated lipid peroxidation (reaction 1-2) (Folcik and others 1995).

$$\text{octadecadienoic acid} \xrightarrow[O_2]{\text{15-lipoxygenase}} \text{13-hydroperoxyloctadecadienoic acid} \qquad (1\text{-}2)$$

As shown in Figure 1-1, LOOHs are unstable molecules and are rapidly reduced to the corresponding hydroxides (LOH) by the action of cellular peroxidases such as glutathione (GSH) peroxidases (reaction 1-3) (Thomas and others 1990). Therefore, 9-HODE and 13-HODE found in cell-mediated (Kaduce and others 1989) or lipoxygenase-mediated (Scheidegger and others 1997) oxLDL as well as in aortic atherosclerotic plaques (Wang and Powell 1991) probably originated from the corresponding less stable HPODE.

$$13\text{-HPODE} + 2\ GSH \xrightarrow{\text{GSH peroxidase}} 13\text{-HODE} + GSSG \quad (1\text{-}3)$$

In the presence of metal ions, LOOHs break down to various aldehydes. Malondialdehyde (MDA) is one of the important products that covalently binds to positively charged lysine residues in apo B-100 and increases the negative charge of oxLDL (Haberland and Fogelman 1985). OxLDL is no longer recognized by the LDL receptor but is a ligand for the scavenger receptors in macrophages and smooth muscle cells (SMC) (Haberland and others 1988; Haberland and others 1992).

Macrophage Lipid Metabolism and Inflammatory Response

Macrophages incubated with oxLDL *in vitro* assume many characteristics typical of foam cells found in early atherosclerotic lesions, whereas native LDL incubated with macrophages does not cause foam cell formation (for reviews see Aviram 1996; Ross 1993). The LDL receptor mediated uptake of LDL by cells is under the regulation of intracellular cholesterol. Increased cellular free cholesterol down-regulates the biosynthesis of the LDL receptor thereby decreasing the further internalization of LDL. Macrophages, however, express scavenger receptors for modified LDL such as acetylated LDL (AcLDL) and oxLDL (Brown and Goldstein 1983). The uptake of modified LDL via scavenger receptors is not down regulated by intracellular cholesterol thereby leading to the accumulation of cholesteryl esters that form intracellular lipid droplets with a

foam-like appearance. There are at least two different kinds of macrophage scavenger receptors: class A and class B (Steinberg 1997). Class A is the original AcLDL receptors which were described by Brown and others (Brown and Goldstein 1983) capable of recognizing both AcLDL and oxLDL. Class A scavenger receptors of type AI and type AII have been cloned (Penman and others 1991). A collagen domain in both type AI and AII receptors binds modified LDL. OxLDL may be the physiologically relevant form of modified LDL since it has been found in atherosclerotic plaques (Haberland and others 1988; Yla-Herttuala and others 1989).

Figure 1-2 shows the transformation of macrophages to foam cells and the effects of oxLDL in the early stages of atherosclerosis. Binding of circulating monocytes to the endothelial surface is the initial step for monocyte migration. Endothelial adhesion molecules such as endothelial leukocyte adhesion molecule 1 (ELAM-1), intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) are found in atherosclerotic plaques (for a review see Holvoet and Collen 1994). OxLDL induces the expression of endothelial adhesion molecules and facilitates the interactions between macrophages and endothelial cells (Berliner and others 1990; Khan and others 1995; Lehr and others 1993). Furthermore, monocytes undergo proliferation and differentiation driven by monocyte chemoattractant protein 1 (MCP-1) and growth factors such as granulocyte-monocyte colony stimulating factor (GM-CSF) and monocyte colony stimulating factor (M-CSF) that are secreted from endothelial and other cells. The expression of MCP-1, GM-CSF and M-CSF are also

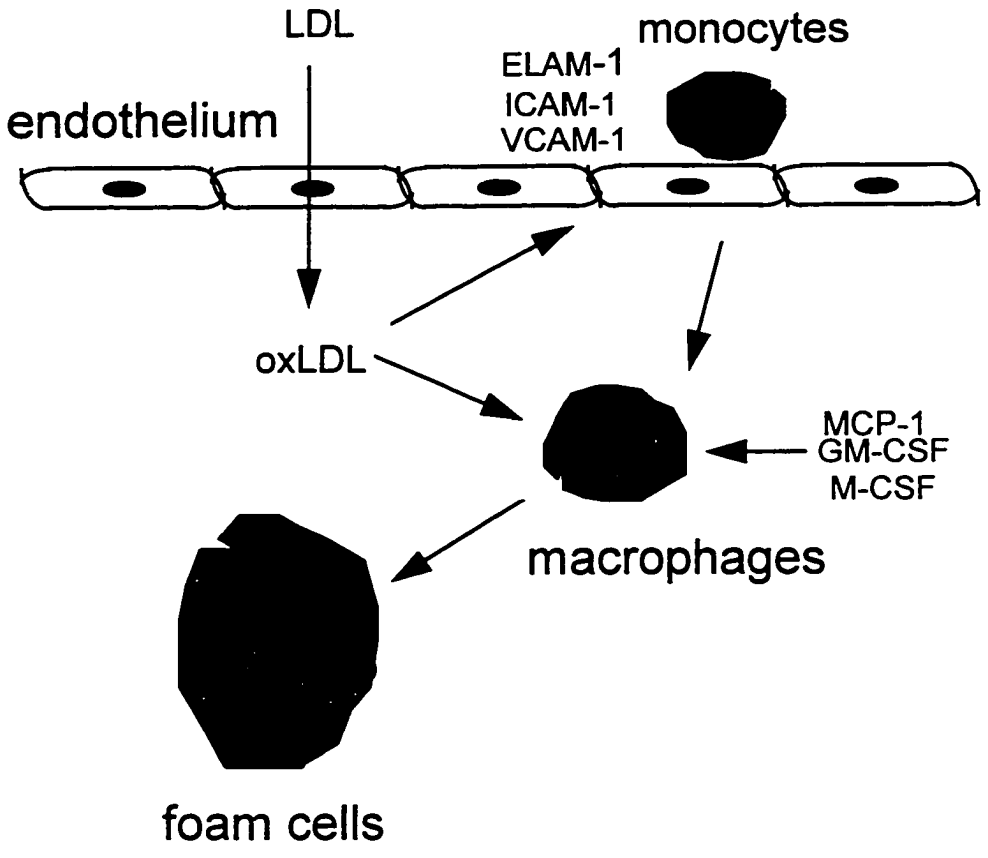


Figure 1-2 A schematic outline showing foam cell formation and the effects of oxLDL. Abbreviations: low density lipoprotein (LDL); oxidatively modified low density lipoprotein (oxLDL); endothelial leukocyte adhesion molecule 1 (ELAM-1); intercellular adhesion molecule 1 (ICAM-1); vascular cell adhesion molecule (VCAM-1). monocyte chemoattratant protein 1 (MCP-1); granulocyte-monocyte stimulating factor (GM-CSF); granulocyte-monocyte stimulating factor (GM-CSF).

induced by oxLDL (Cushing and Fogelman 1992; Rajavashisth and others 1990). Further study has showed that M-CSF enhances the uptake of oxLDL and the formation of foam cells by increasing the expression of scavenger receptors in macrophages (Ishibashi and others 1990).

Nitric Oxide and Atherosclerosis

Palmer and others. (1987) first suggested that nitric oxide (NO) accounts for the biological activity of endothelial-derived relaxing factor (EDRF). NO is derived from L-arginine and oxygen by a family of enzymes termed nitric oxide synthase (NOS). NOS includes three isoforms. Macrophages and many other cell types express inducible nitric oxide synthase (iNOS) upon stimulation by various cytokines and bacterial derived substances such as lipopolysaccharide (LPS). The iNOS enzyme catalyzes the production of large amounts of NO in stimulated macrophages. Endothelial constitutive nitric oxide synthase (ecNOS) and neural constitutive nitric oxide synthase are expressed in endothelial cells and nervous tissues, respectively (Nathan and Xie 1994). NO plays a critical role in maintaining vascular homeostasis. Recent evidence also shows that NO appears to exert anti-atherogenic effects by inhibiting leukocyte adhesion, platelet aggregation and SMC proliferation (for a review see Lloyd-Jones and Bloch 1996). Increased systemic production of NO by supplementation with L-arginine inhibits the progression of atherosclerosis (Boger and others 1996; Wang and others 1994) and promotes the regression of preexisting lesions (Candipan and others 1996) in hypercholesterolemic rabbits. In contrast, long

term supplementation with the NOS inhibitor, N-nitro-L-arginine methylester, promotes atherosclerosis in rabbits (Naruse and others 1994). NO production is also significantly decreased during the early phases of atherosclerosis in humans (Lloyd-Jones and Bloch 1996).

NO shows dual effects on LDL oxidation (see Figure 1-3). Most evidence suggests that NO by itself is an antioxidant capable of inhibiting LDL oxidation *in vitro* (Hogg and others 1995; Jessup and Dean 1993; Rubbo and others 1994; Seccia and others 1996) by quenching $LOO^{\bullet}$ (Hayashi and others 1995). In contrast, NO rapidly reacts with superoxide radicals ($O^{\bullet}_{2}^{-}$) to produce peroxynitrite ($ONOO^{\bullet-}$) that is a potent oxidant capable of oxidizing LDL (Hogg and others 1993; Rubbo and others 1994). 3-Nitrotyrosine is a specific biomarker of $ONOO^{\bullet-}$-mediated protein oxidation. $ONOO^{\bullet-}$-mediated LDL oxidation may occur *in vivo* as indicated by the presence of 3-nitrotyrosine in human atherosclerotic plaques (Buttery and others 1996) and in LDL isolated from human atherosclerotic lesions (Leeuwenburgh and others 1997). Endothelial cells and macrophages are the major cell types that produce NO in atherosclerotic lesions as evidenced by the high levels of 3-nitrotyrosine present in these cell types (Beckmann and others 1994). Furthermore, iNOS protein was found in human atherosclerotic plaques and was co-localized with 3-nitrotyrosine in macrophages (Buttery and others 1996). Inducible NO production from macrophages may, therefore, play a key role in atherosclerosis.

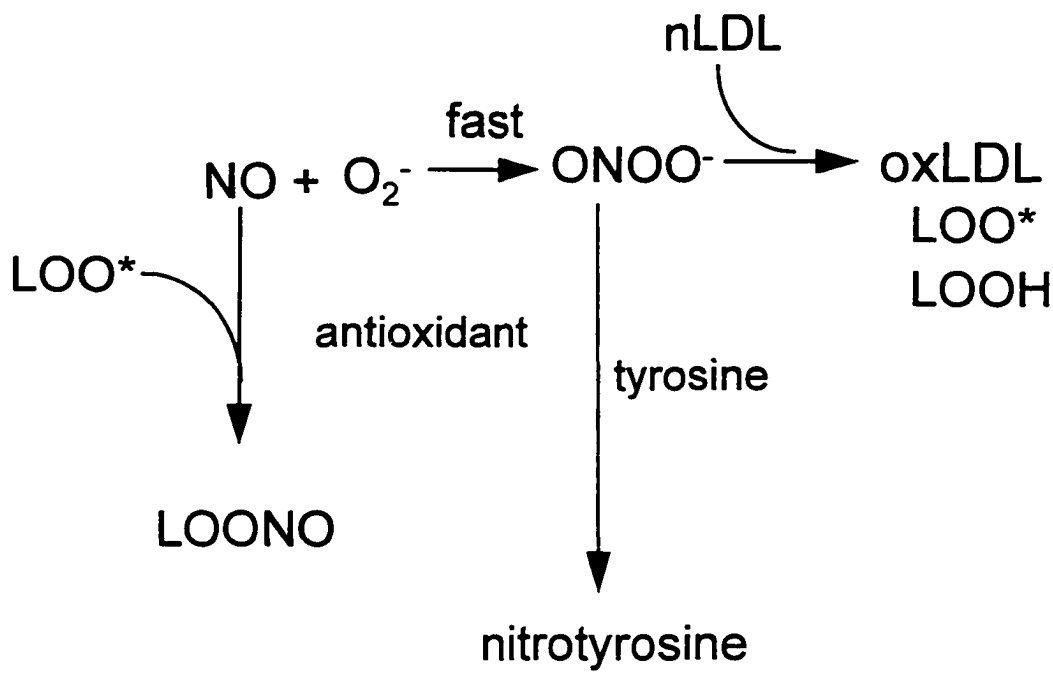


Figure 1-3 The possible roles of nitric oxide and peroxynitrite. Abbreviations: lipid peroxyl radical (LOO$^{\bullet}$); lipid hydroperoxide (LOOH); peroxynitrite (ONOO$^{\bullet-}$); organic peroxynitrite (LOONO); nitric oxide (NO); native low density lipoprotein (nLDL); oxidatively modified low density lipoprotein (oxLDL).

Gene Regulation and Oxidative Stress

OxLDL and NO Production in Macrophages

Endothelial dysfunctions occur at the very early stages of atherosclerosis (for a review see Lloyd-Jones and Bloch 1996). OxLDL was found to decrease constitutive NO production in human vein endothelial cells by inhibiting the transcription of ecNOS mRNA (Liao and others 1995). Inducible NO production in stimulated murine macrophages was also shown to be inhibited by oxLDL (Bolton and others 1994; Thai and others 1995; Yang and others 1994). Recent studies further indicate that oxLDL decreases mRNA for iNOS, tumor necrosis factor α (TNF-α), interleukin 1α (IL-1α) and interleukin 1β (IL-1β) in mouse peritoneal macrophages (Hamilton and others 1995; Ohlsson and others 1996; Schackelford and others 1995). However, the mechanisms whereby oxLDL inhibits the expression of iNOS and other inflammatory related genes in macrophages are not well understood.

Xie and others (Xie and others 1992; Xie 1997) have provided detailed information on the regulation of mouse iNOS gene in RAW264.7 macrophages. Mouse iNOS gene is under quite complicated regulation. There are at least 22 transcription factor binding sites in its promotor sequence (Xie and others 1993). The activation of NF-κB is believed to be required for the induction of iNOS by LPS (Goldring and others 1995; Xie and others 1994). Other transcription factor binding sites include two of each for activating protein (AP-1) and interferon-gamma (IFN-γ). OxLDL has been found to suppress the activation of NF-κB in

LPS and /or IFN-γ stimulated macrophages (Ohlsson and others 1996; Schackelford and others 1995). Schackelford and others (1995) have suggested that the ligation of scavenger receptors by oxLDL activates a pertussis toxin sensitive signaling pathway in macrophages. In this model, oxLDL scavenger receptors are coupled to a Gi protein, which initiates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (IPI_2) and increases intracellular fluxes of Ca^{++}. However, Schackelford and others (1995) are unable to explain why AcLDL has no effect since AcLDL also binds to oxLDL receptors. The role of oxidative stress on the activation of Gi proteins and the suppression of NF-κB are also not addressed by this model.

Yang and others (1994) have found that the lipid extract but not the aqueous extract causes the inhibitory effect of oxLDL. Lysophosphatidylcholine (lysoPC) is considered a bioactive lipid molecule and has been suspected to cause the inhibition. LysoPC, however, causes only slight inhibition of NO production in murine macrophages (Yang and others 1994). The active lipid components in oxLDL that contribute to the inhibition on NF-κB activation and iNOS expression have not been identified.

Oxidative Stress and Gene Regulation

NF-κB and AP-1 are well recognized redox regulated transcription factors (Flohe and others 1997; Schreck and others 1992a). NF-κB is a ubiquitous pleiotropic transcription factor that binds to a specific DNA sequence (Flohe and others 1997). A family of proteins that are referred to as c-Rel proteins form the

NF-κB complex. The c-Rel proteins are classified according to their structural homology: the first class includes p50, p52 and their precursors (i.e., p100 and p105); the second class contains p65 (rel A), c-Rel and Rel B. NF-κB exists as a homodimer such as p50/p50 or heterodimer such as p50/p65. The function of NF-κB is inhibited by another family of proteins called the IκB family. As shown in a simplified model (see Figure 1-4), NF-κB*IκB complex is activated by the phosphorylation and degradation of IκB. NF-κB migrates to the nucleus, binds to a specific DNA sequence and regulates gene expression. The p50/p65 heterodimer contributes to transactivation (Schmitz and Baeuerle 1991), whereas the p50/p50 homodimer may bind to the same κB site thereby inhibiting p50/p65 transactivation (Franzoso and others 1992; Plaksin and others 1993). Furthermore, p100 and p105 proteins containing the IκB domain also inhibit the transactivation of NF-κB. The mechanisms whereby NF-κB regulates genes can be quite complex.

The role of oxidative stress in the activation of NF-κB is controversial. Early studies indicated that H_2O_2 activates NF-κB in T lymphocytes and monocytes (Israel and others 1992; Schreck and others 1991; Suzuki and Packer 1993). This activation was blocked by various antioxidants such as N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate. Minimally oxidized LDL (MM-LDL) has also been shown to activate NF-κB (Parhami and others 1993) and NF-κB regulated genes such as ELAM-1, ICAM-1, VCAM-1, MCP-1, G-CSF and M-CSF in cultured endothelial cells (Collins 1993). Recent evidence

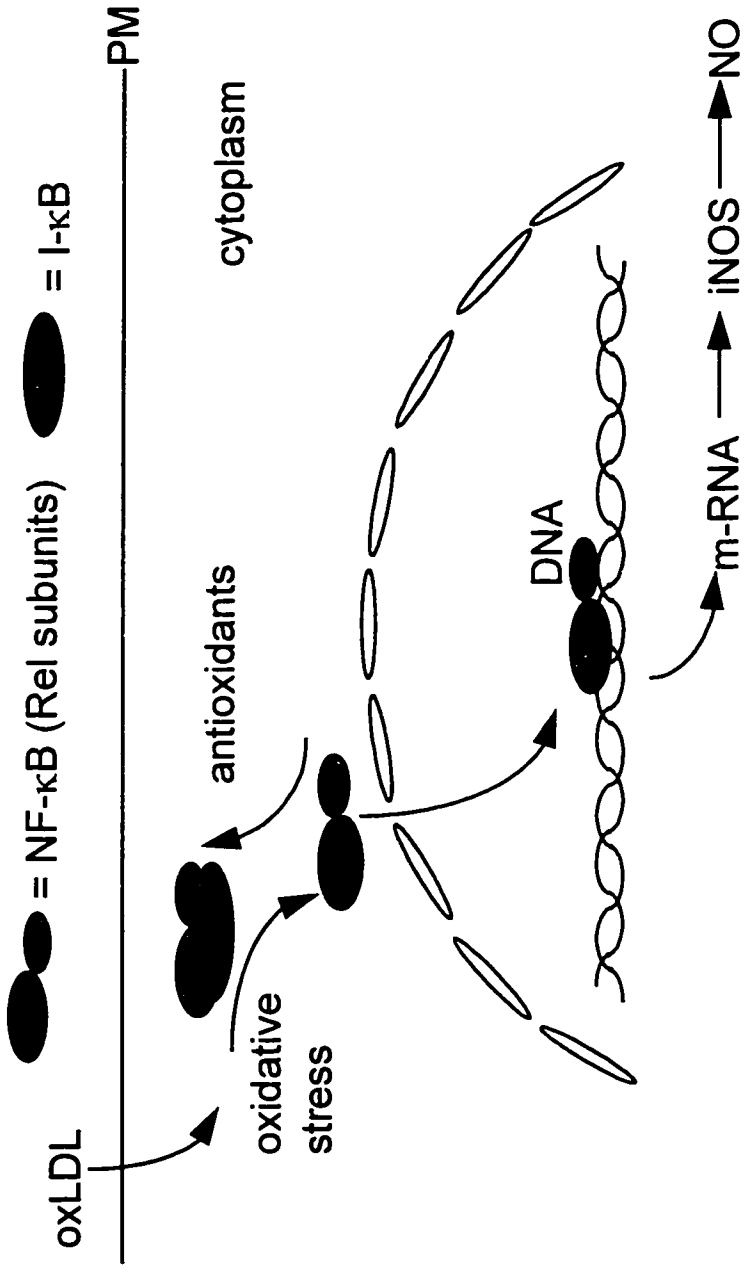
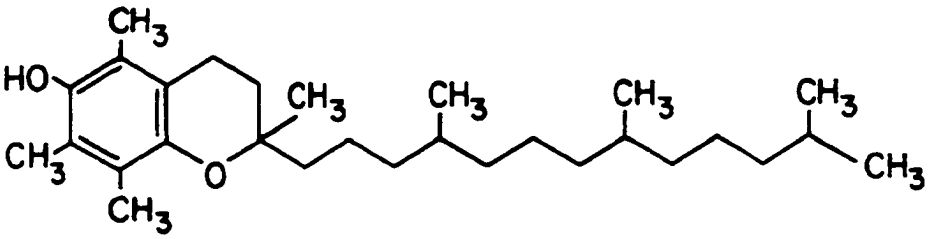


Figure 1-4 A simple model for the activation of NF-κB.

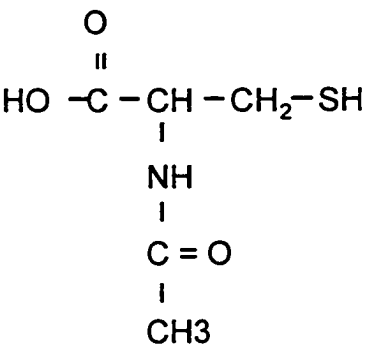
indicates that the activation of NF-κB by oxidants may, however, be limited to certain cell types (Brennan and O'Neill 1995; Hecker and others 1996).

Antioxidants and Atherosclerosis

Epidemiologic studies suggest an inverse relation between coronary arterial disease and antioxidant intake, particularly vitamin E supplementation (Diaz and others 1997; Steinberg 1995). Animal studies have also showed that high dietary intake of antioxidants delays atherogenesis (Parker and others 1995; Williams and others 1992). The benefits of antioxidants in preventing cardiovascular disease have been primarily related to protecting LDL against oxidation (Steinberg 1995). α-Tocopherol, the major lipid soluble antioxidant in plasma (Ingold and others 1987), effectively protects LDL lipids from peroxidation (Steinberg 1995; Ma and others 1994). α-Tocopherol has a chromanol ring with three methyl groups and a phytyl tail (Figure 1-5). In the presence of reactive free radicals, α-tocopherol donates a phenolic hydrogen atom more quickly than PUFA thereby protecting PUFA from peroxidation. In addition, α-tocopherol has been shown to have direct effects on vascular tissues and preserves normal vascular functions (Diaz and others 1997). Loading endothelial cells and macrophages with α-tocopherol enhances their resistance to oxLDL cytotoxicity (Kuzuya and others 1991). The incorporation of α-tocopherol into arterial cells preserves the vascular relaxation impaired by oxLDL (Kugiyama and others 1990). α-Tocopherol inhibits SMC proliferation by inhibiting protein kinase C (PKC) activity (Boscoboinik and others 1994).



VITAMIN E
(α - Tocopherol)



N-Acetylcysteine

Figure 1-5 Chemical structures of α-tocopherol and N-acetylcysteine.

Furthermore, α-tocopherol inhibits monocyte adhesion to human endothelial cells (Devaraj and others 1996). α-Tocopherol may, therefore, reduce the clinical expression of established coronary arterial disease by reducing LDL oxidation as well as by its direct effect on vascular tissues (Stephens and others 1996).

NAC is a non-toxic thiol-containing drug (Figure 1-5) used for the treatment of diseases related to hypersecretion of mucus, pulmonary oxygen toxicity, adult respiratory distress syndrome and potentially for HIV infections (Moldeus and Cotgreave 1994). NAC functions as an antioxidant by scavenging reactive oxygen species such as $O^{\bullet}_2^-$, H_2O_2, $HO^{\bullet}$ and hypochlorous acid. NAC is readily deacetylated in endothelial and other cells to liberate cysteine (Moldeus and Cotgreave 1994). NAC provides cysteine for GSH biosynthesis and is therefore considered to be a GSH precursor. GSH is the main intracellular water soluble antioxidant. Treatment of macrophages and endothelial cells with NAC causes a significant increase in cellular GSH levels (Faruqi and others 1997; van 1995). Recently, NAC has been shown to effectively block the activation of NF-κB in HIV infected lymphocytes (Roederer and others 1993; van 1995) and modulate NF-κB regulated gene expression in leukocytes (Faruqi and others 1997; Schreck and others 1992b). NAC is, therefore, considered to be an excellent intracellular antioxidant for studying cellular redox sensitive mechanisms.

Specific Aims

The purpose of this research was to study the effects of oxLDL on the regulation of murine iNOS gene expression in LPS stimulated RAW264.7 macrophages and to identify the lipid components that contribute to this regulation. In addition, the effects of antioxidants in this model system were also investigated. Our specific aims are:

Specific aim I	To characterize the effects of LOOH in oxLDL on inducible NO production in stimulated RAW264.7 macrophages
Specific aim II	To compare to the effects of synthetic 13-HPODE or lysoPC on NO production in stimulated RAW264.7 macrophages
Specific aim III	To determine whether antioxidants effect on NO production in stimulated RAW264.7 macrophages
Specific aim IV	To study the effects of oxLDL, 13-HPODE and lysoPC on the expression of iNOS gene in stimulated RAW264.7 macrophages.

CHAPTER 2

MATERIALS AND METHODS

Part I -- Effects of Oxidized Low Density Lipoprotein on Nitric Oxide Production in LPS Stimulated RAW264.7 Macrophages

Overall Experimental Design

Human LDL was isolated by sequential ultracentrifugation. Aliquots of the purified LDL were either oxidized by exposure to a water soluble azo-initiator azobis-2-amidinopropane HCl (ABAP) or acetylated by acetic anhydride. OxLDL, AcLDL and LDL were extensively dialyzed and the levels of α-tocopherol as well as LOOH were determined. LDLs were diluted in RPMI-1640 medium supplemented with 0.1% bovine serum albumin (RPMI-0.1%BSA) to give final concentrations of 25-100 μg protein/ml before being added to macrophages. In control experiments, an aliquot of LDL dialysate replaced oxLDL. RAW264.7 macrophage-like cells were co-incubated with LPS and various concentrations of OxLDL, AcLDL or LDL for 24 hours in RPMI-0.1%BSA. The production of NO was determined by measuring NO_2^- accumulation in the medium. At the end of the experiments, cell viability and mitochondrial dehydrogenase activity were determined.

Materials

2,2'-Azobis (2-amidinopropane) HCl (ABAP) was obtained from Polysciences, Inc. (Warrington, PA) and stored at 4 °C. Ethylenediamine-tetraacetic acid (Na_4EDTA), propyl gallate, sodium acetate, acetic anhydride, monobasic, dibasic sodium phosphate, HCl, phosphoric acid (H_3PO_4), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), N-(1-naphthyl)ethylenediamine dihydrochloride, sufanilamide, *Escherichia coli.* lipopolysaccharide serotype 0111:B4 (LPS), and tissue culture grade bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium chlo (NaCl), potassium bromide (KBr), HPLC grade methanol, and hexane were purchased from Fisher Scientific (Fair Lawn, NJ). Absolute ethyl alcohol was obtained from Florida Distillers Company (Lake Alfred, FL). Tocol was a generous gift from Hoffman-La Roche Inc. (Nutley, NJ). RPMI-1640 medium without phenol red, fetal bovine serum (low endotoxin), penicillin, streptomycin, and trypan blue were purchased from Life Technologies (Gaithersburg, MD). Sodium dodecyl sulphate (SDS) was purchased from Hoeffer Scientific Instruments (San Francisco, CA).

Cell Culture

RAW264.7 murine macrophage-like cell line (American Type Culture Collection, Rockville, MD) was cultured at 37 °C in a humidified incubator (95% air with 5% CO_2) in RPMI-1640 medium with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Adherent cells with 10^6/well or 10^7/dish

were subcultured over night in 12 well falcon tissue culture plates or 60 mm petri dishes in serum-free RPMI-0.1%BSA. The experiments were performed the next day after the medium was replaced by fresh RPMI-0.1% BSA.

Lipoprotein Isolation

Human LDL (density 1.019-1.063 g/ml) was isolated from plasma of healthy individuals by sequential ultracentrifugation using a Sorvall OTD-65B ultracentrifuge (Ma and others 1994). Briefly, plasma was centrifuged at 78,303 x g (25,000 rpm) using a Type 30 Beckman rotor for no less than 18 hours at 4 °C. After a layer of chylomicron and very low density lipoprotein (VLDL) was removed, the remaining plasma was adjusted to a density of 1.063 g/ml and centrifuged at 267,800 x g (60,000 rpm) for 24 hours at 4 °C using a Sorvall Ti 865.1 rotor. The LDL layer was collected and dialyzed with bubbling nitrogen gas against 3 liters of 0.15 M NaCl with 3 changes at 4 °C. LDL was filtered (0.22 μm) and stored at 4 °C in sterile containers under N_2 until used within two weeks. This LDL preparation was further dialyzed to remove EDTA before being added to macrophage cultures.

SDS-PAGE electrophoresis

The purity of LDL was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed according to the method of Laemmli (1970) using a 7.5% acrylamide gel at 20 mA in a Mighty Small II slab gel electrophoresis unit (Hoeffer Scientific Instruments, San

Francisco, CA). Gels were stained with Coomassie Blue R-250, and then destained with a solution containing 10% methanol, and 7.5% acetic acid. A single apolipoprotein polypeptide was observed that had an electrophoretic mobility identical to that of apo B-100.

Oxidation of Low Density Lipoprotein

An aliquot of LDL (2-5 mg protein/ml) was oxidized by incubating with 15 mM ABAP in 0.15 M NaCl solution at 30 °C. LDL oxidation was stopped by removal of ABAP by exhaustive dialysis against 3 liters of 0.15 M NaCl with 5 changes for over 24 hours at 4 °C. ABAP was undetectable in the dialysate by UV spectrometry. OxLDL was then filtered (0.22 μm) and stored as described for LDL. LOOH and tocopherol content of oxLDL was determined (as described below) immediately prior to macrophage experiments.

OxLDL is usually prepared by Cu^{++} or Fe^{++} for long lengths of time (Hamilton and others 1995; Schackelford and others 1995). In many cases the transition metal ions are not removed from LDL by dialysis (Yang and others 1994). LDL also has sites that bind Cu^{++} very strongly and dialysis may not be sufficient to completely remove all Cu^{++}. Oxidized LDL with trace amount of metal ions may influence cellular functions (Coffey and others 1995). Furthermore, Cu^{++} or Fe^{++} catalyzes the degradation of LOOH formed during LDL oxidation (O'Brien 1969). In the present study, we oxidized LDL using ABAP rather than transition metal ions so as to prevent nonenzymatic decomposition of LOOH (O'Brien 1969). OxLDL prepared in this manner has been well

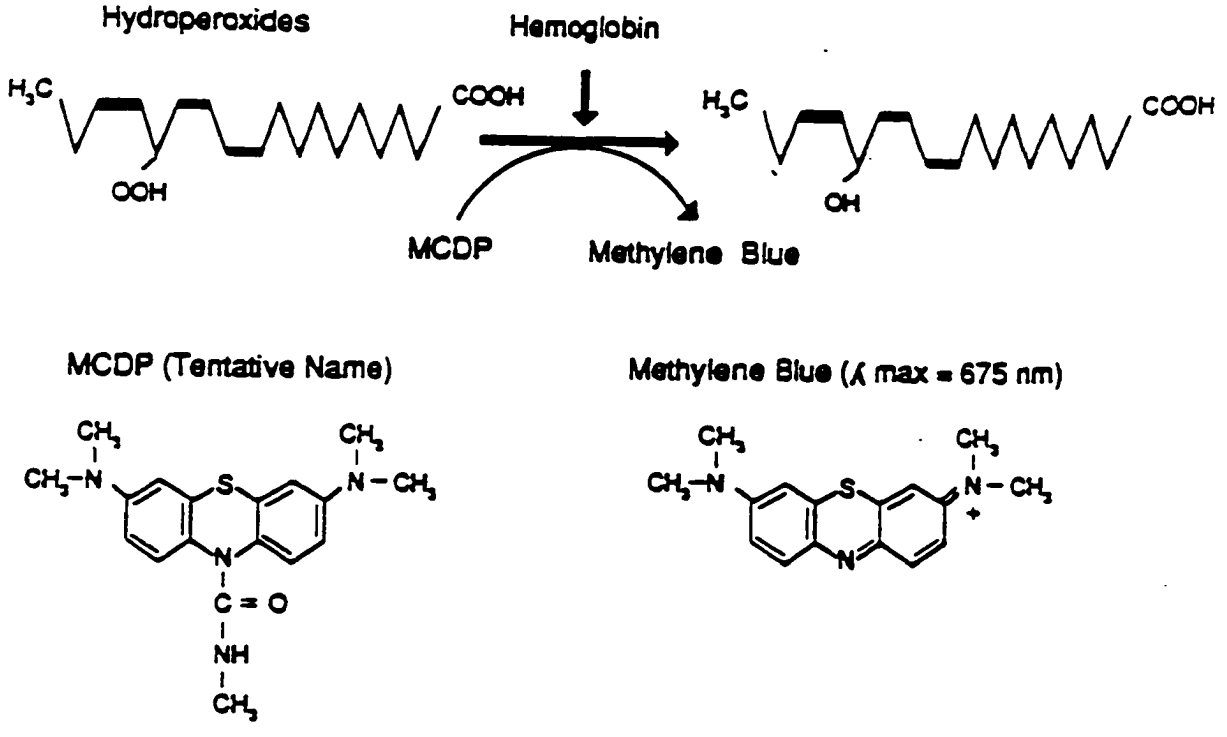
characterized (Ma and others 1994) and is taken up by macrophages via scavenger receptors that also recognizes AcLDL (Kawabe and others 1994).

Acetylation of Low Density Lipoprotein

LDL was acetylated using acetic anhydride as described by Basu and others (1976). Briefly, a 2.0 ml aliquot of LDL was saturated with solid sodium acetate. An 2 μl aliquot of acetic anhydride was added to LDL several times over a one hour period with continuous shaking, until the total amount of acetic anhydride was 1.5 times of LDL (by weight). AcLDL was then dialyzed, filtered, and stored as described for oxLDL.

Measurement of Lipid Hydroperoxides

LOOH levels in oxLDL were determined by a sensitive colorimetric assay as described by Tateishi and others (1987) using a commercial kit obtained from Kamiya Biomedical Co. (Thousand Oaks, CA). In the presence of hemoglobin, LOOH stoichiometrically reacts with the methylene blue derivative 10-N-methylcarbamoyl-3,7-dimethylamino-10-H-phenothiazine to generate methylene blue of which absorbance was determined at 600 nm using a microplate reader (Molecular Device UVmax) (Figure 2-1). Cumene hydroperoxide was used as a standard for determining the concentrations of LOOH in oxLDL.



MCDP: 10-N-Methylcarbamoyl-3, 7-dimethylamino-10 H-phenothiazine

Figure 2-1 Mechanism of the colorimetric assay for the determination of lipid hydroperoxides.

Tocopherol Measurement in Lipoprotein

Tocopherols were extracted into the hexane layer after mixing a 100 ml aliquot of LDL, AcLDL or oxLDL, an equal amount of ethanol (with tocol as internal standard) and 200 ml hexane containing 50 mg/ml BHT. A 150 ml aliquot of hexane was collected, dried under N_2, and redissolved in 100 ml ethanol. A 65 ml aliquot of this ethanol was injected into the HPLC system (ISCO Inc., Lincoln, NE) equipped with a reverse phase C18 ODS (4.6 mm x 25 cm) column (ISCO Inc., Lincoln, NE). Tocopherols were eluted by methanol:H_2O (99.5%:0.5%, v/v) at a flow rate of 1.5 ml/min, and its fluorescence was monitored at an excitation wavelength of 294 nm, and an emission wavelength of 324 nm using a fluorescent detector (McPherson Model FL-750). The amounts of tocopherols were determined using tocol as an internal standard.

Determination of Protein in Lipoprotein

The concentration of LDL protein was determined by a modified Lowry method (Markwell and others 1978) using BSA as standard.

Tocopherol Measurement in Macrophages

Tocopherol extraction was performed using a method modified from Lang and others (1986). After pre-treatment of macrophages with liposomes containing α-tocopherol or control liposomes for 8 hours, cells were harvested and washed three times with phosphate buffer saline (PBS) containing 50 μg/ml BHT by centrifugation at 1500 rpm using ICE national centrifuge for 2 minutes.

Cells were resuspended in PBS to give a cell density of 10^7 cells/ml. A 1.0 ml aliquot of cell suspension was mixed with an equal volume of 0.16 M SDS by vigorously vortexing. A 2.0 ml aliquot of ethanol (with tocol as internal standard) and 2.0 ml hexane containing 50 μg/ml BHT were added to the cell lysate for extraction. A 1.5 ml aliquot of hexane was collected after centrifugation, dried using Virtis spinvac and redissolved in 200 μl ethanol. Cellular tocopherol content was determined by HPLC as described above for the determination of tocopherols in plasma.

Determination of NO Production from Macrophages

The production of NO, reflecting cellular NOS activity, was estimated from the accumulation of nitrite (NO_2^-), a stable breakdown product of NO, in the medium. NO_2^- was measured spectrophotometrically using the Greiss reagent as described by Green and others (1982). Briefly, Greiss reagent was freshly prepared by mixing reagent 1 and reagent 2 prior to experiment. Reagent 1 contains 1.0% sufanilamide and 5% phosphoric acid and reagent 2 contains 0.1% naphthylenediamide hydrochloride. An aliquot of cell cultured medium was mixed with equal volume of Greiss reagent that reacts with NO_2^- to form the azo dye product immediately. The absorbance of the reaction product was recorded at 532 nm using a microplate reader. Sodium nitrite ($NaNO_2$) dissolved in the identical medium was used as a standard for determining the accumulation of NO_2^- in the culture medium.

Determination of Cell Viability by Trypan Blue Dye Exclusion

At the end of the experiment, adherent cells were dissociated by 0.25% trypsin and strained with 0.1% trypan blue solution. Macrophages, including live and stained dead cells, were counted and the percent of viable cells was determined.

MTT Assay for Determination of Cell Viability

This assay is based on the cellular reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenase activity in viable cells to produce a purple formazan product, which is measured spectrophotometrically (Pirillo and others 1997). The MTT assay is widely used to determine cytotoxicity, cell viability and cell proliferation (Clare and others 1995; Pirillo and others 1997). Briefly, at the end of each experiment, cells were washed twice with PBS and incubated with MTT at 37 °C for 4 hours so that MTT was converted to purple formazan crystals. Formazan product was then solubilized by SDS in acidic solution for 16 hours at 37 °C. The mitochondrial dehydrogenase activity was expressed as the absorbance of formazan product at 570 nm.

Statistical Analysis

The comparison between treatments and control was performed by the Student *t*-test. Means among treatments were compared by one-way ANOVA followed by the least significant difference. A $p < 0.05$ was considered as statistically significant.

Part II -- Effects of 13-HPODE and LysoPC on Nitric Oxide Production in LPS Stimulated RAW264.7 Macrophages

Overall Experimental Design

The effects of 13-HPODE and lysoPC on NO production in LPS stimulated macrophages were determined. An aliquot of 13-HPODE in ethanol was dried under nitrogen gas and redissolved in RPMI-0.1% BSA or medium containing 50 μg/ml AcLDL carrier at 4 °C for 4-6 hours with shaking to give final concentrations of 5-40 μM 13-HPODE. 13-HPODE were assayed as described for LOOH in oxLDL prior to cell culture experiments. LysoPC was dissolved in media as described for 13-HPODE to final concentrations of 50-100 μM. RAW264.7 macrophages were co-incubated with 100 ng/ml LPS and the indicated concentrations of 13-HPODE or lysoPC in RPMI-0.1% BSA medium and the production of NO was determined after 24 hour stimulation. In a parallel experiments, 13-HPODE or lysoPC in the AcLDL carrier was added to RAW264.7 macrophages alone with LPS and NO production was determined after 24 hours. At the end of the experiments, cell viability, mitochondrial dehydrogenase activity and DNA content of the cells were determined.

Materials

Soybean lipoxygenase, octadecadienoic acid, boric acid, copper sulphate ($CuSO_4$), acetaldehyde, and chloroform were obtained from Sigma Chemical Company (St. Louis, MO). Ethyl ether was obtained from Aldrich Chemical

Company, Inc. (Milwaukee, WI). Egg lysolecithin (lysophosphatidylcholine) was obtained from Matreya Inc. (Pleasant Gap, PA). Diphenylamine was from J.T. Baker Chemical Co. (Phillipsberg, NJ). Perchloric acid and glacial acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ).

Synthesis and Purification of 13-HPODE

13-HPODE was prepared by a method modified from Egmond and others (1976). Briefly, a 100 ml aliquot of 1 mM octadecadienoic acid in 0.1 M sodium borate buffer (pH 9.0) was incubated with 3.7×10^4 U of soybean lipoxygenase on ice with gentle air bubbling for over 12 hours.

The conversion of octadecadienoic acid to 13-HPODE (see reaction 1-2) was monitored by thin layer chromatography (TLC). A 2.0 ml aliquot of borate buffer reaction mixture was acidified to pH 3.0 and 13-HPODE was extracted into ethyl ether which was then evaporated. 13-HPODE was redissolved in 2.0 ml ethanol and a 100 μl aliquot was applied to a Silica G TLC plate, octadecadienoic acid in ethanol was used as a standard. Octadecadienoic acid and 13-HPODE were then resolved using a solvent system containing ethyl ether : chloroform : acetic acid (100 : 100 : 1 0) (v/v/v) in 1.5 hours. Spots for octadecadienoic acid and 13-HPODE were visualized by charring with 10%$CuSO_4$ and 8% H_3PO_4 at 170-180 °C for 20 minutes. The conversion was determined by comparing the density of spot for 13-HPODE with the total density of spots for both octadecadienoic acid and 13-HPODE.

The reaction was stopped by lowering the pH to 3.0 when the conversion was completed. 13-HPODE was extracted in ethyl ether which was completely evaporated. The resulting 13-HPODE was redissolved in 15 ml absolute ethanol, filtered using an Acrodisc CR PTFE 0.2 μm filter (GelmanSciences, Ann Arbor, MI) and stored at -20 °C. The final concentration reached 7.5 mM as determined from the absorbance at 234 nm using a molar absorptivity of 25,000 $M^{-1}cm^{-1}$. The LOOH value determined by this method was in agreement with the value obtained from the colorimetric assay described in Part I.

Measurement of Cellular DNA Content by the DPA Assay

Cellular DNA content was quantified using diphenylamine (DPA) according to the method described by Natarajan and others (1994). Briefly, at the end of each experiment, cells were washed with PBS and incubated with DPA in glacial acetic acid at 37 °C for 24 hours in the presence of acetaldehyde and perchloric acid. The blue reaction product of DPA was quantified by measuring OD at 650 nm. A standard curve was generated by incubating varying numbers of cells with DPA and the ODs generated correlated fairly well with cell numbers.

Part III -- Effects of α-Tocopherol and N-Acetylcysteine on the Decreased Nitric Oxide Production Caused by OxLDL and 13-HPODE

Overall Experimental Design

The effects of antioxidants such as α-tocopherol and NAC on the

inhibition of NO production caused by oxLDL or 13-HPODE were determined. RAW264.7 macrophages were pre-incubated with either liposomes containing α-tocopherol, control liposomes or NAC for 8 hours before treatment with LPS and oxLDL or 13-HPODE. The pre-incubation time for NAC was within the range used by Irani and others (1997). At the end of the experiments, cell viability and DNA content of the cells were determined.

Materials

N-acetyl-L-cysteine was obtained from Sigma Chemical Company (St. Louis, MO). α-Tocopherol liposomes and control liposomes were generous gifts from SEQUUS™ Pharmaceuticals Inc. (Menlo Park, CA).

Part IV -- Regulation of iNOS Gene Expression by Oxidized Low Density Lipoprotein in LPS Stimulated RAW264.7 Macrophages

Overall Experimental Design

RAW264.7 macrophages were co-incubated with LPS and the indicated concentrations of oxLDL, AcLDL or LDL for 24 hours as described in part I. The accumulation of NO_2^- in the medium was measured and the adherent cells were washed with PBS followed by the extraction of cellular proteins. The iNOS protein was analyzed by the Western blotting technique. Similarly, the effects of 13-HPODE and lysoPC on iNOS protein levels were also determined. The iNOS mRNA levels were analyzed by Northern blotting after macrophages were stimulated with LPS for 6 hours in the presence or absence of oxLDL. For the

detection of NF-κB activation, RAW264.7 macrophages were stimulated for the lengths of time varying from 0 to 120 minutes. Nuclear proteins were extracted and the activation of NF-κB was detected by the electrophoretic mobility shift assay (EMSA). The effects of oxLDL on NF-κB activation was determined by EMSA after 45 minute incubation with LPS in the absence or presence of oxLDL. In control experiments, an aliquot of oxLDL dialysate was used to replace oxLDL.

Materials

NF-κB oligonucleotide, human recombinant NF-κB p50, mouse iNOS cDNA, mouse GADPH cDNA, ^{32}P-dCTP, herring sperm DNA, and random primer labeling system were obtained from Promega (Madison, WI). An electrophoretic gel shift assay (EMSA) kit, and positively charge nylon membrane were purchased from Boehringer Mannheim Corporation (Indianapolis, IN). Anti-p50 and anti-p65 antibodies were obtained from Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-mouse iNOS antibody was from Transduction Laboratory (Lexington, KY). An enhanced chemiluminescent detection kit, the Western nitrocellulose membrane, and nylon membrane for Northern blot were purchased from Amersham Life Science (Arlington Heights, IL). Horseradish peroxidase conjugated anti-rabbit polyclonal antibody, leupeptin, antipain, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), agarose, formamide, formaldehyde, poly dI-dC, isopropanol, MOPS buffer, Tween-20, HEPES, Tris-Glycine SDS buffer, magnesium chloride, TEMED, glycerol, nonidet-P40, and

Kodak X-OMAT AR film were obtained from Sigma Chemical Company (St. Louis, MO). Acrylamide, and bis-acrylamide were purchased from Hoeffer Scientific Instruments (San Francisco, CA).

Cellular Protein Extraction

Cellular proteins were extracted following the instructions provided by Transduction Laboratory (Lexington, KY). Briefly, after stimulation for 24 hours, 1 x 10^6 adherent macrophages were rinsed once with cold PBS, and lysed by 0.1 ml of boiling electrophoresis sample buffer containing 125 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 1% mercaptoethanol, and 0.003% bromophenol blue. The cell lysate was collected into the Eppendorf centrifuge tubes, and boiled for an additional 5 minutes.

Western Blot Analysis

Gel electrophoresis of cellular proteins was performed according to the method of Laemmli (1970). Twenty μg of proteins was separated on a 8% SDS-PAGE with Tris-Glycine SDS running buffer (pH 8.3) at 25 V in a Mighty Small II slab gel electrophoresis unit (Hoeffer Scientific Instruments, San Francisco, CA) for 1.5 hours. Proteins were electrically transferred from the gel to a nitrocellulose membrane with Tris-Glycine transferring buffer (pH 8.3) with 20% methanol for 1.0 hour. The iNOS protein was detected using the enhanced chemiluminescence kit from Amersham Life Science (Arlington Heights, IL). Briefly, the blots were incubated with blocking buffer (PBS pH 7.0, 2% non-fat

milk, 0.1% Tween-20) for 1.0 hour before incubation with the rabbit polyclonal antiserum against the C terminal (961 to 1144 amino acids) of mouse iNOS (Transduction Lab, Lexington, KY) at 1:20,000 dilution. After 60 minute incubation at room temperature, the blots were washed three times with PBS (0.1% Tween-20). Anti-rabbit horseradish peroxidase-conjugated antibody at 60:000 dilution as a secondary antibody was incubated with the blots for 1.0 hour. The blots were then washed twice with assay buffer, and a 2.0 ml aliquot of substrate plus enhancer mixture was evenly added onto the blots. The photograph of iNOS protein was obtained by exposure to Kodak film for 10-25 minutes.

RNA Extraction

Total cellular RNA was isolated from RAW264.7 macrophages by acid-guanidinium phenol-chloroform method using RNAzol™ B RNA isolation kits (TEL-TEST, Inc., Friendswood, TX). Briefly, after removing the medium, 8 x 10^6 adherent cells were lysed in 1.0 ml RNAzol™ B solution by pipetting up and down several times. The lysate was collected and mixed with 100 μl of chloroform by inversion. The aqueous fraction of the mixture was collected after centrifugation at 12,000 g for 15 minutes at 4 °C, and mixed with another 100 μl aliquot of chloroform followed by vigorously vortexing. After three extractions, the aqueous fraction was collected to a fresh tube followed by an additional wash with 500 μl ethanol. RNA was then precipitated with 500 μl isopropanol at -20 °C for at least 30 minutes followed by centrifugation at 7500 g for 8 minutes. After

the supernatant was discarded, RNA pellet was dried, redissolved in 50 μl of DEPC-treated distilled H_2O and stored at -70 °C. The concentration of RNA was quantified by measuring the absorbance at 260 nm (1 OD= 40 μg/ml), and the purity of RNA was determined by the OD ratio at 260 nm/280 nm that was at least I.6 in all samples.

Radio Labeling of DNA Probes

Mouse iNOS and glyceraldehyde-3-phosphodehydrogenase (GAPDH) cDNA probes were radiolabeled with α^{32}P-dCTP using random primer labeling system following the manufactural's instructions (Promega, Madison, WI). Briefly, 30 μg of DNA for iNOS or GAPDH was denatured by incubation at 100 °C for 5 minutes and chilled on ice. Thirty μg of cDNA was mixed with 10 μl of 5 x labeling buffer, 2 μl of a mixture for dATP, dTTP and dGTP, 2.0 μl of nuclear-free BSA, 5 μl of α^{32}P-dCTP (50 μ Ci, 3000 Ci/mmole) and 5 units of DNA polymerase I. The mixture with a total volume of 50 μl was incubated at 37 °C for 1.0 hour until the polymerase reaction was close to complete. The labeled DNA was then denatured by heating to 100 °C for 5 minutes, and chilled on ice before hybridization.

Northern Blot Analysis

Northern blotting was performed as described by Gong and others (1994). Briefly, an aliquot of suspension containing 20 μg of RNA was dried by vacuum centrifugation and lyophilized into a 20 μl aliquot of denaturing and loading buffer

(45% formamide, 6% formaldehyde, 5% glycerol, bromophenol blue in 20 mM MOPS buffer). RNA samples were denatured by incubation at 100 °C for 2 minutes before loading into the gel. RNA was fractionated by electrophoresis on a 1% agarose gel containing 2% formaldehyde using 1 x MOPS running buffer at 100 V for approximately 2 hours until bromophenol blue dye migrated 3/4 of the gel. RNA on the gel was examined, and photographed on UV transilluminator. The eukaryotic RNA of two abundant species 28S rRNA (approximately 5 Kb), and 18S rRNA (approximately 2 Kb) was visualized. After formaldehyde was completely removed from the gel by three washes using 10 x SSC buffer (1.5 M NaCl, 0.15 mM sodium citrate, pH 7.0), RNA was then transferred over night from the gel to a nylon membrane using 10 x SSC buffer.

The blots were pre-hybridized in 0.5 M phosphate buffer (pH 7.2, 7% SDS, 100 μg/ml herring sperm DNA) at 65 °C for 2-4 hours, and hybridized with α^{32}P-dCTP labeled iNOS cDNA at 65 °C for 15 hours. Non-hybridized probes were removed by three washes with 0.1 M phosphate buffer at room temperature (10 minutes for each wash) followed by other three washes with 0.05 M phosphate buffer at 65 °C (15 minutes for each wash). The autoradiography of iNOS mRNA was obtained by exposure to Kodak X-OMAT AR film for 24-48 hours at -70 °C. The α^{32}P-dCTP labeled GAPDH cDNA was used in the second hybridization as an internal standard for normalizing the variation between samples.

Nuclear Protein Extraction

Nuclear extracts were obtained essentially as described by Staal and others (1990). After stimulation, adherent cells (1 x 10^7) were harvested and washed twice in ice-cold PBS by centrifugation for 30 sec at 15,600 x g. The pelleted cells were then washed once in 0.4 ml of low salt buffer [10 mM HEPES, pH 7.8/ 10 mM KCl/ 2 mM $MgCl_2$/ 1 mM (DTT)/ 0.1 mM EDTA] containing protease inhibitors [0.5 mM of phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml of antipain and 5 μg/ml of leupeptin]. After incubation of cells on ice for 15 min, a 25 μl aliquot of 10% nonidet P-40 solution was added to lysis the cells. Nuclei were pelleted at 15,600 x g for 30 seconds, and resuspended in 50 μl of high salt buffer [50 mM HEPES, pH 7.8/ 50 mM KCl/ 300 mM NaCl/ 0.1 mM EDTA/ 1 mM DTT/ 0.1 mM PMSF/ 10% glycerol (v/v)]. The mixture was stand on ice for 20 minutes followed by centrifugation at 15,600 x g for 5 minutes. The clear supernatant containing nuclear proteins was collected, and stored at -80 °C.

Protein Determination

Protein concentrations of cell extract (for Western blotting), and nuclear extract (for EMSA) were determined by the BCA protein assay using a commercial kit from Pierce Chemical Co. (Rockford, IL). Protein samples were diluted with PBS before protein determination in order to minimize the interference from the ingredients in lysate buffer (for Western blotting), and high salt buffer (for EMSA). BSA dissolved in the diluted sample buffer or the diluted high salt buffer was used as a protein standard.

Electrophoretic Mobility Shift Assay (EMSA) for NF-κB Activity

The activation of NF-κB was detected by chemiluminescent EMSA using a commercial kit obtained from Boehringer Mannheim Co. (Indianapolis, IN). The steroid hapten digoxigenin (DIG) was bound to a 26 bp NF-κB oligonucleotide (oligo), and recognized by anti-DIG antibodies (anti-DIG) linked to alkaline phosphatase (AP). Labeled NF-κB oligo was visualized with AP-conjugated anti-DIG, and lumigen PPD chemiluminescent substrate according to the procedure detailed in the Genius System User's Protocol.

The double stranded oligo for NF-κB was labeled at the 3' end by adding the following reagents (on ice): 4 μl of 5 x labeling buffer, 4 μl of $CoCl_2$, (final concentration of 5 μM), NF-κB oligo (final concentration of 0.2 pmol/μl), 1 μl of DIG-11-ddUTP (final concentration of 0.2 μM), 1 μl of terminal transferase (final concentration of 2.5 units/μl) and H_2O to a final volume of 20 μl. After incubation at 37 °C for 15 minutes, DIG labeled oligo was precipitated with 100% chilled ethanol followed by three washes in 70% chilled ethanol by centrifugation at 15.600 x g for 5 minutes. The pelleted oligo was then vacuum dried and redissolved in 25 μl TEN buffer [10 mM Tris-HCl, pH 8.0/1 mM EDTA/0.1 M NaCl].

The reaction of DIG-labeled NF-κB oligo, and NF-κB protein was performed by adding the following reagents (on ice): 3 μl of 5 x binding buffer (20 mM HEPES, pH 7.6, 20 mM NaCl, 30 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM $(NH_4)_2SO_4$, 50 ng/μl poly d(I-C), 0.05% Tween 20), 1 μg of

salmon sperm DNA, 1 μg of BSA, 2 μl of DIG-labeled NF-κB oligo (140 fmol/μl), 15 μg protein of nuclear extract, and H_2O to a final volume of 15 μl. The reaction was performed at room temperature for 15 minutes, and stopped by adding 4 μl of loading buffer.

The oligo-protein complex and free oligo were separated on a 5% non-denatured polyacrylamide gel (20:1 acrylamide: bisacrylamide) electrophoresis in 22.5 mM Tris-borate (pH 8.3, 5 mM EDTA) running buffer at 90 V for 1.5 hours using an electrophoresis unit SE200 (Hoeffer Scientific Instruments, San Francisco, CA). The oligo-protein complex and free oligo were transferred to a positively charged nylon membrane using the PolyBlot transfer system Model SBD-100 (American Bionetics, Hayward, CA), and cross linked to membrane by exposure to UV light. Labeled oligo-protein complex, and oligo were recognized by AP-conjugated anti-DIG. AP catalyzed the conversion of lumigen PPD substrate into a chemiluminescent molecule that was visualized by exposure to Kodak Xomat AR film for 1-2 hours.

EMSA Supershift Assay

This assay detects the specific antibody bound complex by following the procedure for EMSA. Antibodies against NF-κB p50 and p65 subunits bind to the NF-κB dimers resulting in a slower mobility during electrophoresis. Briefly, the experimental procedures were identical to that of EMSA except that 1 μl of antibodies were added to the binding reaction mixture as described above before incubation for 30 minutes instead of 15 minutes.

Densitometry Analysis

The lumigrams of iNOS protein, iNOS mRNA and NF-κB bands from electrophoresis were analyzed using a Scan Maker II flat bed scanner connected to a computer. The TIFF images were quantified using the Sigma gel densitometry software (Jandel Scientific Co., San Rafael, CA)

CHAPTER 3

RESULTS

Part I -- Effects of Oxidized Low Density Lipoprotein on Nitric Oxide Production in LPS Stimulated RAW264.7 Macrophages

The Production of Nitric Oxide in LPS Stimulated RAW264.7 Macrophages

The production of NO by RAW264.7 macrophages, as estimated by the accumulation of NO_2^- in the medium, was dependent on the concentrations of LPS (Figure 3-1). A nearly linear accumulation of NO was observed at concentrations of LPS between 0 and 100 ng/ml. Furthermore, a 5-6 hour lag time was observed before significant amount of NO_2^- was produced, and the subsequent accumulation of NO_2^- continued for over 24 hours. No detectable amount of NO was produced in RAW264.7 macrophages without stimulation.

Oxidative Characteristics of OxLDL and the Inhibition Potential on NO Production in LPS Stimulated RAW264.7 Macrophages

OxLDL was prepared by oxidizing LDL using ABAP that can be removed from LDL preparations by extensive dialysis. Figure 3-2 shows that oxidation of LDL led to the consumption of α–tocopherol, and the formation of LOOH in oxLDL. α-Tocopherol at an initial level of 21.4 nmol/mg LDL protein completely disappeared within 2 hours of oxidation whereas LOOH gradually accumulated as a function of time. OxLDL was extensively dialyzed to completely remove

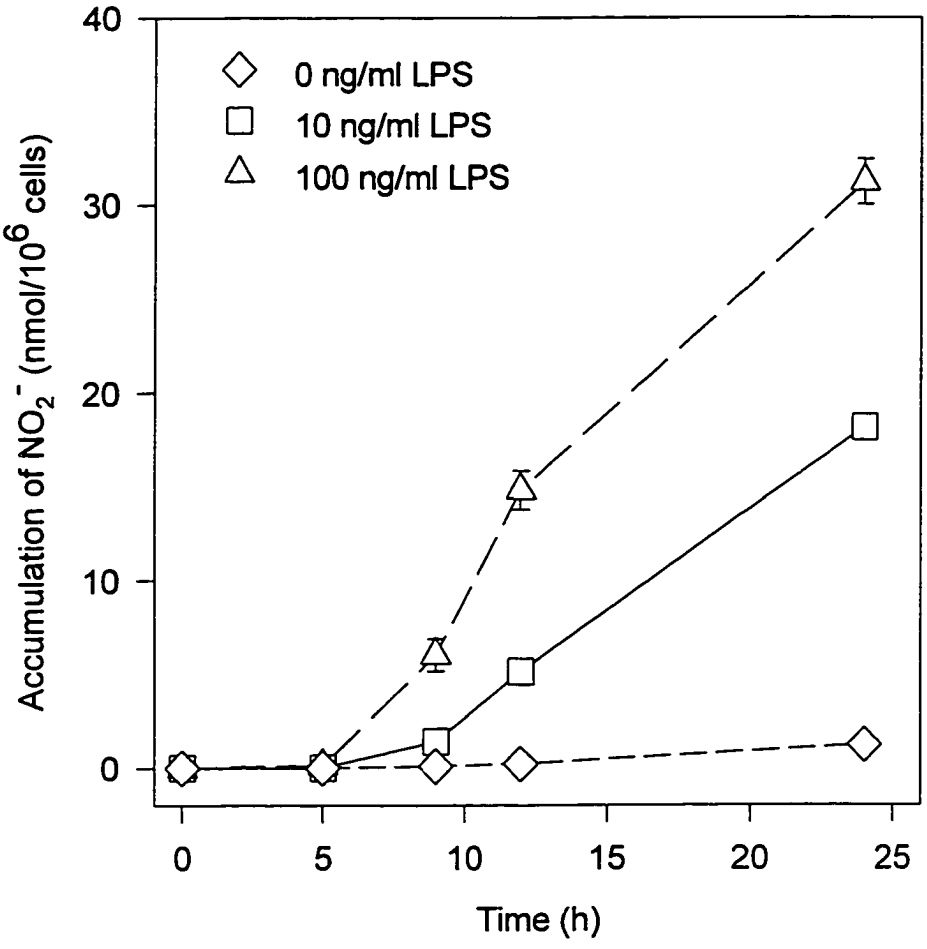


Figure 3-1. Time course of NO production induced by varying concentrations of LPS in RAW264.7 macrophages. Data points represent means ± S.D. of triplicate samples for a single experiment. These results are representative of three separate experiments.

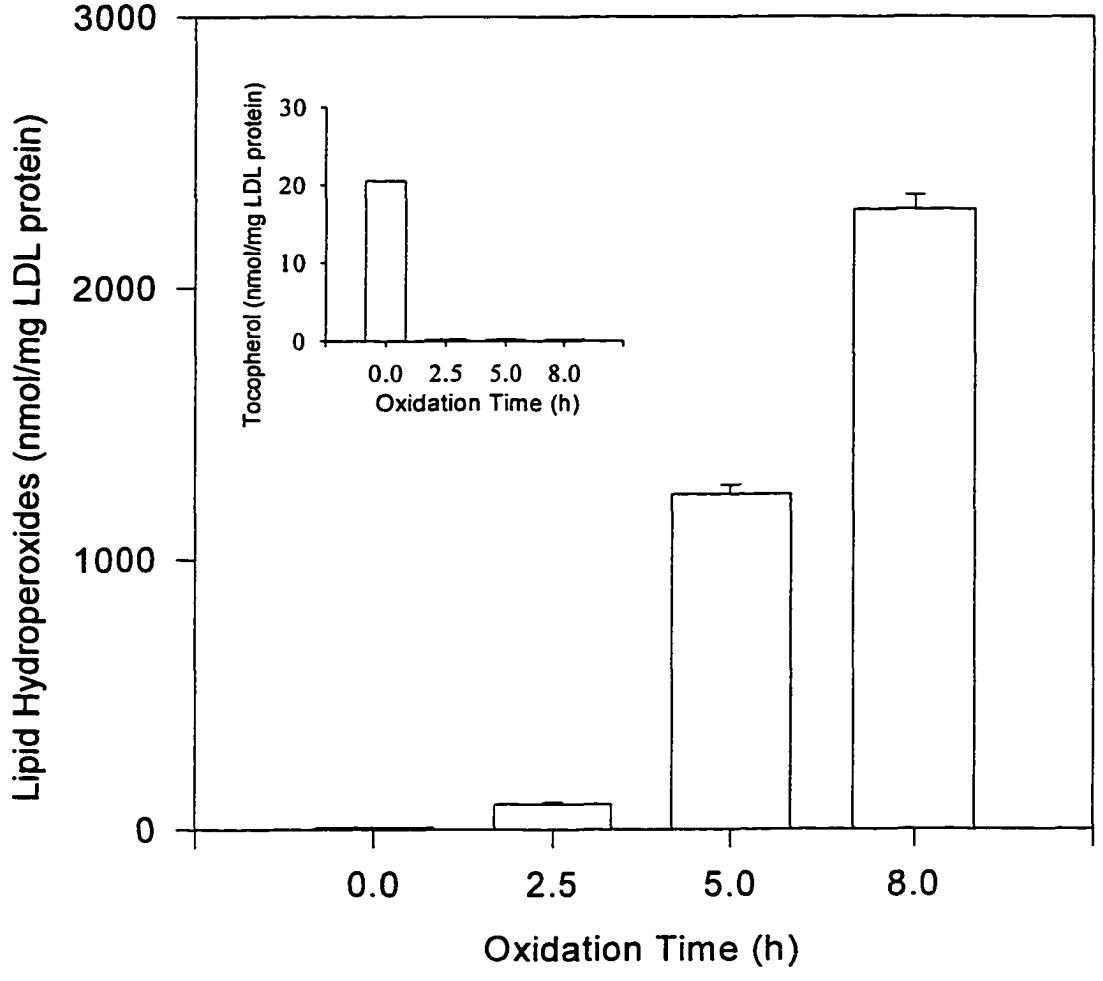


Figure 3-2. Oxidative characteristics of oxLDL. Human LDL was oxidized for 2.5, 5 and 8 hours and the LOOH content was measured by a colorimetric assay and α-tocopherol content was determined by HPLC.

ABAP before being used in the cell culture experiments. The oxLDL dialysate contained no measurable levels of LOOH. Our first series of experiments was designed to examine the extent of LDL oxidation on NO production in LPS stimulated macrophages. Figure 3-3 shows that oxLDL containing higher levels of LOOH exerted stronger inhibition on NO production. The oxLDL dialysate, however, had no effect on NO production in LPS stimulated macrophages (data not shown). Furthermore, oxLDL alone did not induce NO production in unstimulated macrophages (data not shown). These results suggest that LOOH levels determine the inhibition potential of oxLDL on NO production in RAW264.7 macrophages. The concentration dependent experiments, as shown in Figure 3-4, indicate that NO production in LPS stimulated macrophages was markedly inhibited by increasing concentrations of oxLDL in a nonlinear fashion. The rate of inhibition was greater at low concentrations than at high concentrations.

OxLDL on Cell Viability and Cellular Dehydrogenase Activity

To determine whether the inhibition is due to oxLDL cytotoxicity, cell viability was measured. At the end of experiments shown in Figure 3-3, 4, and 5, cell viability was maintained above 95% as accessed by trypan blue dye exclusion method (data not shown). Moreover, oxLDL did not show any cytotoxicity as determined by mitochondrial dehydrogenase activity using the MTT assay (see Table 3-1). Mitochondrial dehydrogenase activity, in fact, was slightly increased by the treatment of high levels of oxLDL, which may indicate a slight mitogenic effect of oxLDL.

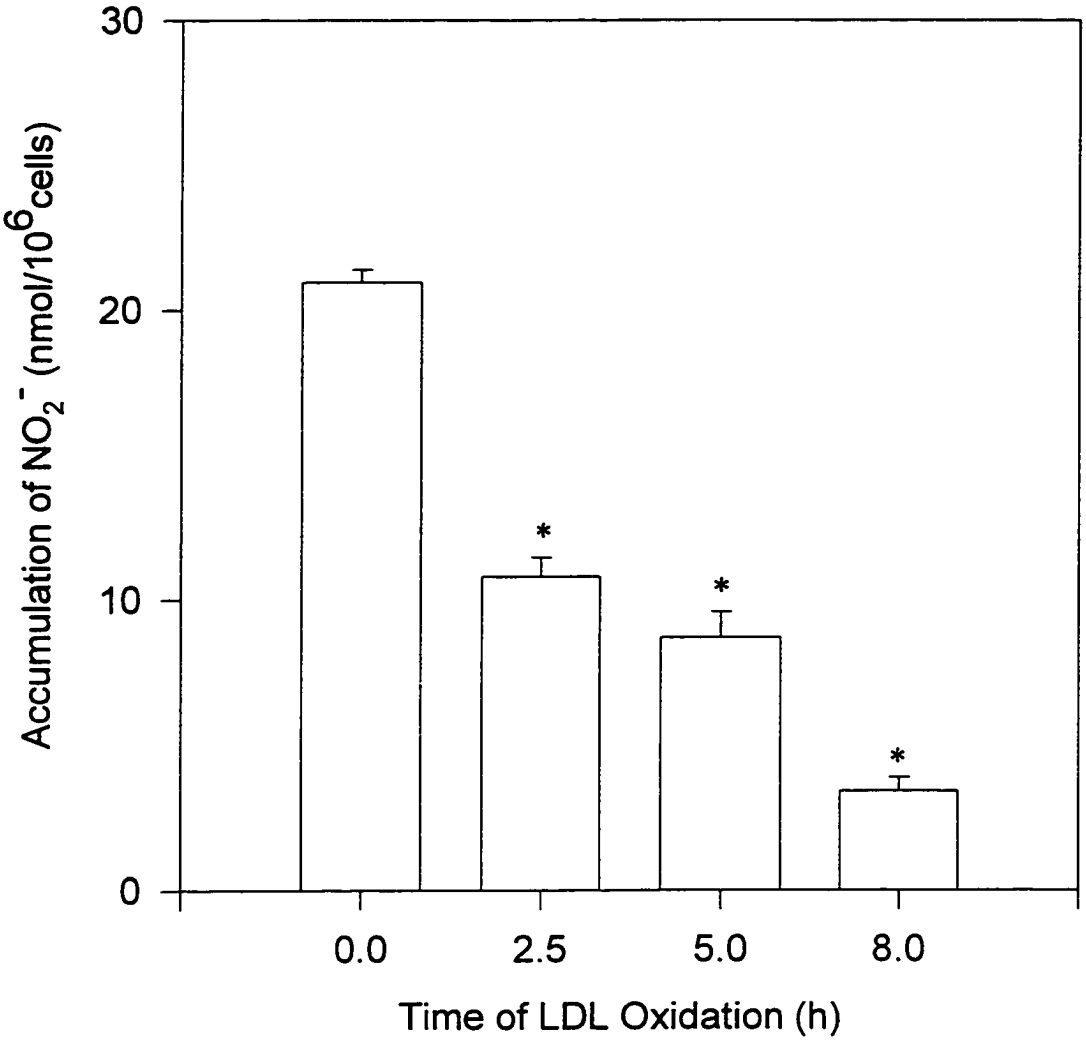


Figure 3-3. Influence of LDL oxidized to varying extent on NO production in LPS stimulated RAW264.7 macrophages. OxLDL preparations (100 μg protein/ml) containing 25, 120 and 230 μM LOOH were added to LPS stimulated RAW264.7 macrophages. Nonoxidized LDL plus LPS was used as a control (no LOOH). Data points represent means ± S.D. of triplicate samples for a single experiment. The means with * are significantly different from treatment with LPS plus nonoxidized LDL (Student's *t* test). These results are representative of three separate experiments.

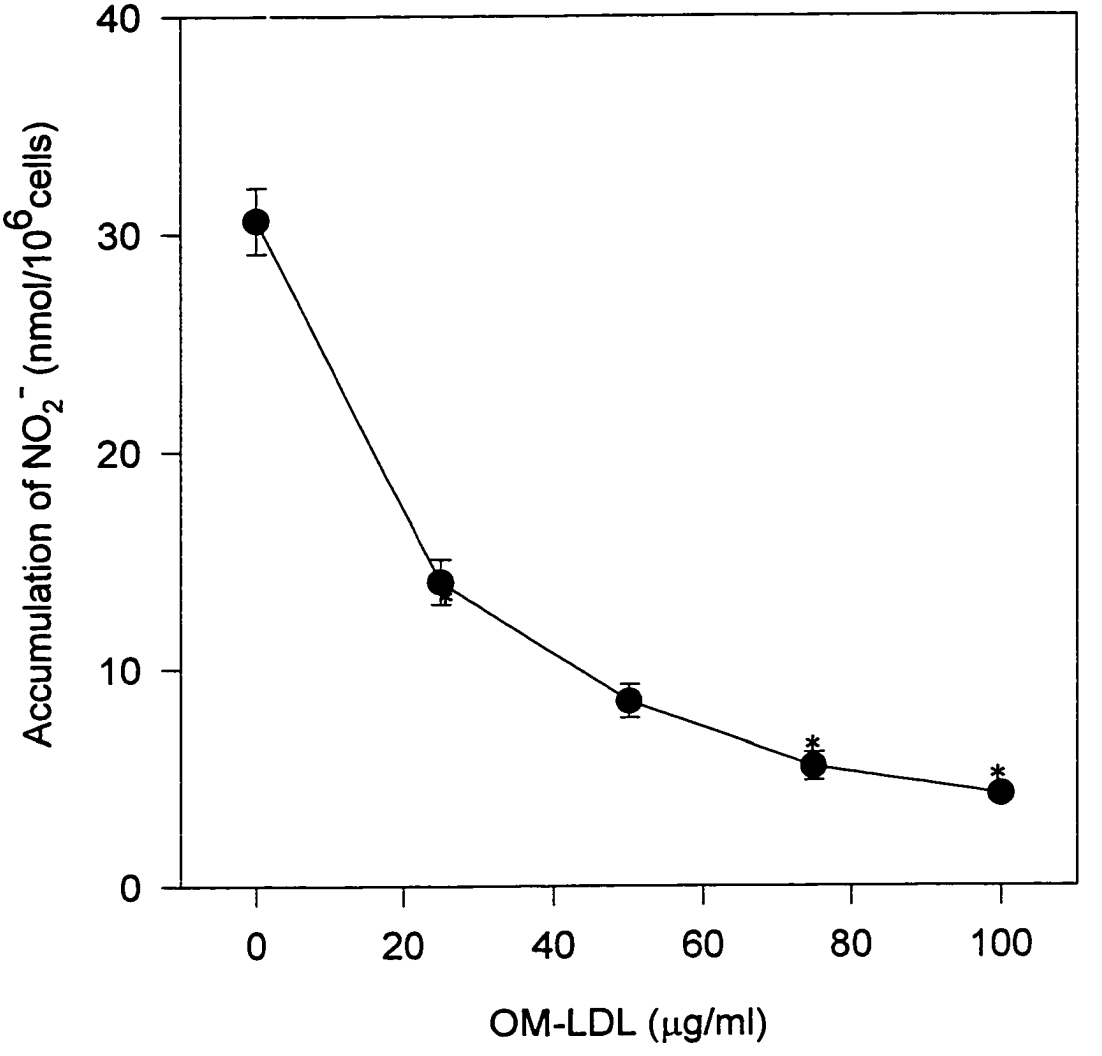


Figure 3-4. Concentration effect of oxLDL on NO production in LPS stimulated RAW264.7 macrophages. OxLDL containing 2.29 μmol LOOH /mg protein was diluted in the medium to give final concentrations of 0, 25, 50, 75 or 100 μg protein/ml before being added to LPS stimulated RAW264.7 macrophages. Data points represent means ± S.D. of triplicate samples for a single experiment. The means with * are significantly different from treatment with LPS (Student's *t* test). These results are representative of three separate experiments.

TABLE 3-1 EFFECT OF OXLDL ON DEHYDROGENASE ACTIVITY

Treatment	Dehydrogenase Activity (OD at 532nm)
Dialysate + LPS	0.41 ± 0.01
OxLDL (50 μg/ml) + LPS	0.41 ± 0.10
OxLDL (75 μg/ml) + LPS	0.42 ± 0.00
OxLDL (100 μg/ml) + LPS	0.43 ± 0.01*

At the end of the experiments shown in Figure 3-4, macrophages were washed with PBS and mitochondrial dehydrogenase activity was measured as 532 nm absorbance with the MTT assay as described in Materials and Methods. Values are means ± S.D. for triplicate samples. The mean with * is significantly different from treatment with LPS plus dialysate ($p<0.05$, Student's *t* test). The data are representative of three separate experiments.

Comparison of OxLDL, AcLDL and LDL on NO Production in LPS Stimulated RAW264.7 Macrophages

OxLDL and AcLDL, but not native LDL, were taken up by scavenger receptors with the accumulation of cholesteryl esters in RAW264.7 macrophages (data not shown). To determine whether AcLDL mediated uptake of modified LDL is required for the inhibition of NO production, we compared the effects of oxLDL, AcLDL, and LDL on NO production in stimulated macrophages. As shown in Figure 3-5, oxLDL (with 750 nmol LOOH/mg protein) inhibited NO production by 61%, whereas AcLDL and LDL with the same protein concentrations showed inhibition of 12% and 18%, respectively. The slight inhibition observed for AcLDL, and LDL may be due to the mild oxidation occurring during the extensive dialysis procedures. In fact, AcLDL and LDL contained LOOH levels of 36 and 42 nmol/mg protein, respectively. These results suggest that oxidative modification of LDL is a key factor causing inhibition on NO production in RAW264.7 macrophages. Furthermore, AcLDL mediated uptake is not sufficient to cause strong inhibition.

Part II -- Effects of 13-HPODE and LysoPC on Nitric Oxide Production in LPS Stimulated RAW264.7 Macrophages

Comparison of 13-HPODE and LysoPC on Inhibiting NO Production in LPS Stimulated RAW264.7 Macrophages

In order to identify the active lipid components of oxLDL that contribute to the inhibition on NO production, the effects of 13-HPODE and lysoPC were

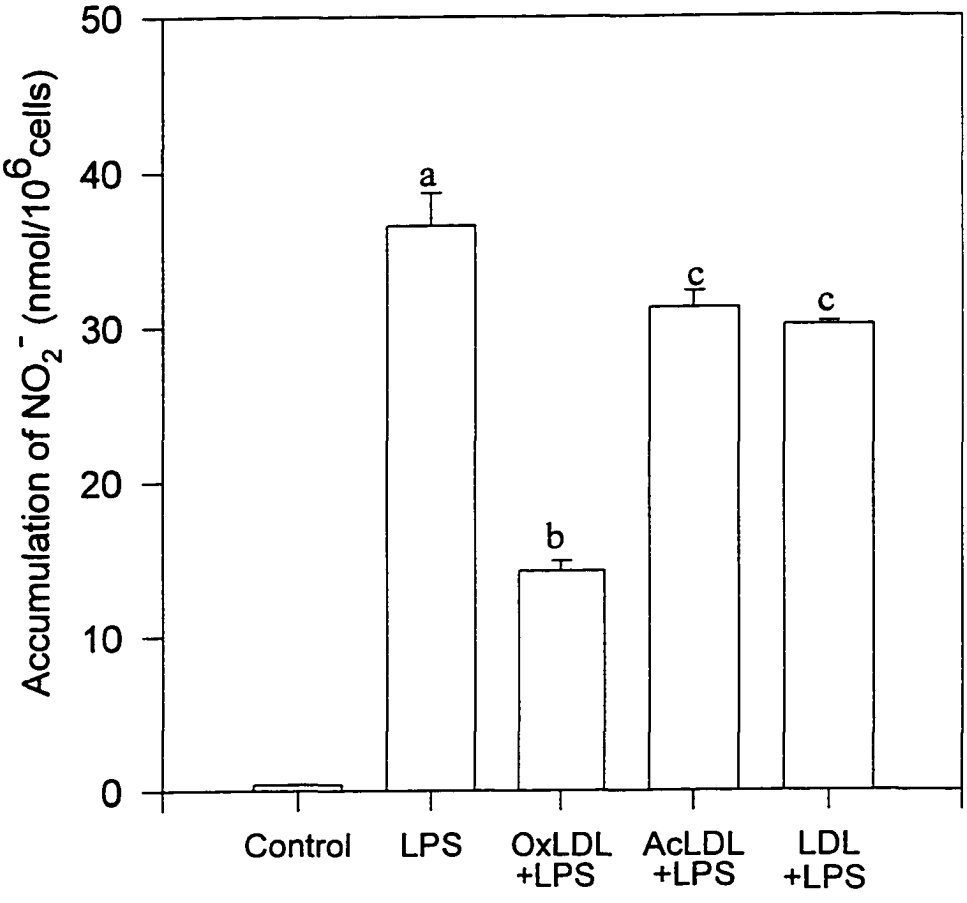


Figure 3-5. Comparison of oxLDL, AcLDL and LDL on NO production in LPS stimulated RAW264.7 macrophages. Data points are means ± S.D. for triplicate samples. The means with different letter superscripts are significantly different ($p<0.05$, one way ANOVA).

studied in LPS stimulated macrophages. 13-HPODE or lysoPC was incorporated into AcLDL carrier before being added to macrophages. Figure 3-6 shows that, in the presence of AcLDL carrier, 35 μM of 13-HPODE markedly inhibited NO production whereas 50 μM of lysoPC was not effective. Surprisingly, a similar inhibition of 13-HPODE was also observed in the absence of AcLDL carrier. LysoPC, at the concentration of 50 μM, was also not effective in the absence of AcLDL. Furthermore, neither 13-HPODE nor lysoPC induced NO production in unstimulated macrophages (data not shown). These results suggest that 13-HPODE is much more potent than lysoPC for inhibiting NO production in LPS stimulated macrophages. In addition, AcLDL carrier mediated uptake may not be required for 13-HPODE, and lysoPC to inhibit NO production in stimulated macrophages.

Concentration Dependence of 13-HPODE and LysoPC on NO Production in LPS Stimulated RAW264.7 Macrophages

Figure 3-7 shows that increasing concentrations of 13-HPODE resulted in a progressive inhibition of NO production in LPS stimulated macrophages. As observed in oxLDL, 13-HPODE showed a nonlinear inhibition of NO production with the rate of inhibition being greater at low concentrations than at high concentrations. 13-HPODE did not induced the production of NO in unstimulated macrophages (data not shown). Compared to 13-HPODE, lysoPC moderately inhibited NO production induced by LPS, and significant inhibition was observed only above 80 μM lysoPC (Figure 3-8). Cell viability as

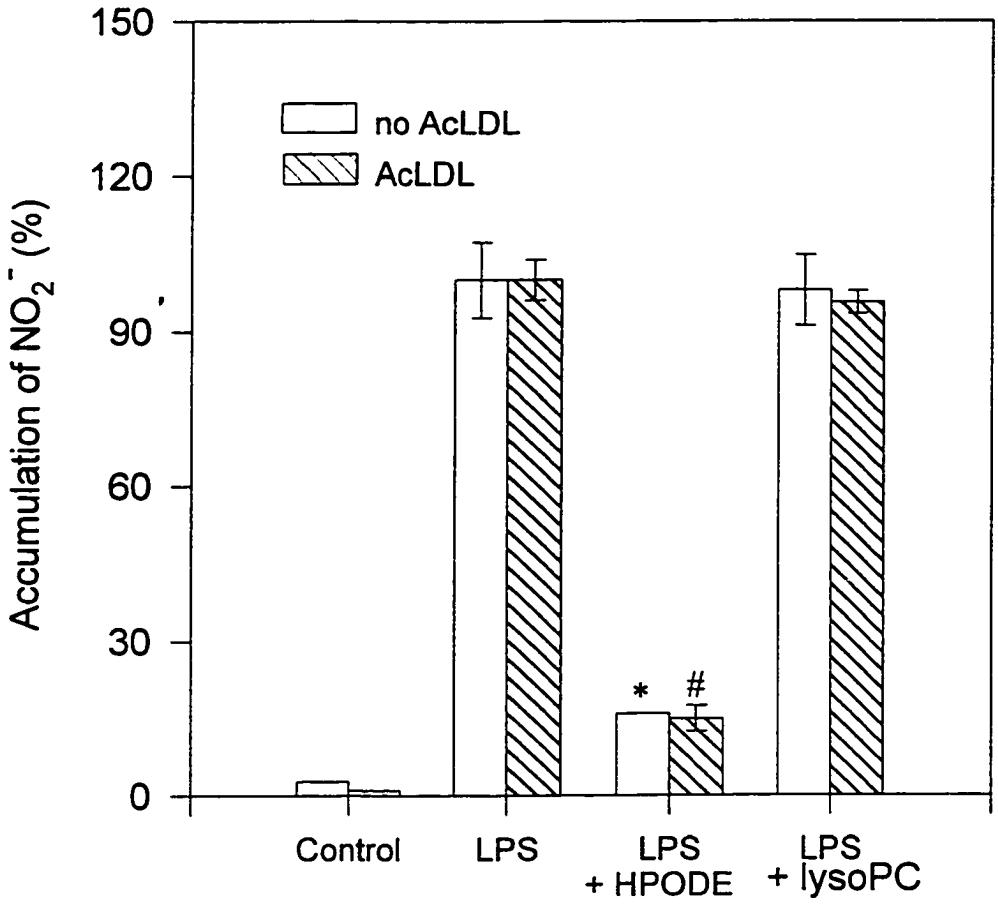


Figure 3-6. Comparison of 13-HPODE and lysoPC on NO production in LPS stimulated RAW264.7 macrophages in the absence or presence of AcLDL. 13-HPODE or lysoPC was incorporated into AcLDL carrier (50 μg protein/ml) before being added to macrophages. Results are expressed as percent accumulation of NO_2^- compared to LPS treatment. Data points represent means ± S.D. of triplicate samples for a single experiment. Treatments with 13-HPODE plus LPS are significantly different from treatments with LPS alone in the absence (*) or presence (#) of AcLDL (Student's *t* test). These results are representative of three separate experiments.

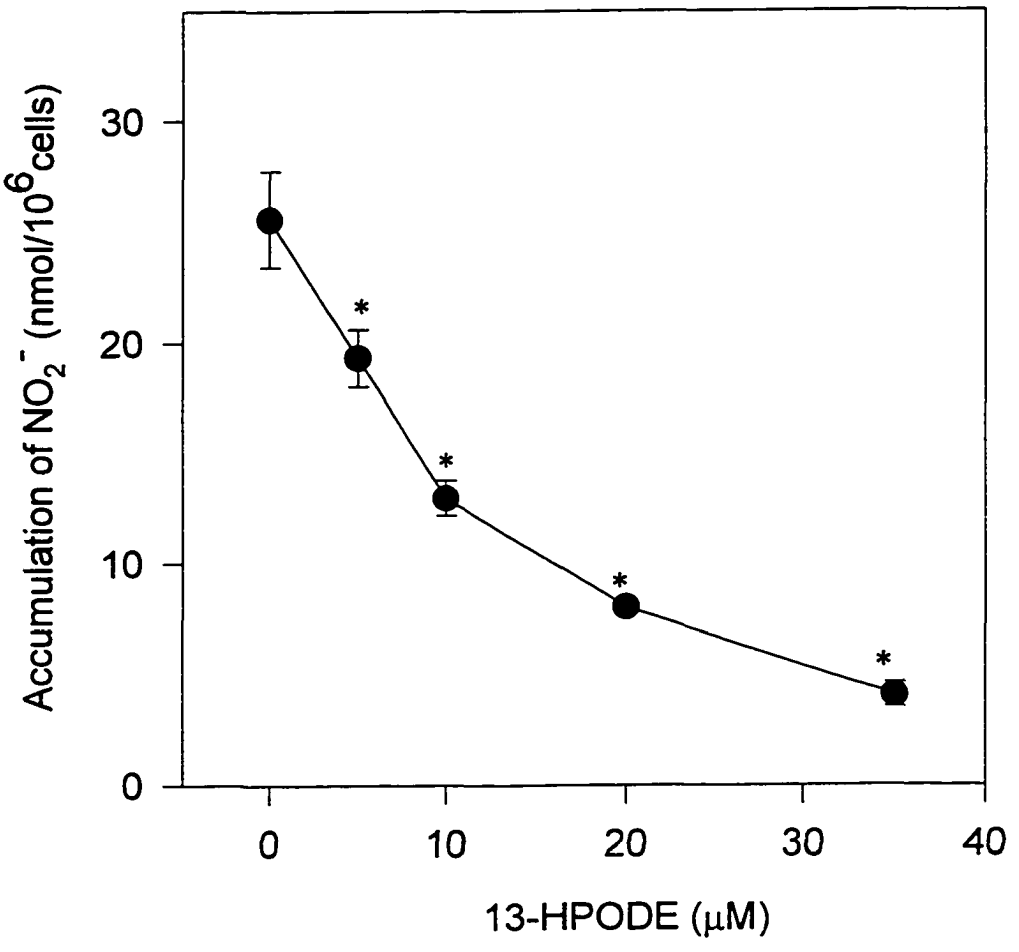


Figure 3-7. Concentration effect of 13-HPODE on NO production in LPS stimulated macrophages. Data points represent means ± S.D. of triplicate samples for a single experiment. The means with * are significantly different from treatment with LPS (Student's *t* test). These results are representative of three separate experiments.

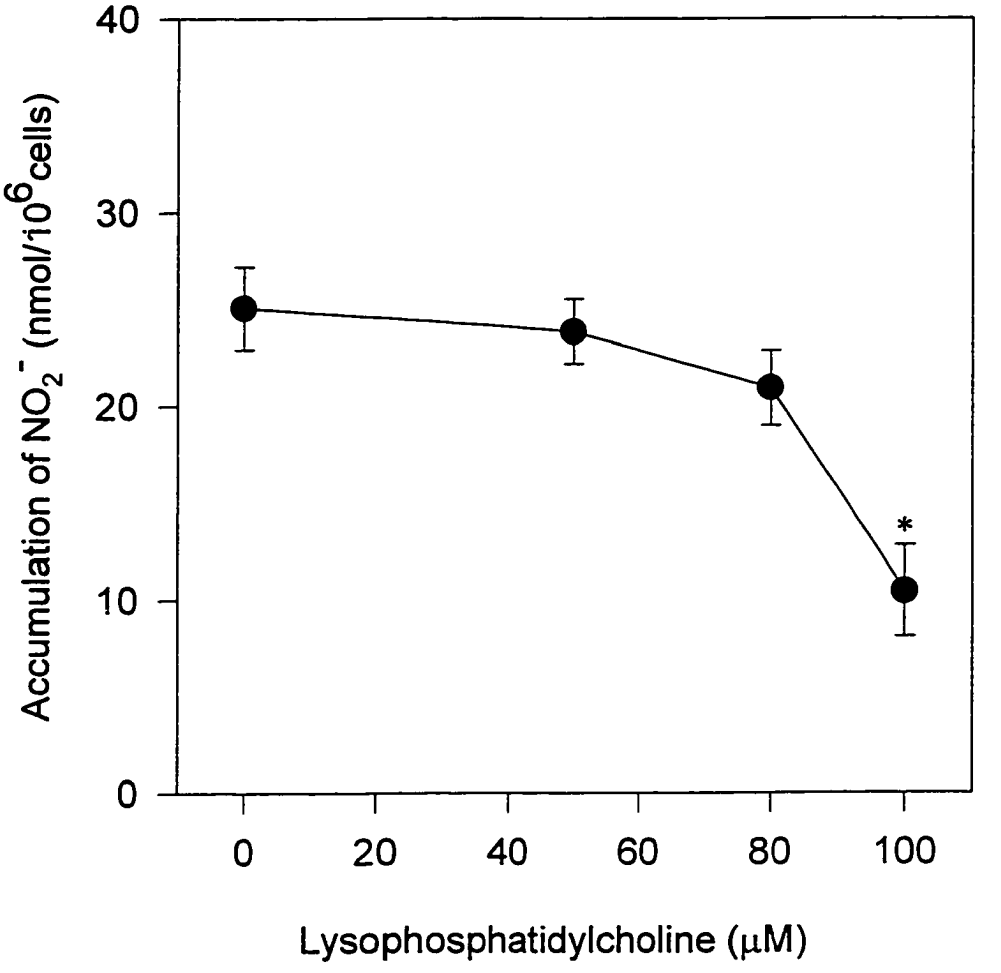


Figure 3-8. Concentration effect of lysoPC on NO production in LPS stimulated macrophages. Data points represent means ± S.D. of triplicate samples for a single experiment. The means with * are significantly different from treatment with LPS (Student's *t* test). These results are representative of three separate experiments.

determined by the MTT assay, and cell number as measured by the DPA assay, however, were not significantly decreased by 13-HPODE or lysoPC in the concentrations used in these experiments (see Table 3-2). These data suggest that the marked inhibition of NO production observed for 13-HPODE is not a general property of detergent like lipids.

Part III -- Effects of Antioxidants on the Decreased Nitric Oxide Production Caused by OxLDL and 13-HPODE in RAW264.7 Macrophages

Pre-treatment of RAW264.7 Macrophages with α-Tocopherol Attenuates the Inhibition of NO Production Caused by OxLDL

To determine whether an increased cellular content of α-tocopherol influences NO production, RAW264.7 macrophages were enriched with α-tocopherol by pre-incubation with α-tocopherol containing liposomes before treatment. After 8 hours of pre-incubation, cellular levels of α-tocopherol increased to 16.56 $\pm$ 2.1 nmol/10^7cells with treatment of α-tocopherol liposomes. α-Tocopherol was undetectable in cells treated with control liposomes. As shown in Figure 3-9, the increased cellular α-tocopherol attenuated the inhibition caused by oxLDL by about 30%. α-Tocopherol, however, neither induced NO production in unstimulated macrophages nor altered NO production in stimulated macrophages (data not shown). These data suggest that the inhibition caused by oxLDL can be partially reversed by increasing cellular levels of α-tocopherol.

TABLE 3-2 EFFECTS OF 13-HPODE AND LYSOPC ON DEHYDROGENASE ACTIVITY AND CELL DENSITY

Treatment	10^6cells/well (DPA DNA Assay)[1]	Dehydrogenase Activity (MTT assay OD at 532nm)[2]
Control	1.49 ± 0.07	0.42 ± 0.01
LPS	1.33 ± 0.03*	0.43 ± 0.01
50 μM LysoPC + LPS	1.21 ± 0.07*	0.43 ± 0.01
100 μM LysoPC + LPS	1.30 ± 0.02*	0.43 ± 0.01
5 μM HPODE + LPS	ND	0.42 ± 0.02
10 μM HPODE + LPS	ND	0.42 ± 0.02
15 μM HPODE + LPS	1.34 ± 0.03*	ND
20 μM HPODE + LPS	ND	0.42 ± 0.01
30 μM HPODE + LPS	1.22 ± 0.05*	0.43 ± 0.01
45 μM HPODE + LPS	1.29 ± 0.03*	ND

The DPA and MTT assay were performed at the end of the experiments shown in Figure 3-6, 3-7, 3-8. [1] Macrophage DNA contents were determined by the DPA assay. Cell numbers were calculated from 532 nm absorbance using a standard curve generated from varying numbers of cells. [2]Macrophages mitochondrial dehydrogenase activity was measured as 532 nm absorbance with the MTT assay. Values are means ± S.D. for triplicate samples. The means with * are significantly different from control ($p<0.05$, Student's *t* test). ND, not determined. The data are representative of three separate experiments.

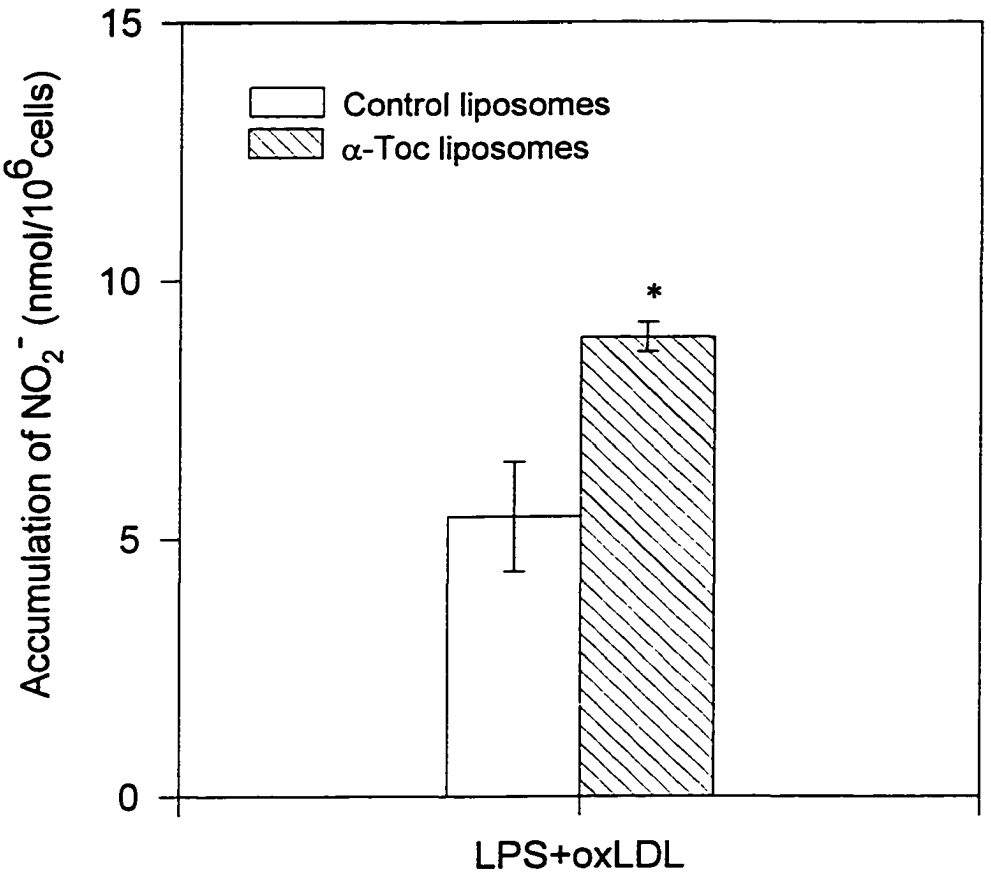


Figure 3-9. Effect of α-tocopherol pre-treatment on the inhibition caused by oxLDL in LPS stimulated RAW264.7 macrophages. Liposomes containing α-tocopherol or control liposomes were pre-incubated with RAW264.7 macrophages for 8 hours before treatment with 100 μg/ml oxLDL and 100 ng/ml LPS. Data points represent means ± S.D. of triplicate samples for a single experiment. *The mean of pre-treatment with α-tocopherol containing liposomes is significantly different from pre-treatment with blank liposomes (Student's *t* test). These results are representative of three separate experiments.

Pre-treatment of RAW264.7 Macrophages with NAC Attenuates the Inhibition of NO Production Caused by OxLDL

To determine whether an increased intracellular water soluble antioxidant NAC influences NO production, RAW264.7 macrophages were pre-incubated with NAC for 8 hours before treatment. Figure 3-10 shows that pre-incubation of macrophages with NAC significantly attenuated the inhibition of NO production caused by oxLDL. The effects of NAC were dose dependent. NAC became effective at 5.0 mM and completely reversed the inhibition at a concentration of 10.0 mM. Cell viability was maintained above 95%. Cell number was not increased by NAC treatment as assessed by the DPA assay (Table 3-3). NAC, however, showed cytotoxicity when the concentration reached 20.0 mM. Furthermore, NAC at a concentration of 10.0 mM potentiated NO production in LPS stimulated macrophages by about 25%. NAC alone, however, did not induce NO production in RAW264.7 macrophages. These data suggest that the inhibition caused by oxLDL was completely reversed by pre-treatment with 10.0 mM NAC.

Pre-treatment of RAW264.7 Macrophages with NAC Attenuates the Inhibition Caused by 13-HPODE

To determine whether an increased intracellular water soluble antioxidant NAC influences NO production, RAW264.7 macrophages were pre-incubated with NAC for 8 hours before treatment. Figure 3-11 shows that pre-treatment of macrophages with NAC significantly attenuated the inhibition of NO production

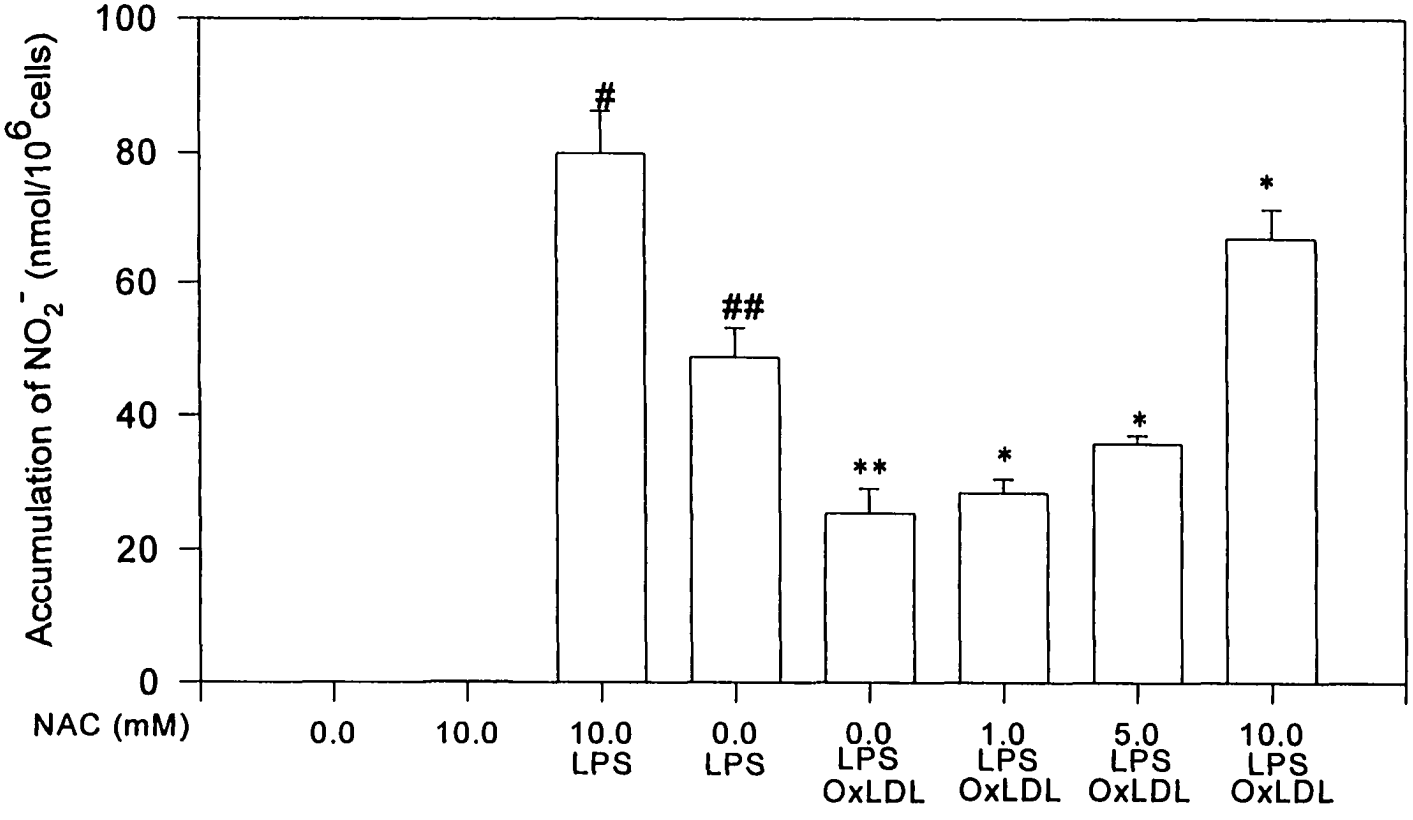


Figure 3-10. Effect of NAC pre-treatment on the inhibition caused by oxLDL in LPS stimulated RAW264.7 macrophages. RAW264.7 macrophages were pre-incubated with indicated concentration of NAC for 8 hours before treatment with 100 μg/ml oxLDL and 100 ng/ml LPS. Data points represent means ± S.D. of triplicate samples for a single experiment. Treatment with LPS (##) is significantly different from that pre-treated with NAC (#). Treatments with LPS and oxLDL (**) are significantly different from that pre-treated with NAC (*) (Student's *t* test). These results are representative of three separate experiments.

TABLE 3-3 CELL DENSITY AFTER TREATMENT WITH NAC AND OXLDL

Treatment	10^6cells/well (DPA DNA Assay)
Control	1.48 ± 0.09^a
10 mM NAC	1.46 ± 0.02^a
OxLDL + LPS	1.25 ± 0.06^b
LPS	1.22 ± 0.11^b
1 mM NAC + OxLDL + LPS	1.30 ± 0.15^b
5 mM NAC + OxLDL + LPS	1.24 ± 0.05^b
10 mM NAC + OxLDL + LPS	1.25 ± 0.05^b
20 mM NAC + OxLDL + LPS	0.29 ± 0.14^c

At the end of the experiments shown in Figure 3-10, macrophages were washed with PBS and cellular DNA content was determined by the DPA assay as described in Materials and Methods. Cell numbers were calculated from 532 nm absorbance using a standard curve generated from varying numbers of cells. Values are means $\pm$ S.D. for triplicate samples. The means with different letter superscripts indicate significantly different ($p<0.05$, one way ANOVA). The data are representative of three separate experiments.

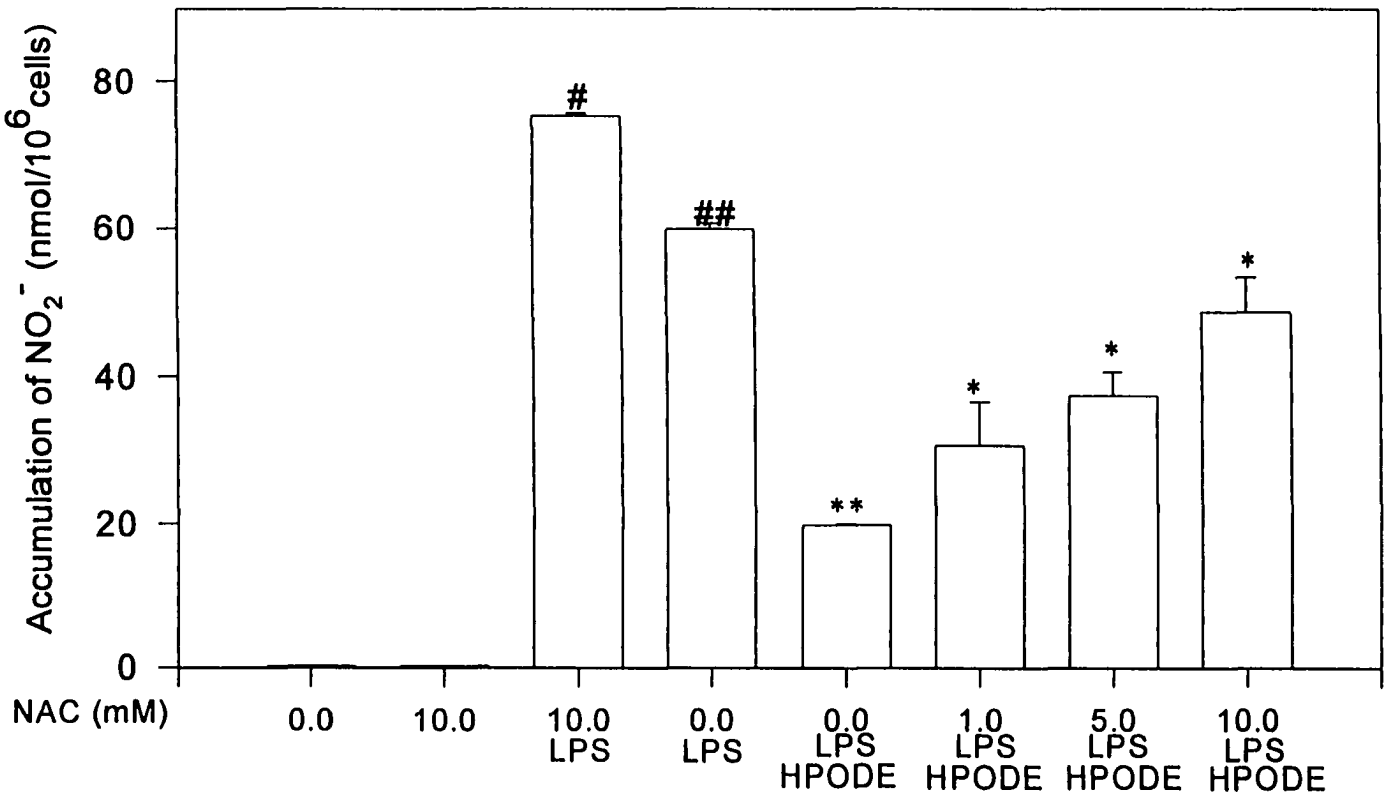


Figure 3-11. Effect of NAC pre-treatment on the inhibition caused by 13-HPODE in LPS stimulated RAW264.7 macrophages. RAW264.7 macrophages were pre-incubated with indicated concentration of NAC for 8 hours before treatment with 40 μM 13-HPODE and 100 ng/ml LPS. Data points represent means ± S.D. of triplicate samples for a single experiment. Treatment with LPS (##) is significantly different from that pre-treated with NAC (#). Treatments with LPS and 13-HPODE (**) are significantly different from that pre-treated with NAC (*) (Student's *t* test). These results are representative of three separate experiments.

TABLE 3-4 CELL DENSITY AFTER TREATMENT WITH NAC AND 13-HPODE

Treatment	10^6cells/well (DPA DNA Assay)
Control	1.97 ± 0.20^a
10 mM NAC	1.81 ± 0.11^a
LPS	1.55 ± 0.04^b
HPODE + LPS	1.35 ± 0.13^b
0.1 mM NAC + HPODE + LPS	1.46 ± 0.14^b
0.5 mM NAC + HPODE + LPS	1.46 ± 0.18^b
1.0 mM NAC + HPODE + LPS	1.53 ± 0.12^b
5.0 mM NAC + HPODE + LPS	1.52 ± 0.02^b
10 mM NAC + HPODE + LPS	1.33 ± 0.03^b
20 mM NAC + HPODE + LPS	1.18 ± 0.03^c

At the end of the experiments shown in Figure 3-11, macrophages were washed with PBS and cellular DNA content was determined by the DPA assay. Cell numbers were calculated from 532 nm absorbance using a standard curve generated from varying numbers of cells. Values are means $\pm$ S.D. for triplicate samples. The means with different letter superscripts indicate significantly different ($p<0.05$, one way ANOVA). The data are representative of three separate experiments.

caused by 13-HPODE. The effects of NAC were concentration dependent. NAC became effective at a concentration of 1.0 mM. Cell viability was maintained above 95%. Cell numbers were not increased by NAC as assessed by DPA DNA assay (Table 3-4). NAC, however, showed cytotoxicity when the concentration reached 20.0 mM. NAC alone, however, did not induce NO production in unstimulated macrophages. These data suggest that the inhibition caused by 13-HPODE was attenuated by pre-treatment with NAC.

Part IV – The Regulation of Inducible Nitric Oxide Synthase Gene Expression in RAW264.7 Macrophages

OxLDL Decreases iNOS Protein Induced by LPS in RAW264.7 Macrophages

To determine whether oxLDL decreases cellular iNOS levels, iNOS protein was analyzed by Western blotting. After treatment of LPS stimulated RAW264.7 macrophages with oxLDL, AcLDL, or LDL for 24 hours, NO production by macrophages was determined in the medium (see Figure 3-5) and cellular proteins were extracted. Figure 3-12 shows Western blotting of iNOS protein in LPS stimulated RAW264.7 macrophages treated with oxLDL, AcLDL, or LDL. A 130 kDa band identical to the iNOS protein standard (shown in Figure 3-13) was observed in LPS stimulated macrophages but not in unstimulated macrophages. OxLDL significantly decreased iNOS protein levels induced by LPS in RAW264.7 macrophages, whereas AcLDL and LDL only minimally decreased iNOS protein levels. These results are consistent with the results

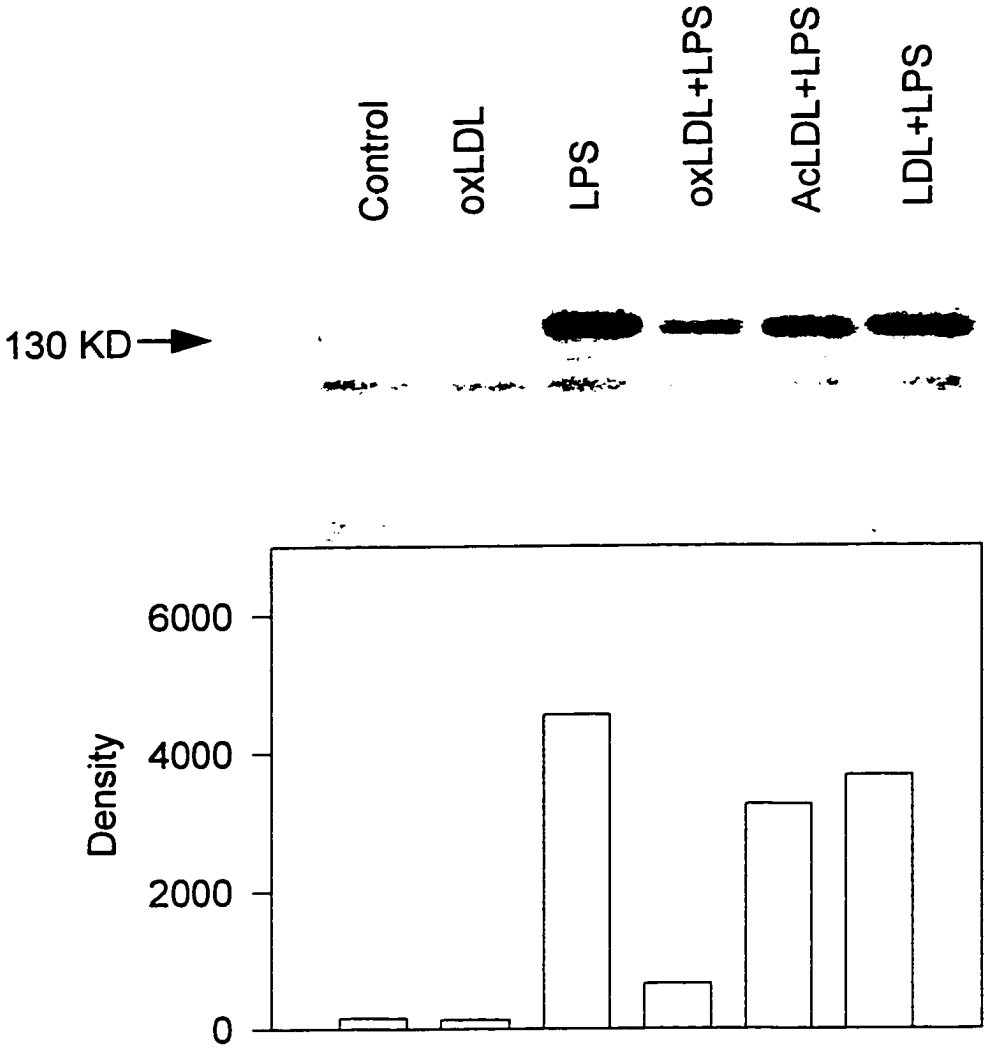


Figure 3-12. Western blot of iNOS from LPS stimulated RAW264.7 macrophages treated with oxLDL. RAW264.7 macrophages were incubated with 100 ng/ml LPS and oxLDL dialysate, 50 μg/ml oxLDL, AcLDL or LDL for 24 hours. Cellular proteins were extracted and analyzed. Top panel shows the photograph of the iNOS protein. Arrow indicates the approximate molecular mass of 130 KDa from the standards run in parallel. The bottom panel is the densitometry of iNOS. A representative of Western blot from three separate experiments is shown.

shown in Figure 3-5, which suggest that the decreased NO production caused by oxLDL is due to the decreased iNOS protein levels.

13-HPODE Decreases iNOS Protein Induced by LPS in RAW264.7 Macrophages

To determine whether 13-HPODE, and lysoPC have similar effects of oxLDL in decreasing cellular iNOS protein levels, iNOS protein was analyzed by Western blotting after treatment of LPS stimulated RAW264.7 macrophages with 13-HPODE or lysoPC for 24 hours. As shown in Figure 3-13, increasing concentrations of 13-HPODE progressively decreased iNOS protein levels in stimulated macrophages. LysoPC, at a concentration of 50 μM, had no significant effect on iNOS protein levels compared to 13-HPODE (Figure 3-14). Neither 13-HPODE nor lysoPC induced iNOS protein expression in unstimulated RAW264.7 macrophages (data not shown).

OxLDL Decreases iNOS mRNA Induced by LPS in RAW264.7 Macrophages

To determine whether the decreased iNOS protein caused by oxLDL is due to the decreased iNOS mRNA, Northern blots were performed on RNA extracted from RAW264.7 macrophages stimulated with LPS for 6 hours in the absence or presence of oxLDL. As shown in Figure 3-15, LPS induced iNOS mRNA transcription whereas iNOS mRNA was undetectable in unstimulated macrophages. OxLDL significantly decreased iNOS mRNA induced by LPS, without altering GAPDH mRNA levels in RAW264.7 macrophages.

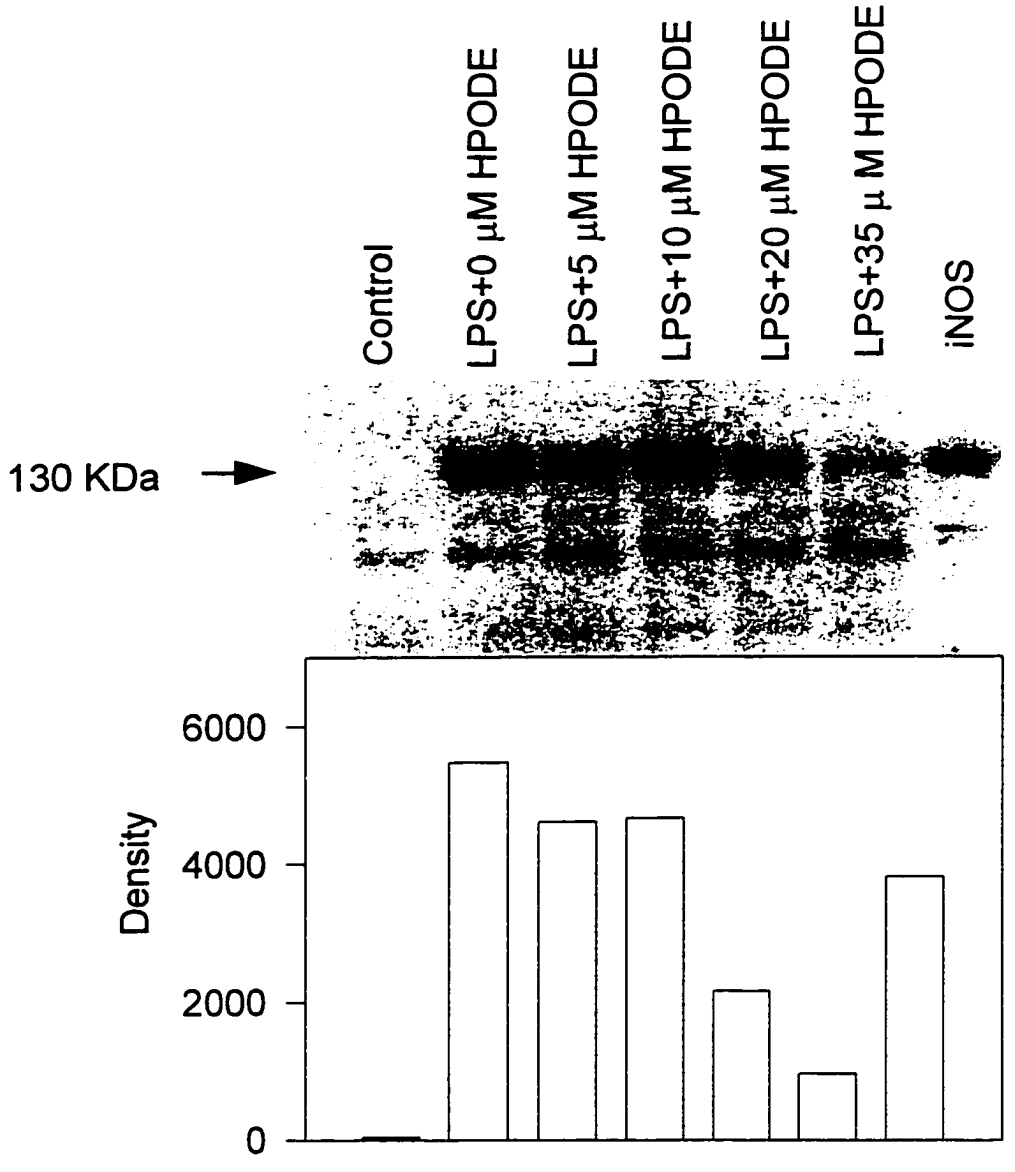


Figure 3-13. Western blot of iNOS from LPS stimulated RAW264.7 macrophages treated with 13-HPODE. RAW264.7 macrophages were incubated with 100 ng/ml LPS alone or plus 0, 5, 10, 20 or 35 μM 13-HPODE for 24 hours. Cellular proteins were extracted analyzed. Top panel shows the photograph of the iNOS protein. Arrow indicates the approximate molecular mass of 130 KDa from the standards run in parallel and the iNOS protein standard is also shown on the right. The bottom panel is the densitometry of iNOS. A representative of Western blot from four separate experiments is shown.

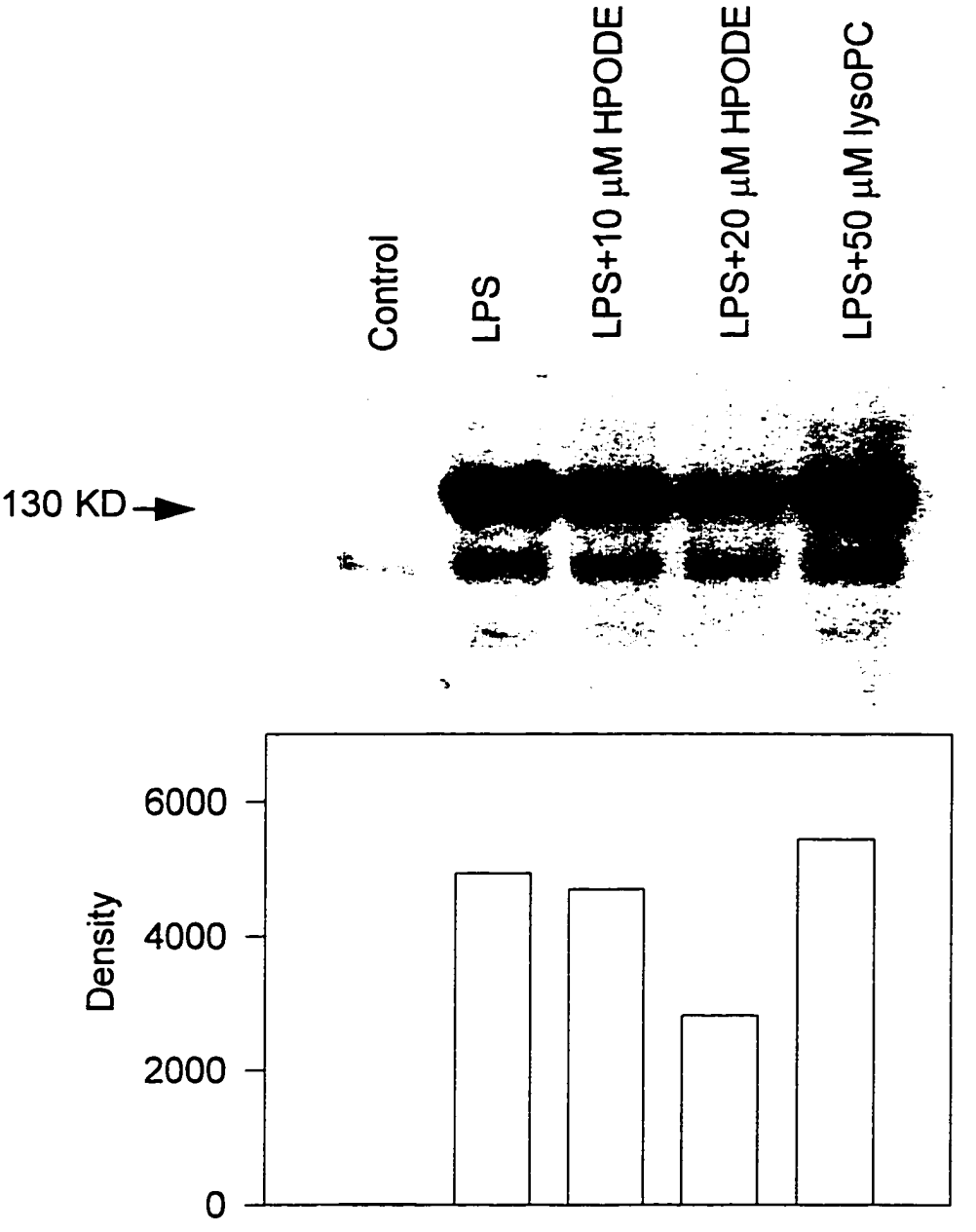


Figure 3-14. Western blot of iNOS from LPS stimulated RAW264.7 macrophages treated with 13-HPODE or lysoPC. RAW264.7 macrophages were incubated with 100 ng/ml LPS alone or plus 10, 20 μM 13-HPODE or 50 μM lysoPC for 24 hours. Cellular proteins were extracted and analyzed. Top panel shows the photograph of iNOS. Arrow indicates the approximate molecular mass of 130 KDa from the standards run in parallel. The bottom panel is the densitometry of iNOS. A representative of Western blot from four separate experiments is shown.

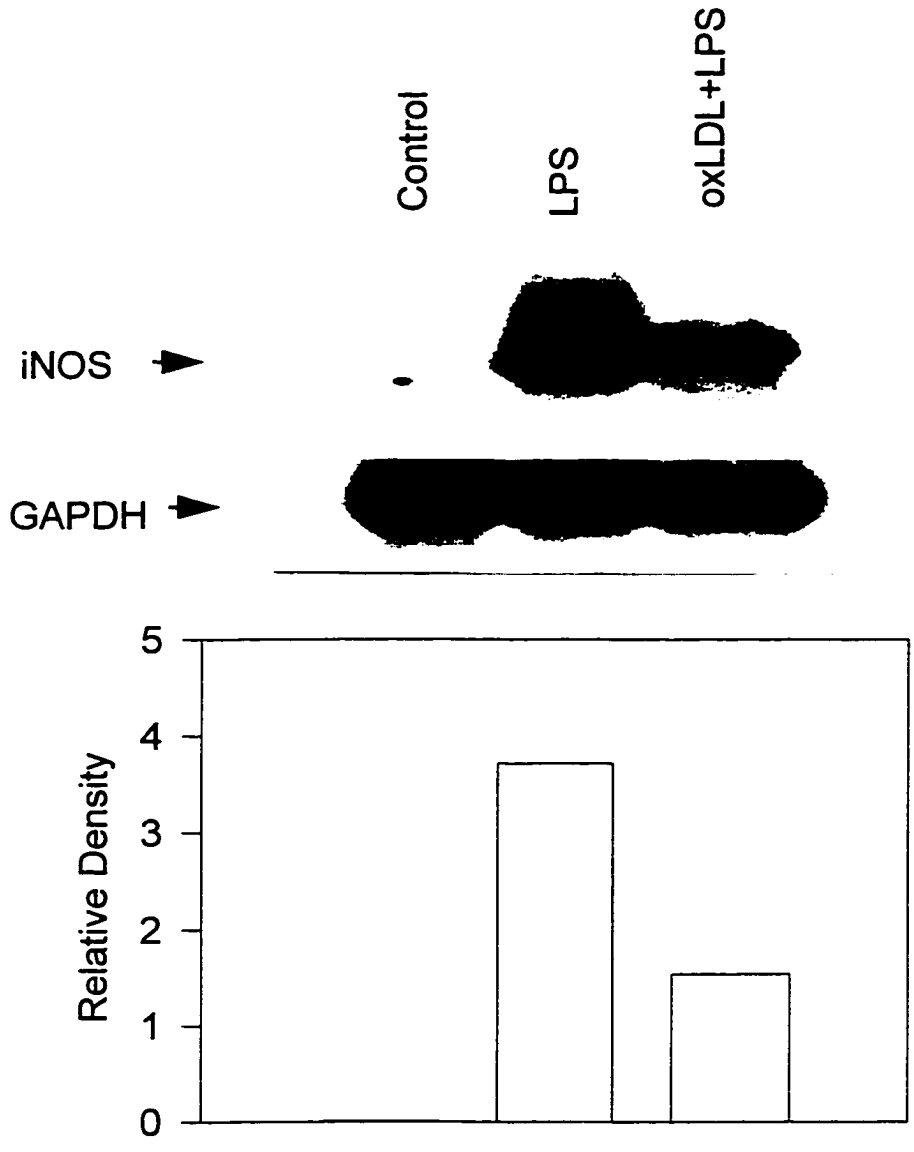


Figure 3-15. Northern blot of iNOS mRNA from LPS stimulated RAW264.7 macrophages treated with oxLDL. RAW264.7 macrophages were stimulated with 100 ng/ml LPS in the absence or presence of 50 μg/ml oxLDL for 6 hours. Total mRNA was isolated and analyzed. Top panel shows the autoradiograph of mRNA for iNOS and GAPDH, respectively. The bottom panel is the densitometry of mRNA. A representative of Northern blot from three separate experiments is shown.

Activation of NF-κB Induced by LPS in RAW264.7 Macrophages

To determine whether the induction of iNOS mRNA, and iNOS protein was accompanied by the activation of NF-κB in macrophages, EMSA was performed on nuclear proteins extracted from LPS stimulated RAW264.7 macrophages. Figure 3-16 shows the time course of NF-κB activation induced by LPS. Nuclear extracts from unstimulated RAW264.7 macrophages showed basal levels of NF-κB activity. The components of NF-κB complex were identified by supershift assay using Rel antibodies against p50 (anti-p50) and p65 (anti-p65) NF-κB subunits. As shown in Figure 3-17, the band labeled as p50/p65 was shifted toward the origin by both anti-p50 and anti-p65 and therefore being identified as the p50/p65 heterodimer, whereas the band labeled as p50/p50 was shifted by anti-p50 but not anti-p65 and therefore being identified as the p50/p50 homodimer. The bottom band being shifted by neither anti-p50 nor anti-p65 was considered to be non-specific DNA binding. The top band appeared in mixture with anti-Rel antibodies and considered supershift band. Results shown in Figure 3-16 indicate that LPS markedly induced p65/p50 DNA binding activity which appeared after 30 minutes and reached a maximum activation at 45 minutes. The activation of NF-κB lasted for at least 2 hours.

Effects of Oxidized Low Density Lipoprotein on the Activation of NF-κB in RAW264.7 Macrophages

To determine whether oxLDL influences the activation of NF-κB in

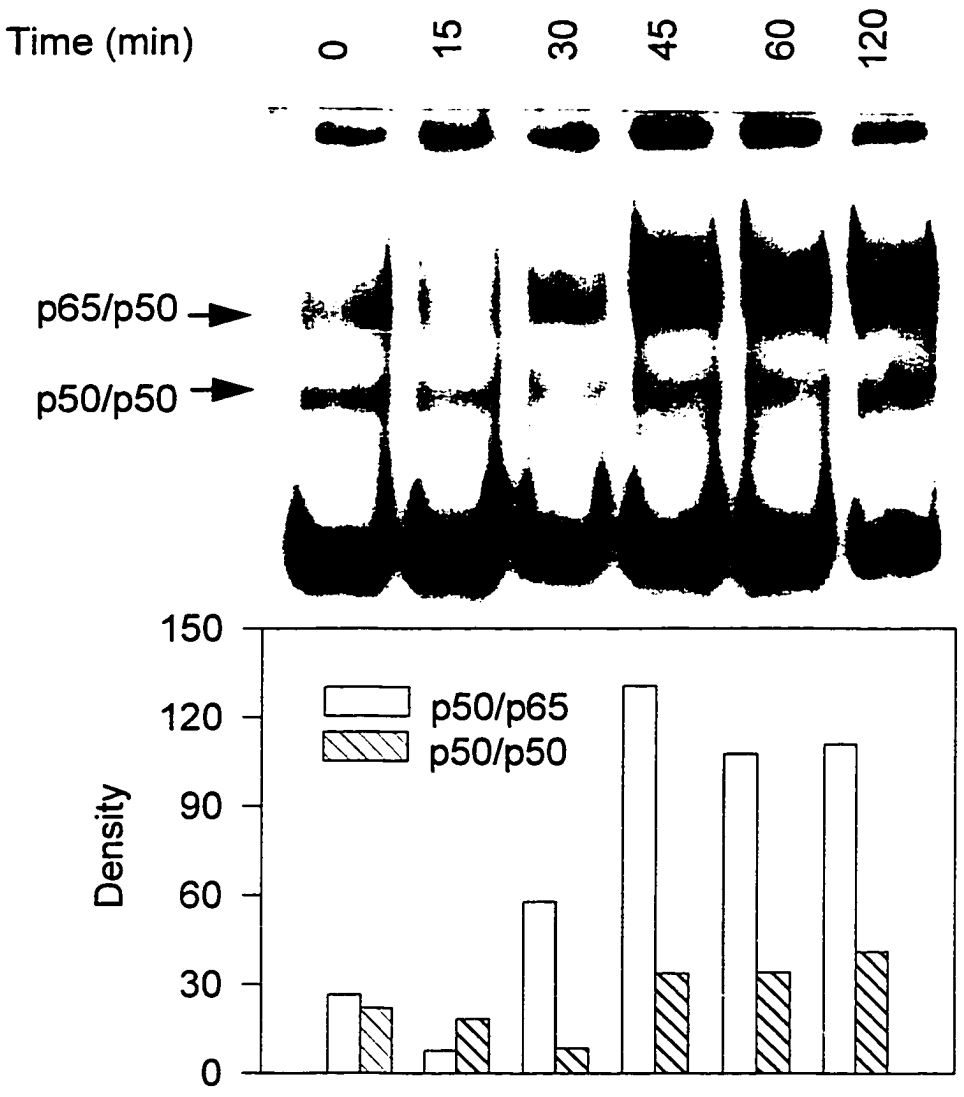


Figure 3-16. Activation of NF-κB induced by LPS in RAW264.7 Macrophages. Macrophages were stimulated with 100 ng/ml LPS for varying legths of time. The nuclear proteins were extrated and analyzed by the EMSA. Top panel shows the NF-κB DNA binding activity. Arrows at the p50/p50 and p50/p65 indicate NF-κB homodimer and heterodimer proteins, respectively. The bottom panel is the densitometry of the NF-κB bands. These results are representative of two experiments.

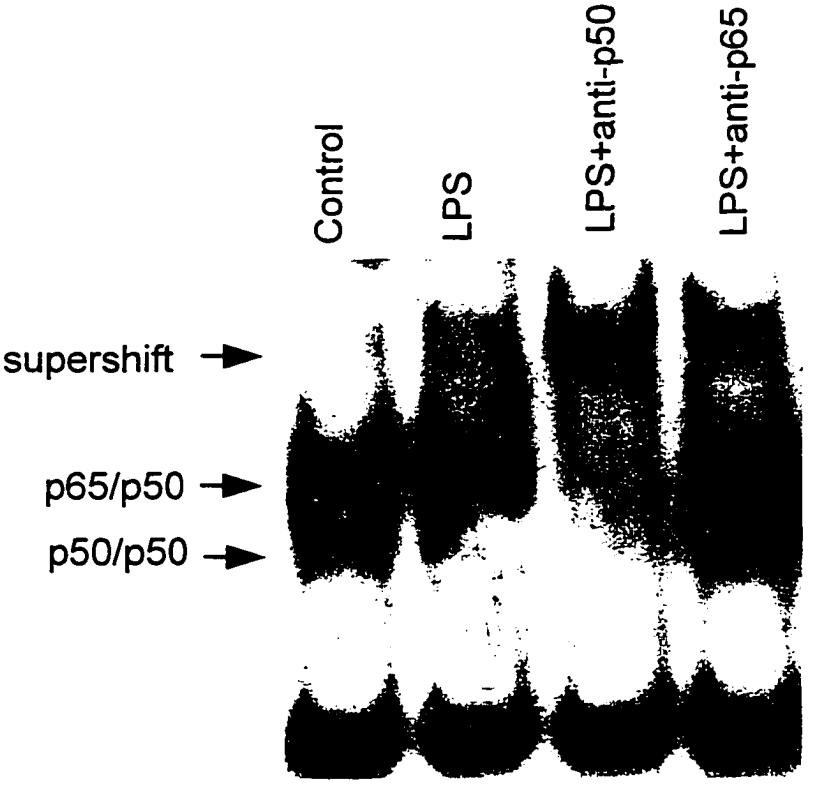


Figure 3-17. Supershift assay for identifying NF-κB proteins. Antibodies specific to NF-κB p50 or p65 was added to the binding reaction mixture before the precedures of EMSA. Arrows at the p50/p50 and p50/p65 indicate NF-κB homodimer and heterodimer proteins. Respectively. These results are representative of two experiments.

RAW264.7 macrophages, nuclear proteins were extracted from macrophages after co-incubation with LPS and oxLDL for 45 minutes. Figure 3-18 shows that oxLDL alone did not induce p50/p65 NF-κB DNA binding activity in unstimulated RAW264.7 macrophages. In contrast, the basal levels of p50/p65 activity were suppressed by oxLDL. The activation of p50/p65 NF-κB DNA binding activity induced by LPS was slightly suppressed by oxLDL in RAW264.7 macrophages.

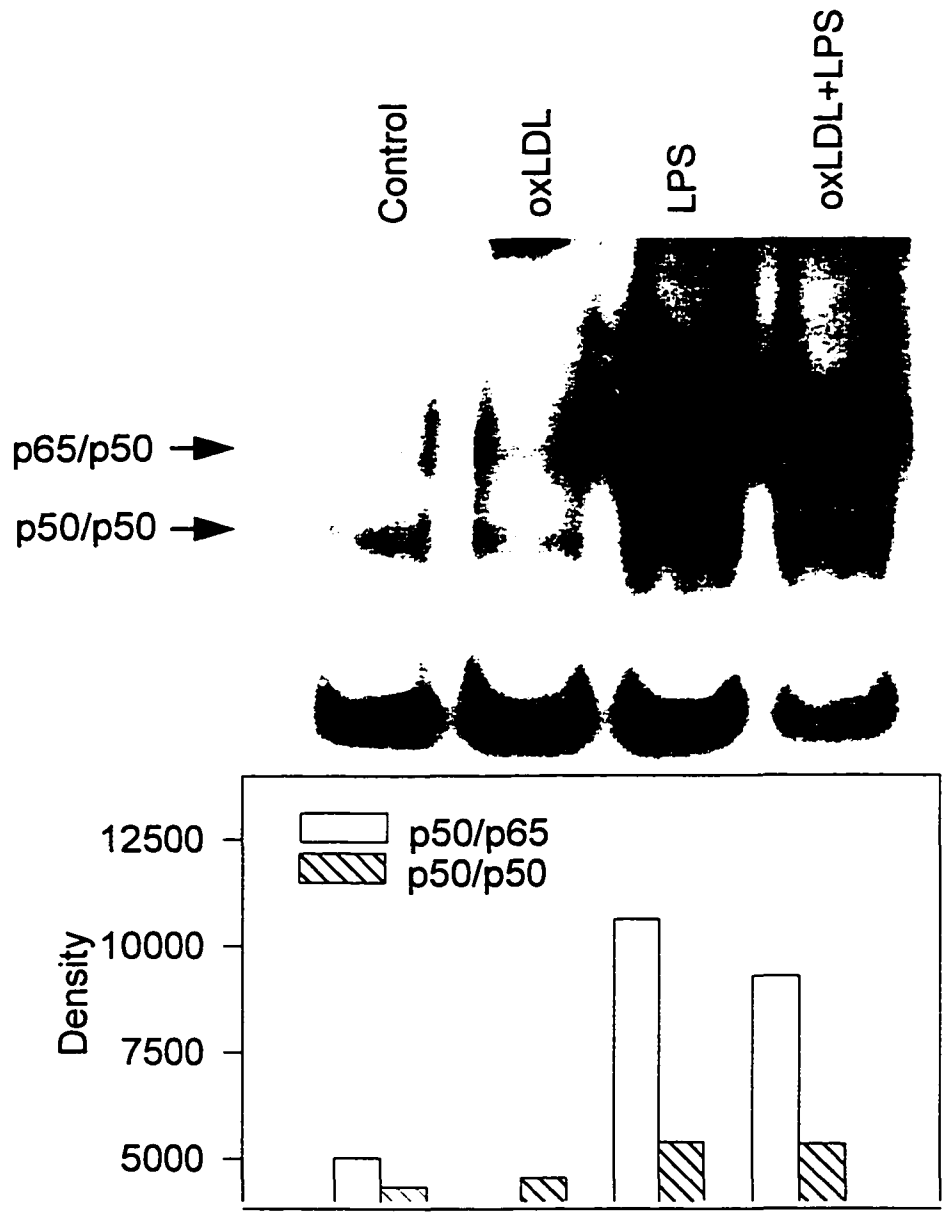


Figure 3-18. The influence of OxLDL on the activation of NF-κB induced by LPS in RAW264.7 Macrophages. Macrophages were incubated with 100 ng/ml LPS and 100 μg/ml oxLDL for 45 minutes. The nuclear proteins were extrated and analyzed by the EMSA. The top panel shows NF-κB DNA binding activity. Arrows at the p50/p50 and p50/p65 indicate NF-κB homodimer and heterodimer proteins, respectively. The bottom panel is the densitometry of NF-κB bands. These results are representative of three experiments.

CHAPTER 4

DISCUSSION

Atherosclerosis appears to be a chronic inflammation of the vasculature as indicated by the presence of macrophages, lymphocytes, immunoglubulins, adhesion molecules, cytokines, colony stimulating factors as well as inflammatory mediators such as NO and prostacyclin (PGI_2) in atherosclerotic lesions (Ross 1993). The activation of NF-κB (Brand and others 1996) and the expression of iNOS in atherosclerotic plaques (Buttery and others 1996) further confirm the presence of an active inflammatory process. Macrophages may produce NO by iNOS in atherosclerotic lesions (Buttery and others 1996; Leeuwenburgh and others 1997). Alterations in NO production by macrophages may modulate atherogenesis (Lloyd-Jones and Bloch 1996).

The Inhibition Potential of Oxidized Low Density Lipoprotein on NO Production

RAW264.7 macrophages produced large amounts of NO upon stimulation with LPS (see Figure 3-1). The production of NO depended on the concentrations of LPS, induction time and L-arginine supply. Under our experimental conditions, RAW264.7 macrophages produced NO in a nearly linear fashion. NO produced by cultured macrophages reacts with oxygen to form unstable intermediates which decompose to NO_2^- and nitrate (NO_3^-) (see

reaction 3-1) (Marletta and others 1988). NO_2^- and NO_3^- have been shown to be at a constant ratio of 2:3 in the medium of culture macrophages (Marletta and others 1988). The iNOS gene from RAW264.7 macrophages has been cloned and well characterized by Xie and others (1992). Goldring and others (1995) also observed the activation of NF-κB in RAW264.7 macrophages after stimulation with LPS. Furthermore, RAW264.7 cells had macrophage characteristics in phagocytosis of latex beads and accumulation of oxLDL lipids intracellularly as determined by O-red oil staining (data not shown). Via and others (1985) also showed that RAW264.7 macrophages have AcLDL scavenger receptors which take up AcLDL with the accumulation of cellular lipids. These information suggest that RAW264.7 macrophages provide a useful model for studying the effects of oxLDL on iNOS gene expression.

$$NO + O_2 \text{ ----------> } NO_2^- + NO_3^- \qquad (3\text{-}1)$$

We have previously studied the oxidation kinetics of human LDL (Ma and others 1994). In the presence of tocopherols, the accumulation of LOOH in oxLDL is very slow until all tocopherols were consumed. LOOH then accumulates very rapidly in the absence of tocopherols. In the present study, LDL was oxidized to the extent that all tocopherols were completely consumed (see Figure 3-2). Jessep and others (1990) demonstrated that LDL is not converted to a form that were recognized by scavenger receptors until all

endogenous tocopherols are completely oxidized. LOOH accumulated in oxLDL to increasing levels as a function of time (see Figure 3-2). The oxLDL used in experiments described here would be taken up by macrophage scavenger receptors. LDL contains 2332 nmol PUFA/mg protein with an octadecadienoic acid content of about 2000 nmol/mg protein (for a review see Esterbauer and others 1992). Most investigators determine the extent of LDL oxidation by measuring MDA as a LOOH degradation product (Haberland and others 1982). In most cases, it is not clear whether MDA is free or bound to oxLDL. We, however, measured LDL associated LOOH directly used a colorimetric method (Ma and others 1994; Tateishi and others 1987). We found that PUFAs are almost completely oxidized to form a maximum of about 2300 nmol LOOH/mg protein (see Figure 3-2). LOOH is the most abundant primary lipid peroxidation product in LDL oxidized *in vitro* and probably in atherosclerotic lesions (Folcik and Cathcart 1994, Folcik, 1995 #115; Noguchi and others 1993; Steinbrecher and Pritchard 1989). LOOH exists as cholesterol hydroperoxides, phospholipid hydroperoxides and free LOOH in oxLDL. The native LDL used in our experiments contained very low concentrations of LOOH and had tocopherol (mainly α-tocopherol) levels of about 21.4 nmol/mg protein. This LDL would be taken up by the LDL receptor but not the scavenger receptors.

In agreement with Yang and others (1994), our data show that oxLDL was a more effective inhibitor of NO production in LPS stimulated macrophages than AcLDL or LDL (see Figure 3-5). Nonetheless, macrophages take up both AcLDL

and oxLDL with an almost equal accumulation of intracellular lipids (Yang and others 1994). Furthermore, the effects of AcLDL and LDL on NO production are very similar, even though AcLDL is taken up by AcLDL scavenger receptors whereas LDL is taken up by the LDL receptor. Therefore, neither the binding of modified LDL to AcLDL scavenger receptors nor lipid loading are likely to account for the observed inhibition of NO production. These results further support the notion that the inhibition potential of oxLDL may be determined by LOOH levels in oxLDL.

The slight inhibitory effect of AcLDL and LDL may be due to several factors. Both AcLDL and LDL contained trace amounts of LOOH that would also increase slightly during the 24 hours of incubation with macrophages (Aviram and Rosenblat 1994). The LOOH in AcLDL and LDL, even as low as 30-50 nmol/mg protein may cause inhibition. Suzuki and others (1997b) suggested in a recent review that the ligand-receptor coupling such as TNF-α receptor binding would produce reactive oxygen species. We cannot exclude the possibility that ligation of AcLDL with AcLDL scavenger receptors or LDL with the LDL receptor would also produce reactive oxygen species, which give rise to a slight inhibition. Furthermore, whether class B scavenger receptors that only recognize oxLDL but not AcLDL (Sparrow and others 1989) are involved in the strong inhibitory effect of oxLDL has not been explored.

OxLDL at the concentrations used in this study did not decrease cell viability or mitochondrial dehydrogenase activity as determined by the MTT

assay. Furthermore, oxLDL prepared by our method is free of Fe^{++} and Cu^{++} ions, and may have lower cytotoxicity compared to oxLDL prepared with these transition metal ions. Coffey and others (1995) suggest that Fe^{++} increases oxLDL cytotoxicity by causing the breakdown of LOOH to toxic $LO^{\cdot}$ and $^{\cdot}OH$ radicals and aldehydes.

13-HPODE and LysoPC on NO Production in LPS Stimulated Macrophages

13-HPODE and lysoPC have been shown by other investigators to be the major products formed during LDL oxidation (Folcik and others 1995; Noguchi and others 1993; Steinbrecher and others 1990). We are the first to find that 13-HPODE was markedly more effective than lysoPC for inhibiting NO production by stimulated RAW264.7 macrophages (see Figure 3-6). 13-HPODE is a product formed when octadecadienoic acid at 2-position of phosphatidylcholine (PC) is oxidized and hydrolyzed by the PLA_2 activity of oxLDL (see reaction 1-1). Compared to 13-HPODE, LOOH in oxLDL was found to be less effective as an inhibitor of NO production (see Figure 3-4 and 3-7), which suggests that unesterified LOOH may be a better inhibitor than esterified LOOH.

As shown in Figure 3-6, lysoPC at 50 μM had no significant effect on NO production (see Figure 3-6). LysoPC is the second major product formed during LDL oxidation. LDL contains about 1500 nmol phospholipids/mg protein with PC at a level of 818 nmol/mg protein (Esterbauer and others 1992). LysoPC reaches 400-600 nmol/mg protein in extensively oxidized LDL (Steinbrecher and others 1990). Extensively oxidized LDL at a concentration of 100 μg protein/ml

has lysoPC about 40-60 μM, which is the concentration we used in our study. Therefore, lysoPC is unlikely to be a major contributor for the inhibitory effect of oxLDL on NO production.

The data in Figure 3-6 also show that the inhibitory effects of 13-HPODE and lysoPC on NO production were very similar in AcLDL carrier or BSA carrier. In the concentration dependent experiments shown in Figure 3-7 and 3-8, both 13-HPODE and lysoPC in BSA carrier showed inhibition of NO production, with 13-HPODE being more potent than lysoPC. These results suggest that the effects of 13-HPODE and lysoPC did not require an AcLDL carrier. In the present study, we have not determine the mechanisms for the uptake of 13-HPODE by macrophages. However, we found that 13-HPODE levels decreased when 13-HPODE was incubated with macrophages and were undetectable after 24 hours (data not shown). In the Fe^{++} free RPMI medium, 13-HPODE is unlikely to decompose to $LO^{\bullet}$, and $^{\bullet}OH$ radicals, and aldehydes. Cellular GSH peroxidases would be expected to reduce 13-HPODE to 13-HODE with a transient consumption of intracellular GSH (see reaction 1-3) (Coffey and others 1995; Schmitt and others 1995).

Antioxidants on Nitric Oxide Production

In the experiment shown in Figure 3-5, AcLDL and LDL only slightly inhibited NO production in macrophages compared to oxLDL. AcLDL and LDL contained not only low levels of LOOH but also high levels of α-tocopherol. Macrophages take up AcLDL with the accumulation of lipids as well as

intracellular α-tocopherol. Whether the high in intracellular α-tocopherol also contributes to modulating NO production has not been previously investigated. We loaded macrophages with α-tocopherol by pre-incubation with α-tocopherol containing liposomes. Macrophages took up liposomes by phagocytosis and the cellular α-tocopherol in macrophages increased dramatically compared to macrophages loaded with control liposomes containing no tocopherol. As shown in Figure 3-9, increased cellular α-tocopherol attenuated the inhibition due to oxLDL by about 35% compared to cells treated with control liposomes. α-Tocopherol has been shown by Schmitt and others (1995) to inhibit the GSH and ATP depletion caused by oxLDL in cultured endothelial cells. Kuzuya and others (1991) also observed that loading of endothelial cells and macrophages with α-tocopherol or probucol reduced oxidative injury due to oxLDL. The intracellular content of α-tocopherol may, therefore, be important for reducing cellular oxidative stress. Recently, increased evidence also shows that intracellular α-tocopherol prevents the impairment of EDRF mediated relaxation due to oxLDL as well as inhibits SMC proliferation and monocyte adhesion on endothelium (Devaraj and others 1996; Keaney and others 1996; Ozer and others 1993). The inhibition on cellular PKC activity has been suggested to contribute, in part, to the cellular effects of α-tocopherol (Devaraj and others 1996; Keaney and others 1996; Ozer and others 1993). These results suggest that α-tocopherol containing liposomes provide an effective way to increase tissue tocopherol

levels and would therefore provide a unique approach for pharmacological intervention.

The data in figure 3-10 and 3-11 show that pre-treatment of macrophages with NAC also attenuated the inhibitory effects of oxLDL and 13-HPODE on NO production. NAC serves as an antioxidant by scavenging radicals and by increasing intracellular GSH levels (Moldeus and Cotgreave 1994; van 1995). Both oxLDL and LOOH has been shown to deplete intracellular GSH levels (Hennig and others 1996; Schmitt and others 1995; Thomas and others 1993). Phospholipid hydroperoxide glutathione peroxidase (PHGPX) is a 18kDa selenoprotein expressed in cells (Maiorino and others 1991). PHGPX reduces LOOH in oxLDL to LOH and 13-HPODE to 13-HODE using GSH as a substrate. OxLDL and 13-HPODE, therefore causing the consumption of intracellular GSH. Treatment of macrophages with NAC reversed the inhibition of NO production due to oxLDL and 13-HPODE. These results further suggest that the inhibition of oxLDL and 13-HPODE on NO production may be mediated by redox sensitive mechanisms. Increased intracellular α-tocopherol or NAC may preserve NO production in macrophages. The present study also suggest a possible pharmacological strategy for intervention in atherosclerotic process.

Regulation of iNOS Gene

The data in Figure 3-12 and 3-15 show that LPS induced the expression of iNOS in RAW264.7 macrophages, whereas oxLDL significantly decreased iNOS expression by decreasing iNOS protein and iNOS mRNA. Yang and

others (1994) found that oxLDL inhibits isolated cytosolic iNOS activity. Nevertheless, our study indicates that a decreased expression of iNOS caused by oxLDL may be the major contributor for the decreased NO production. OxLDL decreased iNOS mRNA levels, presumably by inhibiting the transcription of iNOS gene in macrophages as found in endothelial cells by Liao and others (1995).

The induction of mouse iNOS gene is regulated by NF-κB, AP-1 and other transcription factors (Xie and others 1994; Xie 1997). As shown in Figure 3-16, LPS was found to activate NF-κB in RAW264.7 macrophages. These results confirm that the activation of NF-κB is important for the expression of iNOS as suggested by others (Goldring and others 1995; Xie and others 1994). We also showed that oxLDL slightly suppressed the activation of NF-κB 45 minutes after incubation (see Figure 3-18). The suppression of NF-κB activation was in agreement with the decreased iNOS mRNA and iNOS protein shown in Figure 3-13 and 3-15, respectively. The slight suppression of NF-κB, however, is not sufficient enough to explain the dramatic decrease of iNOS mRNA levels in this experiment. An increased post-transcriptional degradation of iNOS mRNA can not be excluded. Others investigators (Ohlsson and others 1996; Schackelford and others 1995) have found that oxLDL almost completely suppresses NF-κB activation in mouse peritoneal macrophages. Furthermore, several studies (Hamilton and others 1995; Ohlsson and others 1996; Schackelford and others 1995) also show that oxLDL inhibits the expression of other NF-κB regulated

genes such as TNF-α, IL-1α and IL-1β. These further support the notion that oxLDL inhibits iNOS expression at least in part at the transcriptional level.

If the suppression of iNOS gene expression was due to the suppression of NF-κB then we would expect to see a marked suppression of NF-κB with oxLDL, but this was not observed as shown in Figure 1-18. Compared to study of Ohlsson and others (1996) who treated macrophages with oxLDL for 24 hours before stimulation with LPS, we only treated cells with oxLDL plus LPS for 45 minutes. It might be possible that macrophages were not exposure to oxLDL sufficient long to elicit a strong inhibition in our experiments. It is also possible that the activation of NF-κB is required but not sufficient for the expression of iNOS, as suggested by others (Flodstrom and others 1996; Kuo and others 1997). Additional transcription factors such as AP-1 may also regulate iNOS transcription (Flodstrom and others 1996; Kuo and others 1997; Xie and others 1994). NF-κB probably acts in concert with adjacent cis-elements that are activated by extracellular signals to form an effective initiation transcription complex (Collins 1993). In other words, the suppression of AP-1 or other transcription factors could also inhibit iNOS gene expression. Interestingly, AP-1 is also a redox sensitive transcription factor (Flohe and others 1997). Unlikely NF-κB, AP-1 is inhibited by oxidants (Flohe and others 1997). Therefore, oxLDL may suppress AP-1 activation thereby inhibiting iNOS expression. At the present time, only very limited information is available on the role of AP-1 on the regulation of the iNOS gene in macrophages.

The results shown in Figure 3-12, 3-15 and 3-18 clearly show that oxLDL neither induced iNOS nor activated NF-κB in macrophages. Our results, along with that of others (Hamilton and others 1995; Ohlsson and others 1996; Schackelford and others 1995; Thai and others 1995) do not fit the simple model shown in Figure 1-3 in which oxidants activate NF-κB and antioxidants block this effect. Regulation of gene expression by NF-κB is complex, the activation of p50/p65 activates gene expression, whereas the activation of p50/p50 may be inhibitory thereby suppressing gene expression (Franzoso and others 1992; Plaksin and others 1993). Schackelford and others (1995) have discussed the possibility that oxLDL induces an unidentified complex that competes with p50/p65 binding to the NF-κB DNA sequence thereby inhibits TNF-α expression. However, results from the present study did not show a dramatic activation of p50/p50 dimer that could inhibit p50/p65 binding.

Recent evidence suggests that the induction of NF-κB by oxidative stress may be cell type specific (Brennan and O'Neill 1995; Grigoriadis and others 1996; Hecker and others 1996). In endothelial cells, oxLDL indeed induces NF-κB as well as NF-κB regulated genes such as adhesion molecules (i.e., VCAM-1, ELAM-1, ICAM-1), colony stimulating factors (i.e., GM-CSF, M-CSF), chemoattratant (i.e., MCP-1), and cytokines (i.e., IL-6, IL-8, IFN-β) (for a review see Collins 1993; Collins and others 1995). This is a typical example of the model shown in Figure 1-4. However, macrophages behave differently compared to endothelial cells in that intracellular GSH depletion due to oxLDL

continues in endothelial cells but rebounds, and then exceeds the resting GSH levels in macrophages by increasing GSH biosynthesis (Darley-Usmar and others 1991; Faruqi and others 1997). Furthermore, NF-κB has been suggested to have dual effects on macrophages (Sato and others 1995). Macrophages may have superior abilities to deal with oxidative stress because they can generate oxidative bursts upon stimulation by pathogens. Endothelial cells may be more susceptible to oxidative stress than macrophages.

Oxidative stress also induces heat shock proteins (HSP) that protect against environmental stress. HSP70, for instance, was induced in cultured human endothelial, and SMC by treatment with oxLDL (Pirillo and others 1997; Zhu and others 1994; Zhu and others 1995). Significantly, several studies (Berberian and others 1990; Johnson and others 1993) have found an increased expression of HSP70 in human, and rabbit atherosclerotic arteries. Furthermore, HSP70 has been shown to inhibit the activation of NF-κB, and the expression of iNOS in arterial SMC, and astroglial cells (Feinstein and others 1996; Wong and others 1995). Suppression of NF-κB caused by oxLDL may be a secondary response of HSP induction. This may explain the slight inhibition of NF-κB caused by oxLDL as shown in Figure 3-18, because the secondary suppression of NF-κB due to the induction of HSP70 may not be obvious as early as 45 minutes. HSP70 is, therefore, a possible link between oxLDL and decreased iNOS mRNA levels in macrophages.

We are the first to find that 13-HPODE mimicked the effect of oxLDL on

decreasing iNOS protein in LPS stimulated macrophages (see Figure 3-13). LysoPC, however, at almost twice of the concentration for 13-HPODE, had no significant effect (see Figure 3-14). These results further suggest the involvement of oxidative stress in iNOS gene expression because both 13-HPODE and oxLDL would induce intracellular oxidative stress by the consumption of GSH but lysoPC would not have this effect (Darley-Usmar and others 1991; Faruqi and others 1997; Hennig and others 1996). Furthermore, intracellular α-tocopherol or pre-treatment with NAC attenuated the inhibition on NO production caused by oxLDL or 13-HPODE (see Figure 3-9, 3-10 and 3-11). Oxidative stress, therefore, is a plausible mechanism for inhibiting NO production in stimulated macrophages.

Physiological Significance

Inducible Nitric Oxide Production and Atherosclerosis

Inducible NO production from macrophages may be important for modulating *in vivo* LDL oxidation (Leeuwenburgh and others 1997; Rubbo and others 1994). Macrophages produce NO by iNOS using L-arginine and O_2 as substrates and NADPH as a co-factor. $O^{\bullet}_2{}^-$ is thought to be produced by NADPH oxidase. The potential physiological significance of diminished NO production by macrophages in the arterial intima due to oxLDL may be partly dependent upon the localized $O^{\bullet}_2{}^-$ production (Rubbo and others 1994). NO may act as an antioxidant under conditions where local production of $O^{\bullet}_2{}^-$ is low (Jessup and

Dean 1993; Rubbo and others 1994). In this case, the diminished production of NO by oxLDL could promote atherosclerosis. In contrast, if the localized $O^{\bullet}_{2}^{-}$ level is high, then a diminished production of NO would decrease $ONOO^{\bullet-}$ levels and prevent the pro-atherogenic effects caused by this oxidant (Hogg and others 1993). A recent study indicates that in L-arginine depleted macrophages, iNOS produces both NO and $O^{\bullet}_{2}^{-}$ (Xia and Zweier 1997). Significantly, low L-arginine levels are associated with increased atherogenesis (Naruse and others 1994). The co-production of NO and $O^{\bullet}_{2}^{-}$ by iNOS leads to the production of $ONOO^{\bullet-}$ which may be important in atherosclerosis (Leeuwenburgh and others 1997; Rubbo and others 1994). The induction of iNOS in macrophages, therefore, may play an important role in initiating or modulating the process of atherosclerosis.

Antioxidants and Nitric oxide Production

Antioxidants have been implicated in decreasing the incidence of cardiovascular disease. Antioxidants, specially α-tocopherol, protects LDL against lipid peroxidation. The present study provides evidence that α-tocopherol preserved NO production in macrophages treated with oxLDL. Our results support the hypothesis of Diaz (Diaz and others 1997) that α-tocopherol has direct effects on tissues in preventing atherosclerosis. We also found that NAC reversed the inhibition of NO production caused by oxLDL or 13-HPODE. Therefore, α-tocopherol and NAC may modulate the process of atherosclerosis by preserving NO production that otherwise would be impaired by oxLDL.

Atherosclerosis and Infection

Accumulating evidence suggests that infectious microorganisms may play a role in atherosclerosis. The most prevalent pathogen related to atherosclerosis is chlamydia (Mlot 1996). Chlamydia was consistently found with high incidence in human atherosclerotic plaques using in PCR techniques (Jackson and others 1997; Kuo and others 1993). Chlamydia is a special type of bacteria that lives as a parasite in macrophages, and contains LPS (Toman and others 1997). Although we do not understand how chlamydia is related to atherosclerosis, chlamydia is unlikely to be an accidental deposit in pre-existing atherosclerotic lesions (Mlot 1996). Chlamydia infection could produce signals that modulate macrophage functions such as the induction of iNOS.

Similarly, infectious viruses such as cytomegalovirus (CMV) and herpes simplex virus (HSV) have been linked to atherosclerosis (for a review see Hajjar and Nicholson 1997). CMV and HSV have also been found in human atherosclerotic lesions and transplanted hearts. Infection of cultured endothelial cells, or animal models with HSV produces alterations similar to those observed in chronic atherosclerotic lesions (Jacob and others 1992). HSV infected macrophages have been shown to adhere to endothelium via scavenger receptor class B (Suzuki and others 1997a). Furthermore, both CMV and HSV are able to activate NF-kB, and may initiate an atherosclerotic-like process (Speir and others 1996).

CONCLUSIONS

Our study indicates that the levels of LOOH in oxLDL determine the inhibition potential of oxLDL on NO production in LPS stimulated RAW264.7 macrophages. This inhibition is not due to oxLDL cytotoxicity. 13-HPODE markedly inhibits NO production compared to lysoPC. These data suggest that LOOH in oxLDL may be more important than lysoPC for inhibiting NO production. Both α-tocopherol and NAC attenuate the inhibitory effects of oxLDL and 13-HPODE. OxLDL also decreases iNOS protein and mRNA levels. OxLDL suppresses the expression of iNOS gene, possibly at the transcriptional level. The present study, therefore, suggests that the atherogenic properties of oxLDL may, in part, be mediated by modulating NO production in macrophages. Oxidative stress may be a possible mechanism for regulation of iNOS gene expression by oxLDL. Preservation of NO production may contribute to the anti-atherosclerotic effects of antioxidants. This study also suggests that the regulation of NO production may be a target for future intervention studies of atherosclerosis.

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Manuscripts:

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IMAGE EVALUATION TEST TARGET (QA-3)

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