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Some aspects of the cell biology of lipid S-nitrosothiols.

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NOTE TO USERS

Some aspects of the cell biology of lipid S-nitrosothiols

By

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ABSTRACT

Nitric Oxide is an important messenger and mediator in biological systems. Due to its short half life, S-nitrosothiols (RSNOs) have been suggested to be one of the important biomolecules for the storage and transport of NO. RSNOs also exhibit NO-like bioactivities. In the present study, we focus on the study of some aspects of cell biology of lipid S-nitrosothiols.

In the first part of the study, we focus on the comparison of the cellular uptake of native LDL and chemically modified (homocysteinylation and S-nitrosation) LDL by Normal Human Fibroblasts (NHFs). Here, S-nitroso homocysteinylated LDL (LDL-SNO) was synthesized through the reaction of homocysteinylated LDL (LDL-SH) and S-nitrosocysteine. The effects of homocysteinylation and subsequent S-nitrosation on the uptake of LDL were examined in NHFs. The present results demonstrated that LDL-SH could be S-nitrosated to form LDL-SNO in vitro. Under our experimental conditions, homocysteinylation and subsequent S-nitrosation did not affect the specific uptake of LDL through LDL receptor mediated endocytosis. In addition, insulin exerts the same stimulatory effect on the uptake of chemically modified (homocysteinylation and S-nitrosation) LDL as that of native LDL. These results suggested that S-nitrosation of LDL-SH might be a mild and favorable modification to reverse the detrimental actions of LDL-SH in atherosclerosis. The potential beneficial effects of LDL-SNO in biological systems were discussed.

In the second part of the study, we focus on the comparison of the role of lipid S-nitrosothiols in apoptosis under light and dark conditions. Here, S-nitroso-octadecanethiol, a novel and lipophilic S-nitrosothiol, was synthesized to explore its apoptotic role in the SK-MEL-28 human melanoma cells under dark and light conditions. The present study demonstrated that S-nitroso-octadecanethiol could induce apoptosis in SK-MEL-28 human melanoma cells. The apoptosis induced by S-nitroso-octadecanethiol involved the release of cytochrome C from mitochondria and caspase 3 activation. Furthermore, the apoptotic efficiency is substantially enhanced approximately 30% along with more cytochrome c released from mitochondria and higher caspase-3 activity upon exposure to the light with respect to dark condition. Therefore, the present study suggested that S-nitroso-octadecanethiol might have a potential to act as a photochemotherpeutic agent for melanoma.

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LIST OF ABBREVIATIONS

AH^-	Ascorbate monoanion
Apaf-1	Apoptotic protease-activating factor-1
ASNO	S-nitroso-albumin
CaM	Calmodulin
cGMP	Cyclic guanosine monophosphate
Cys	Cysteine
CySNO	S-nitroso-cysteine
DD	Death domain
DiI	3,3'-dioctadecylindocarbocyanine
DISC	Death-inducing signaling complex
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial Nitric Oxide Synthase
FAD	Flavin adenine dinucleotide, oxidized form
FADD	Fas-associated protein with death domain
FasL	Fas ligand
FMN	Flavin mononucleotide
GSH	Glutathiol
GSNO	S-nitrosoglutathiol
GSSG	Oxidized Glutathiol
GTx	Glutathione peroxidase
H_4B	Tetrahydrobiopterin
Hcy	Homocysteine
HcyTL	Homocysteine thiolactone
Homocysteinylated LDL	LDL-SH
iNOS	Inducible nitric oxide synthase
LDL	Low density lipoprotein
LSH	1-octadecanethiol
ODTSNO	S-nitroso-octadecanethiol

N_2O_3	Dinitrogen trioxide
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NHFs	Normal Human Fibroblasts
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO^-	Nitroxyl anion
NO_2	Nitrogen dioxide
NOSs	Nitric oxide synthases
O_2	Oxygen
O_2^-	Superoxide anion
$ONO_2CO_2^-$	Nitrosoperoxycarbonate
$ONOO^-$	Peroxynitrite
oxLDL	Oxidized low density lipoprotein
PDI	Protein Disulfide Isomerase
PTP	Permeability transition pore
RS^-	Thiolate anion
RSNO	S-nitrosothiol
SAPKs	Stress activated protein kinases
sGC	Soluble guanylate cyclase
SNAP	S-nitroso-N-acetylpenicillaime
S-nitrosated LDL-SH	LDL-SNO
XO	Xanthine oxidase

Part I

S-nitrosation of homocysteinylated low density lipoproteins and comparison of the uptake of native and modified (homocysteinylated and S-nitrosated) LDL by Normal Human Fibroblasts

1.0 Abstract

Background and objectives: Oxidized low density lipoprotein (oxLDL) is a key element for the development of atherosclerosis. Homocysteinylation of LDL by homocysteine thiolactone (HcyTL) has been suggested to increase the susceptibility of LDL to oxidative modification. The extent of oxidation of homocysteinylated LDL (LDL-SH) has been shown to relate to the levels of free thiol groups incorporated into the LDL through the interaction with HcyTL (Ferretti et al., 2004). Nitric oxide (NO) is a good inhibitor for the oxidative modification of LDL. Protein thiol groups are the potential cellular targets for S-nitrosation reaction to form S-nitrosothiols (RSNOs), the most important biomolecules for the storage and transport of short-lived NO. RSNOs also possess NO-like bioactivities. Therefore, in the present study, whether free SH groups of LDL-SH could be susceptible to S-nitrosation to form favorable S-nitroso homocysteinylated LDL (LDL-SNO) was examined. Normal levels of LDL in plasma play a key role in vascular diseases such as atherosclerosis. Insulin plays a regulatory role in LDL homeostasis. Thus, the uptake of native LDL and chemically modified LDL (LDL-SH and LDL-SNO) by Normal Human Fibroblasts (NHFs) was compared. The effect of insulin on the uptake of chemically modified LDL was also examined.

Methods: S-transnitrosation was used to study the nitrosation of LDL-SH. Fluorescently labeled LDL, LDL-SH and LDL-SNO with 3,3'-dioctadecylindocarbocarbocyanine (DiI) were used to compare the cellular uptake of native and chemically modified LDL.

Results and conclusions: LDL-SH could be S-nitrosated to form LDL-SNO in vitro. Under our experimental conditions, homocysteinylation and subsequent S-nitrosation did

not affect the specific uptake of LDL through LDL receptor mediated endocytosis. There is no significant difference among the uptake of LDL, LDL-SH and LDL-SNO by NHFs. Insulin exerts the same stimulatory effect on the uptake of chemically modified (homocysteinylation and S-nitrosation) LDL as that of native LDL. These results suggested that S-nitrosation of LDL-SH might be a mild and favorable modification to reverse the detrimental actions of LDL-SH in atherosclerosis. The potential benefical effects of LDL-SNO in biological systems are discussed.

1.1 Introduction

1.1.1 Nitric oxide (NO) chemistry

NO is a small and hydrophobic molecule that is synthesized in biological systems (Moncada et al., 1991). It is one of the most important signaling molecules in biological systems. It plays various roles in physiology and pathophysiology such as stimulation of vascular smooth muscle relaxation and atherosclerosis (Kerwin et al., 1995).The biological actions of NO are dependent on its chemical reactivity. Thus, understanding the chemical reactions of NO in vivo is very important.

NO is a free radical which contains an unpaired electron. Therefore, in biological systems, NO will react with those substances that have unpaired electrons. Such typical examples include the reactions with oxygen (O_2) and superoxide anion ($O_2^{\bullet -}$). Another important reaction of NO in biological systems is the interaction with metal complexes such as heme-containing protein soluble guanylate cyclase (sGC) (Krumenacker et al., 2004).

1.1.1.1 Chemical reactions with O_2

NO is unstable in an oxygen environment because it undergoes autooxidation. In the gas phase, the reaction of NO with O_2 forms nitrogen dioxide (NO_2) (Eq.1.1.1) and dinitrogen trioxide (N_2O_3) (Eq.1.1.2).

$$2\,NO + O_2 \longrightarrow 2\,NO_2 \quad (1.1.1)$$

$$NO_2 + NO \longrightarrow N_2O_3 \quad (1.1.2)$$

N_2O_3 is responsible for the S-nitrosation reaction, the most important pathway for the formation of the biologically important molecules such as S-nitrosothiols (RSNOs). However, in the aqueous phase, the resultant intermediate N_2O_3 is not stable and undergoes hydrolysis to form nitrite (Eq. 1.1.3), which accounts for the short half- life of NO (reported to be only 0.1 seconds [Kelm et al., 1990]) (Wink et al., 1998).

$$N_2O_3+H_2O \longrightarrow 2\ HNO_2 \quad (1.1.3)$$

NO and O_2 are 9 times more soluble in hydrophobic solvents than in the water (Shaw et al., 1977; Malinski et al., 1993). Thus increased concentrations of NO and O_2 in the hydrophobic phase can accelerate reaction of NO with O_2 up to 300- fold with respect to the aqueous phase (Liu et al., 1998). Therefore, it has been proposed that in biological systems the reaction of NO and O_2 and subsequent S-nitrosation reactions mediated by N_2O_3 occur predominantly in the hydrophobic compartments such as lipid membranes, hydrophobic cores of the lipoproteins or protein interiors (Wink et al., 1998; Rafikova et al., 2002).

1.1.1.2 Chemical reactions with superoxide anion ($O^{\bullet}_2^-$)

The reaction of NO with $O^{\bullet}_2^-$ occurs at near diffusion controlled rates with a rate constant of 4.3 to 6.7 $x10^9 M^{-1}S^{-1}$ to produce a powerful oxidant, peroxynitrite ($ONOO^-$) (Eq.1.1.4) (Beckman et al.,1996).

$$NO + O_2^{\bullet -} \longrightarrow ONOO^- \quad (1.1.4)$$

Peroxynitrite can undergo various chemical reactions. These reactions exhibit detrimental effects on biological systems. For example, once protonated, it will rapidly decompose to a potently cytotoxic intermediate-peroxynitrous acid (Beckman et al.,

1996). Peroxynitrous acid has shown to mediate most of the oxidation and nitration reactions of peroxynitrite (Koppenol et al., 1992). However, in vivo, the detrimental actions of peroxynitrite are greatly reduced due to the relatively high concentrations of reduced glutathiol (GSH) which exist in most cells (Wink et al., 1998). In addition, the reaction of peroxynitrite with CO_2 produces a potent oxidizing and nitrating agent: nitrosoperoxycarbonate ($ONO_2CO_2^-$) (Lymar et al., 1995) which causes the tyrosine nitration seen in some pathological conditions such as Alzheimer's disease (AD) (Mayer et al., 1997; Reynolds et al., 2005.).

1.1.1.3 Chemical reactions with soluble guanylate cyclase (sGC)

A well-studied reaction of NO with metal complexes in biological systems is the reaction with soluble guanylate cyclase (sGC), one of the heme-containing proteins. sGC has been suggested to be the major receptor in vivo for NO (Denninger et al., 1999). Furthermore, the interaction of NO with sGC is one of the most important mechanisms underlying the biological effects of NO. NO binds to the heme iron of sGC at diffusion-controlled rates to form iron-nitrosyl complex (Mayer et al., 1997). The resultant iron-nitrosyl complex is pentacoordinate so as to activate sGC (Stone et al., 1995). Activation of sGC stimulates the production of an important second messenger cGMP, which is responsible for numerous physiological actions of NO such as vasodilation, anti-platelet aggregation and neurotransmission (Krumenacker et al., 2004).

1.1.2 NO biochemistry

1.1.2.1 NO formation in biological systems

1.1.2.1.1 Enzymatic pathway for NO biosynthesis

It has been shown that NO synthesis in biological systems is catalyzed by a family of enzymes NO synthases (NOS). The reaction involves sequential oxidation of L-arginine to L-citrulline to produce NO, which utilize NADPH and O_2 as cosubstrates. It also involves the formation of intermediate N-hydroxyl-L-arginine, which can act as a substrate for NOS (Korth et al., 1994; Griffith et al., 1995) (Figure 1.1.1).

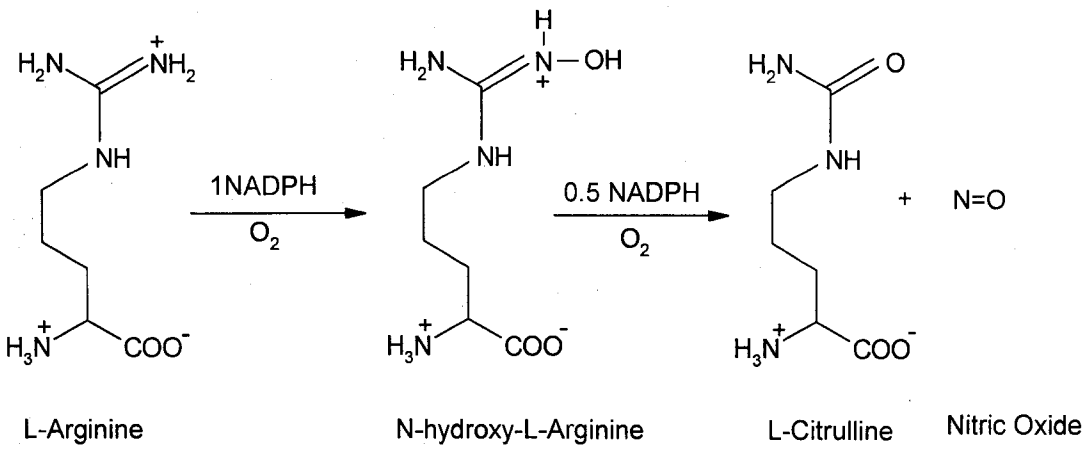


Figure 1.1.1: Enzyme-catalyzed NO biosynthesis (Griffith et al., 1995)

1.1.2.1.2 Non-enzymatic pathway for NO synthesis

In addition to the most established pathway for enzymatic NO synthesis in biological systems, a recent study (Zweier et al., 1999) reported that enzyme-independent NO formation also occured in biological systems. Under acidic and highly reduced

conditions, NO can be generated in tissues by either direct disproportionation or reduction of nitrite to NO. This pathway becomes the major source of NO in important disease states such as ischemia or shock, where acidosis and marked hypoxia occur, although this pathway for NO synthesis can occur in all biological tissues.

1.1.2.2 NOS structure and function

Three NOS isoforms have been shown to be the products of separate genes (Nathan et al., 1994). Two of three NOS are constitutive enzymes known as neuronal NOS (nNOS) and endothelial NOS (eNOS) (Fleming et al., 1998). They synthesize NO in response to the increase of Ca^{2+} in the cell. Another one is inducible NOS (iNOS). It synthesizes NO upon stimulation by signals such as cytokines (Kroncke et al., 1995; Hierholzer et al., 1998).

Although different isoforms, each NOS isoform monomer has a similar bidomain structure: a C-terminal reductase domain containing binding sites for NADPH, FAD, and FMN, and a N-terminal oxygenase domain for binding heme, tetrahydrobiopterin (H_4B) and the substrate L-Arginine. Each domain can exist and function independently. An amino acid recognition sequence for the Ca^{2+}- binding protein calmodulin (CaM) is located between the two domains (Hemmens et al., 1998) (Figure 1.1.2).

All three NOS isoforms are homodimeric enzymes (Stuehr, 1997). However, the interactions between oxygenase and reductase domains of the subunits are different for inducible NOS and constitutive NOS. Venema et al., (1997) reported that iNOS subunit association consists of only head to head interactions of the oxygenase domains, however, eNOS and nNOS subunit association involves not only the oxygenase domain

interactions but also the reductase domain interactions as well as the interactions between oxygenase and reductase domains.

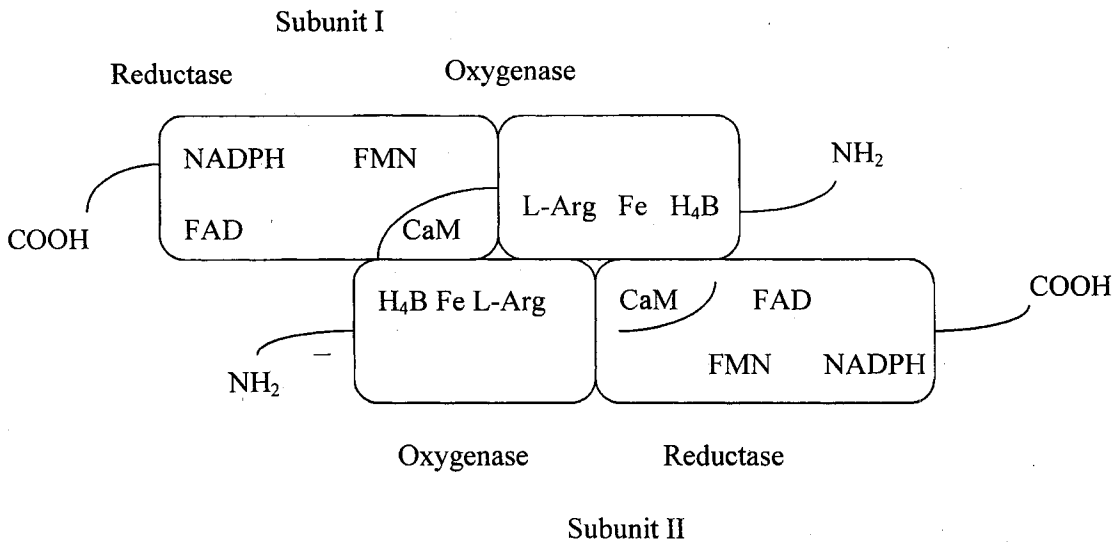


Figure1.1.2: Domain structure of NOS homodimer, showing cofactor and substrate binding sites (Andrew et al., 1999).

1.1.2.2.1 NOS reductase domain structure

The NOS reductase domain has binding sites for FMN, FAD and NADPH (Stuehr et al., 1997). It has been suggested that the NOS reductase domain is homologous to other FMN- and FAD-containing reductases such as cytochrome P-450 reductase (Wang et al., 1997). An isolated NOS reductase domain can transfer electrons from NADPH through the flavins FMN and FAD to cytochrome *c* as do cytochrome P-450 reductase (Gachhui et al., 1996; Chen et al., 1996). However, with respect to cytochrome P-450 reductase, CaM-binding domain is a requirement for the electron transfer of NOS reductase domain

(Abu-Sound et al., 1994), since Calmodulin activates not only intradomain electron transfer, but also interdomain electron transfer from NADPH via flavins to the heme, so as to contribute to the synthesis of NO (Abu-Sound et al., 1994; Stuehr et al., 1997).

1.1.2.2.2 NOS oxygenase domain structure

The human iNOS oxygenase domain structure is mainly exemplified as a model for studying the structure of NOS isoforms oxygenase domains. Crane et al. (1997) reported that the human iNOS oxygenase domain has an elongated shape. It contains many β-sheets, considerably different from those heme-containing oxygenases. It also contains a novel α-β fold. Therefore its structure is described to be analogous to a baseball glove with the heme group in the palm of the glove.

Zinc ion has been discovered to tetrahedrally coordinate to two pairs of cysteine (Cys) residues through the examination of the crystal structure of eNOS (Raman et al., 1998). It has been proposed that zinc metal center functions as maintaining the integrity of H_4B-bindig site. In addition, the zinc binding motif, Cys-$(X)_4$-Cys, is found to be conserved in all known NOS isoforms to date, suggesting that the zinc ion exits in all of physiologically relevant NOS structures. For example, zinc metal center has also been found to exist in human iNOS (Fischmann et al., 1999) and nNOS (Hemmens et al., 2000). In addition to maintain the integrity of H_4B-bindig site, a zinc metal center might favour dimer stabilization in iNOS (Li et al., 1999) and nNOS (Hemmens et al., 2000)

1.1.2.3 Regulation of NOS activity

Although these three NOS isoforms can be regulated by differentially specific mechanisms, there are some regulation mechanisms that can be shared by all three NOS isoforms.

1.1.2.3.1 Feedback inhibition of NOS by NO

NO may be a negative feedback modulator of NOS. This is because NO can readily and reversibly bind the heme iron to form catalytically inactive Ferrous/Ferric-nitrosyl complex with NOS. Therefore, NOS enzymatic activity can be recovered only upon NO release from the heme iron (Hurshman et al., 1995; Abu-Soud et al., 1995). The extent of the inhibition of NOS enzymatic activity by NO is different for the different isoforms of NOS. It has been shown that ecNOS and nNOS are more susceptible to inhibition by NO than iNOS since NO has a higher reactivity toward heme in cNOS than iNOS (Wink et al., 1998). Moreover, the resultant Fe-NO complex is more stable in cNOS than iNOS.

1.1.2.3.2 H_4B

NOS activity can be regulated by NOS cofactor H_4B levels. H_4B is essential for coupling O_2 activation to NO synthesis (Mayer et al., 1995). The NOS homodimer has two binding sites for H_4B which are located on each of the two subunits. However, the binding of H_4B to NOS showes strong negative cooperatives. The binding of the first H_4B lowers the enzyme's affinity for binding the second H_4B by at least an order of

magnitude (Gorren et al., 1996). Thus insufficient H_4B levels can limit NO biosynthesis (Harrison, 1997).

1.1.2.3.3 Calmodulin (CaM)

CaM is necessary for the enzymatic activities of all three NOS isoforms, but functions differently in these three NOS isoforms. In eNOS and nNOS, CaM binding leads to the increase in the rate of electron transfer from NADPH to the reductase domain flavins (Gachhui et al.; 1996 Gachhui et al., 1998.). At the same time, it also triggers electron transfer from the reductase domain to the heme center (Abu-Sound et al., 1994). Thus, CaM helps to increase the rate of NO synthesis caused by eNOS and nNOS. However, for iNOS, CaM binding is not related to enzyme catalytic activity, but is essential to maintain the integrity of iNOS structures such as folding and stabilizing (Fossetta et al., 1996; Wu et al., 1996).

1.1.3 The physiological roles of NO in low density lipoprotein (LDL) peroxidation

NO has been characterized as an endothelium-derived relaxing factor (EDRF) (Gruetter, et al., 1979). The role of NO in vascular systems has been extensively explored. NO plays a key role in the stimulation of vascular smooth muscle relaxation (Ignarro, 1989a; Ignarro, 1989b) and inhibition of platelet aggregation and adhesion through a cGMP-dependent mechanism (Furlong et al., 1987; Radomski et al., 1987). Any damage to the endothelial cell surface will affect the normal functions of NO in vascular systems, resulting in numerous pathophysiological disorders such as atherosclerosis (Ignarro et al., 1999). Among the factors contributing to atherosclerosis, oxidized low density lipoprotein

(oxLDL) is a key element (Napoli et al., 1997). NO has been suggested to be a good inhibitor for LDL peroxidation, thus reducing the possibility of the development of the atherosclerosis initiated by oxLDL. However, NO regulates lipid peroxidation by a mechanism independent of cGMP (Bloodsworth et al., 2000).

1.1.3.1 Regulation of lipoprotein lipid peroxidation by NO

1.1.3.1.1 Mechanism of lipid peroxidation

Lipid peroxidation occurs as bis-allylic hydrogen of the unsaturated fatty acid is easily susceptible to be abstracted by initial free radicals to produce lipid free radicals (Eq. 1.1.5). Lipid free radicals then react with oxygen to produce lipid peroxyl radicals. Lipid peroxyl radicals then propagate the lipid peroxidation since these radicals can oxidize adjacent unsaturated fatty acid to produce lipid hydroperoxide and a second lipid radical (Eq.1.1.7). Thus propagation of the lipid peroxidation (Hogg et al., 1999b; Ignarro 2000).

$$LH + R^{\cdot} \longrightarrow L^{\cdot} + RH \qquad \text{(Eq.1.1.5)}$$

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \qquad \text{(Eq.1.1.6)}$$

$$LOO^{\cdot} + LH \longrightarrow L^{\cdot} + LOOH \qquad \text{(Eq.1.1.7)}$$

1.1.3.1.2 Mechanism of inhibition of the LDL peroxidation by NO

LDL is one of the lipid-carrier blood lipoproteins. Its density ranges from 1.039 g/ml to 1.062 g/ml. It comprises a non-polar core of triacylglycerols and cholesteryl esters, surrounded by an amphiphilic coat of phospholipids and cholesterol molecules and a single molecule of apolipoprotein B-100 (Voet et al., 1999a). About 50% of fatty acid molecules

in the LDL are unsaturated, thus rendering LDL susceptible to oxidative modification (Esterbauer et al., 1992). LDL enters certain cell types by LDL-specific receptor-mediated endocytosis and is subsequently transported to the lysosomes for degradation (Brown et al., 1979).

The most extensively examined mechanism for inhibition of LDL peroxidation by NO is the termination of the lipid peroxidation propagation reaction via scavenging of lipidperoxyradical ($LOO^{\bullet}$) (Eq. 1.1.8) (Rubbo et al., 1995; Goss et al., 1995; Goss et al., 1997.)

$$LOO^{\cdot} + NO \longrightarrow LOONO \quad (1.1.8)$$

Recent studies have shown that NO inhibits LDL peroxidation initiated by enzymatic or non-enzymatic pathways (Bloodsworth et al., 2000). For example, Rubbo (1995) demonstrated that pure NO gas can inhibit LDL peroxidation initiated by lipoxygenase. Hogg et al., (1993) reported that NO donor compounds S-Nitroso-N-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP) could inhibit LDL peroxidation initiated by Cu^{2+}. Termination of lipid peroxidation propagation reaction by NO has been shown to protect the cells from the cytotoxicity induced by lipidperoxy radical (Wink et al., 1994; Wink et al., 1995).

NO is diffusible and lipophilic, and as a result it can diffuse into and concentrate in the hydrophobic core of LDL as a lipophilic antioxidant for LDL (Denicola et al., 2002). It can react with lipid peroxyl radical ($LOO^{\bullet}$) at near-diffusion limited rates ($k=1.0\text{-}3.0\times10^{9} M^{-1}S^{-1}$) (Padmaja et al., 1993), 10^{4}-10^{5} times higher than that of the reaction with α-tocopherol (Hogg et al., 1999b). α -tocopherol is one of the low molecular weight lipophilic antioxidants present in LDL (Esterbauer et al., 1992). Therefore, NO can protect these low

molecular weight antioxidants from oxidation (Goss et al., 1997) and act in synergy with these endogenous antioxidants to protect LDL from oxidation (Ignarro, 1989b; Rubbo et al., 2002).

1.1.3.1.3 Mechanism of stimulation of the LDL peroxidation by NO

In contrast to its well-established direct role in protecting LDL from oxidation, NO can also promote LDL peroxidation indirectly by forming reactive nitrogen species such as powerful oxidant $ONOO^-$. NO reacts with O_2^- at diffusion-limited rates [4.3 to 6.7 $x10^9 M^{-1}S^{-1}$] to form peroxynitrite ($ONOO^-$). $ONOO^-$ is such a powerful oxidant that it can directly initiate lipid peroxidation reactions by abstracting a hydrogen atom from a polyunsaturated fatty acid molecule (Darley-Usmar et al., 1992). It has been suggested that peroxynitrite can have free access to hydrophobic compartments in cells. Therefore, it can readily accumulate in the hydrophobic phase to regulate membrane and lipoprotein lipid oxidation reactions (Davis et al., 2001).

As mentioned above, NO can act as both antioxidant and prooxidant for lipid peroxidation. However, whether NO serves as antioxidant or pro-oxidant depends on the relative concentrations of NO and O_2^- (Goldstein et al., 2000; Jourd'heuil et al., 2001; Rubbo et al., 2002). NO only stimulates peroxynitrite-induced lipid peroxidation when the concentration of NO is less than or equivalent to the concentration of O_2^-. If the concentration of NO is more than that of O_2^-, NO can inhibit peroxynitrite-induced lipid peroxidation (Rubbo et al., 1994). Therefore, NO could modulate LDL peroxidation in a concentration-dependent manner.

1.1.4 S-nitrosothiols (RSNOs)

S-nitrosothiols are thiol-esters of nitrite. They have the generic structure R-S-N=O (Oae et al., 1983). RSNOs have long half-lives with respect to NO; they have been suggested to be one of the important biomolecules in vivo for the storage and transport of NO (Stamler et al., 1992a). RSNOs also exhibit NO-like bioacitivities such as stimulation of smooth muscle relaxation, inhibition of platelet aggregation and adhesion (Stamler et al., 1992a; 1992b). Therefore, understanding how RSNOs are formed in biological systems and their mechanisms of actions are of great importance.

1.1.4.1 Mechanism of RSNOs formation in biological systems

1.1.4.1.1 Oxygen-dependent route

RSNOs cannot be formed by the simple reaction of NO with free thiol groups. The reaction of NO with thiols generates thiol disulfide rather than RSNOs (Eq. 1.1.9) (DeMaster ,1995).

$$NO+2RSH \longrightarrow RSSR+2NO^{-}+H^{+} \quad (1.1.9)$$

The well-characterized mechanism for RSNO formation in biological systems is N_2O_3-mediated S -nitrosation of free thiols (e.g. Glutathiol [GSH]). N_2O_3 can be formed through autooxidation of NO (Kharitonov, 1995).

1.1.4.1.2 Nitrous acid-dependent route

RSNOs can be easily synthesized in the laboratory via the reaction between thiols and nitrite at acidic conditions (Eq. 1.1.10).

$$HNO_2+RSH \rightarrow RSNO+H_2O \quad (1.1.10)$$

This route doesn't occur at physiological pH (pH~7.0), and could only be relevant under extreme acidic conditions (pH< ~3) (Hogg, 2002).

1.1.4.1.3 Hydrophobic phase-dependent route

Actually, this route is crossed over with the oxygen-dependent route, but the hydrophobic interiors of protein (Nedospasov et al., 2000) and membranes (Liu et al., 1998) may facilitate the formation of the nitrosating agent N_2O_3 from NO due to the enhanced solubility of NO and O_2 in the hydrophobic phase with respect to the water phase (Malinski et al., 1993; Shaw et al., 1997). Rafikova et al., (2002) reported that the hydrophobic phase of extracellular albumin catalyze the formation of RSNOs. The mechanism involves micellar catalysis of NO oxidation in the albumin hydrophobic core and specific transfer of NO^+ to low-molecular-weight thiols.

1.1.4.2 Mechanism of biological action of RSNOs

1.1.4.2.1 NO release

1.1.4.2.1.1 Photodecomposition

RSNOs are photosensitive, especially to UV light. Irradiation of RSNOs leads to the homolytic cleavage of the S-NO bond, resulting in the release of NO and formation of thiyl radicals (Eq.1.1.11).

$$RSNO \xrightarrow{h\nu} RS\cdot + \cdot NO \quad (1.1.11)$$

For example, we have shown that irradiation of S-nitrosoglutathione (GSNO) at 340nm or 545nm results in the release of NO (Sexton et al., 1994). Singh et al. (1995) also demonstrated that irradiation of GSNO with light (λ=550nm) resulted in the homolytic decomposition of GSNO to generate NO and glutathionyl radical.

1.1.4.2.1.2 Metal-ion-catalyzed decomposition

RSNOs can be decomposed in a solution containing metal ions such as Cu^{+}, Fe^{2+}, Hg^{2+} and Ag^{+} (Williams et al., 1996a; Dicks et al., 1997).The well-studied decomposition of RSNOs catalyzed by metal ions is the reaction with copper ion. The possible decomposition mechanism catalyzed by copper ion is shown as follows (Figure 1.1.3):

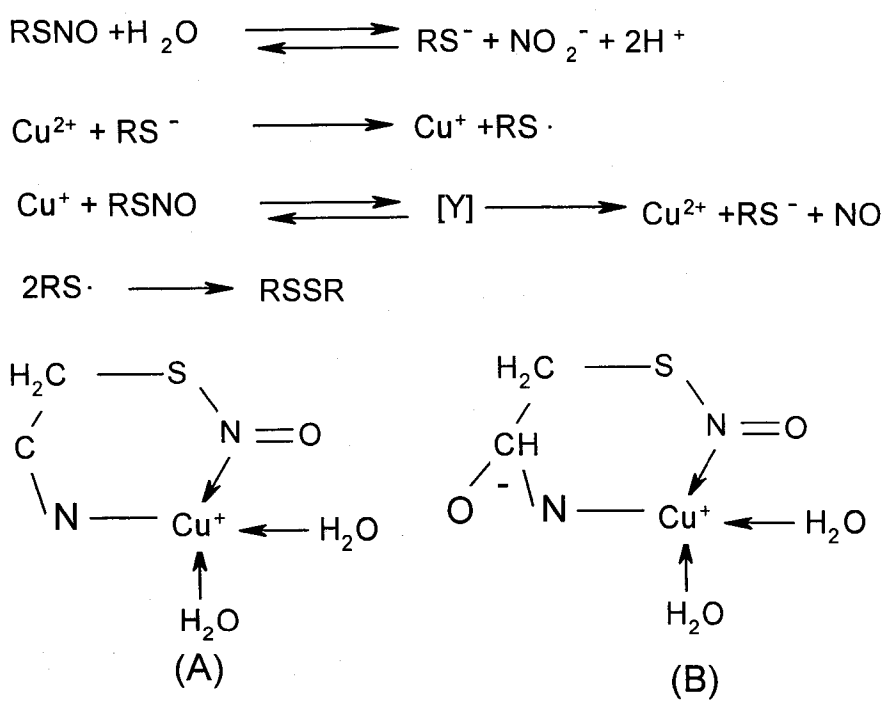


Figure 1.1.3: RSNO decomposition catalyzed by copper ion (Wang et al., 2002)

It has been suggested that Cu^{+} is the true effective catalyst, because the specific Cu^{+} chelator neocuproine stops the decomposition of RSNO completely (Williams, 1996b). Cu^{+} results from the reduction of Cu^{2+} by thiolate ion generated by hydrolysis of RSNO or free thiols. Cu^{+} catalyzes RSNO decomposition by forming a complex intermediate Y. Its structure is shown as A and B. Both Cu^{2+} and RS^{-} can be regenerated and are present in catalytic quantities (Wang et al., 2002).

In addition, previous studies demonstrated that large concentrations of GSNO (e.g. 1mM) are resistant to Cu^{2+}- catalyzed decomposition. This is because oxidized glutathione (GSSG) is an effective copper ion chelator as shown in Figure 1.1.4. At higher GSNO concentration, GSSG is high enough to chelate Cu^{2+} to prevent the release of NO from GSNO. However, at lower GSNO concentration, GSSG is not enough to chelate Cu^{2+}. Thus, Cu^{2+} promotes the NO release from lower concentrations of GSNO such as 1μM (Noble et al., 1999, Singh et al., 1999).

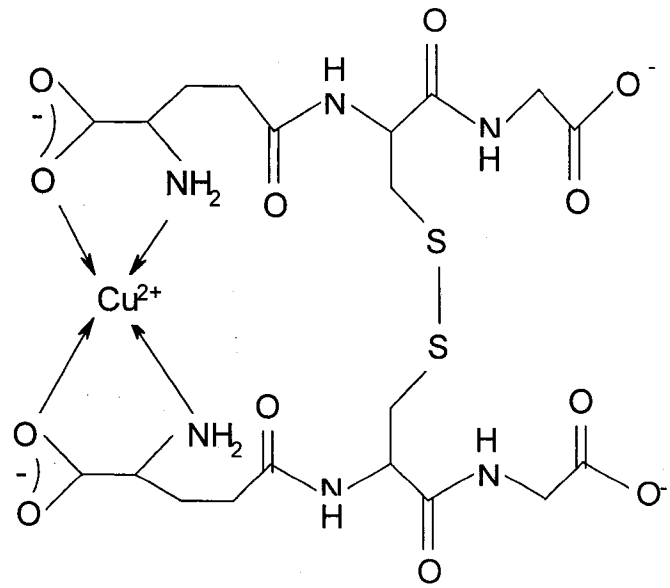


Figure 1.1.4: The structure of complex of GSSG with Cu^{2+} (Noble et al., 1999)

1.1.4.2.1.3 Reductant –dependent decomposition

Ascorbate and protein thiols such as Cys residues in the albumin have been demonstrated to promote the release of NO from RSNOs such as S-nitroso-albumin (ASNO) and GSNO. The mechanism involves the NO^+ transfer to thiolate anion (RS^-) to form RSNO (Eq.1.1.12). Subsequent release of NO from RSNO depends on a reductive activation of NO^+ (RSNO) to NO, which could be catalyzed by ascorbate monoanion (AH^-) or free thiol groups of albumin (Eq.1.1.13 &Eq.1.1.14). Compared with metal ion-catalyzed the release of NO from RSNO, ascorbate or protein thiols can cause only a slow release of NO from RSNO (Scorza et al., 1997).

$$R'SNO + RS^- \rightleftharpoons RSNO + R'S^- \qquad \text{(Eq.1.1.12)}$$

$$RSNO \xrightleftharpoons{RSH} RSSR + NO \qquad \text{(Eq.1.1.13)}$$

$$RSNO + AH^- \rightleftharpoons RSH + A + NO \qquad \text{(Eq. 1.1.14)}$$

1.1.4.2.1.4 Enzyme-catalyzed decomposition

Recent studies have shown several enzymes that can break down low molecular weight RSNOs in vitro. For example, Hogg et al. (1997) reported that GSNO can act as a substrate for γ-glutamyl transpeptidase (γ-GT), leading to the formation of S-nitrosocysteinyl glycine (CG-SNO) from GSNO (Figure 1.1.5). CG-SNO is more susceptible to transition metal ion-catalyzed decomposition to release NO with respect to GSNO. In addition, γ-GT dependent decomposition of GSNO depends on the type of tissues, because this reaction occurs in the kidney, but not in the heart.

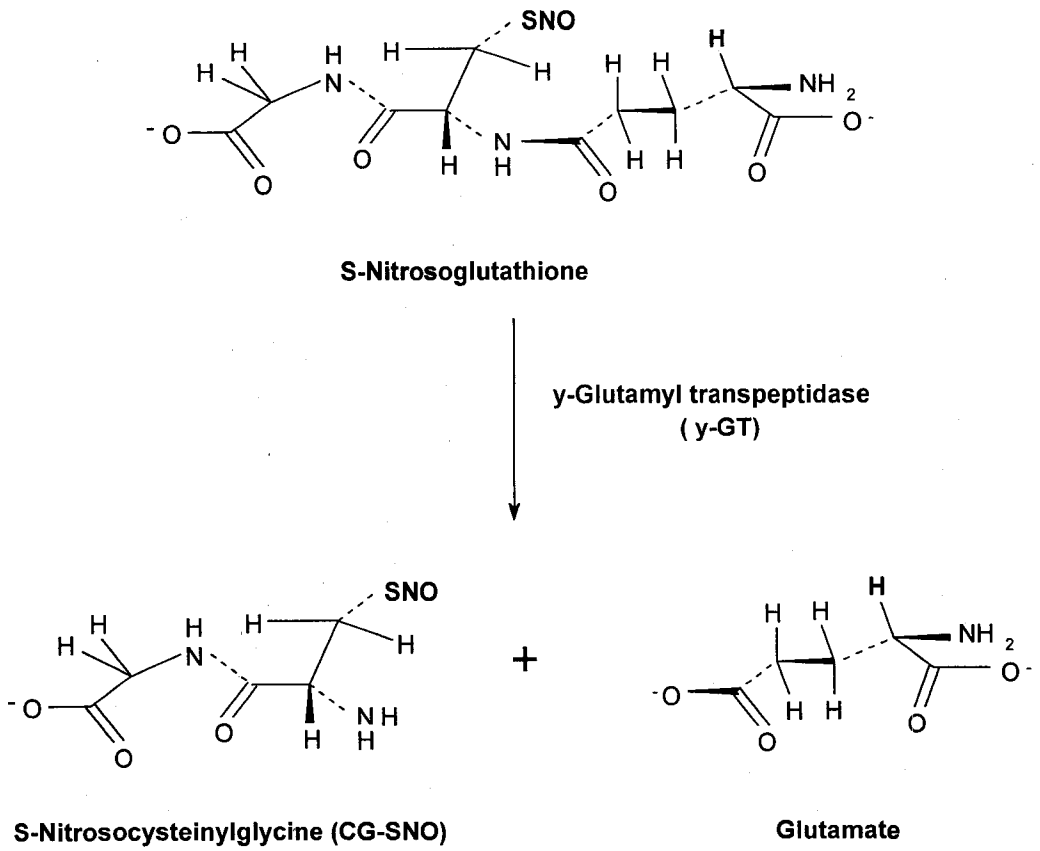


Figure 1.1.5: Proposed mechanism of γ-GT-dependent GSNO decomposition (Hogg et al., 1997)

Trujillo et al., (1998) reported that xanthine oxidase (XO) can catalyze the decomposition of low molecular weight RSNOs, such as GSNO and CysNO in an $O_2^{\cdot-}$-dependent or independent pathway to release NO. Glutathione peroxidase (GTx) has been implicated in the decomposition of GSNO to release NO (Freedman et al., 1995; Hou et al., 1996) Later, Liu et al. (2001) reported that glutathiol-dependent formaldehyde dehydrogenase is a GSNO reductase. Although it is specific for GSNO, it can affect the metabolism of other RSNOs such as S-nitrosylated protein. This enzyme is critical for RSNOs homeostasis and safeguards against nitrosative stress in biological systems.

1.1.4.3 S-transnitrosation

S- transnitrosation refers to the transfer of nitroso group from a S-nitrosothiol to a thiol (Eq.1.1.15), more specifically, the nitrosyl (NO^+) transfer from RSNO to the thiol target in the protein, peptides or amino acids so as to form a new R'SNO (Arnelle et al., 1995).

$$RSNO+R'SH \rightleftharpoons R'SNO+RSH \quad \text{(Eq.1.1.15)}$$

This reaction is a reversible second order reaction (Hogg, 1999a). It has been suggested that S-transnitrosation reactions may represent an important mechanism in vivo for the biological actions of RSNO (Park et al., 1993). In addition, S-transnitrosation reaction might be an alternative pathway for the release of NO from stable RSNO (Al-sa'doni et al., 2000). For example, recent studies demonstrated that transnitrosation reaction with low molecular weight thiols, such as L-cysteine, is required for the release of NO from S-nitrosoalbumin (ASNO) and GSNO, because S-nitroso-cysteine (CySNO) is easily subject to the decomposition catalyzed by Cu^{2+} to release NO (Scharfstein et al., 1994; Pietraforte et al., 1995).

1.1.4.4 *S*-thiolation

Thiol groups are also the targets for the *S*-thiolation reaction. *S*-thiolation may occur either by nucleophilic attack of sulfur of the RSNO by thiolate anion or by transnitrosation, followed by nucleophilic attack of sulfur of the protein S-nitrosothiol. Both pathways for *S*-thiolation result in the formation of a mixed disulfide and nitroxyl anion (NO^-) (Figure 1.1.6) (Konorev et al., 2000). *S*-thiolation has been suggested to be a physiologically cellular response to the oxidative stress (Hogg, 2000). Recent study,

however, demonstrated that *S*-thiolation may also be a cellular response to nitrosative stress (Padgett et al., 1998). Furthermore, it has been suggested that *S*-thiolation might be a thiol redox control mechanism for regulation of protein by RSNOs (Ji et al., 1999; Konorev et al., 2000).

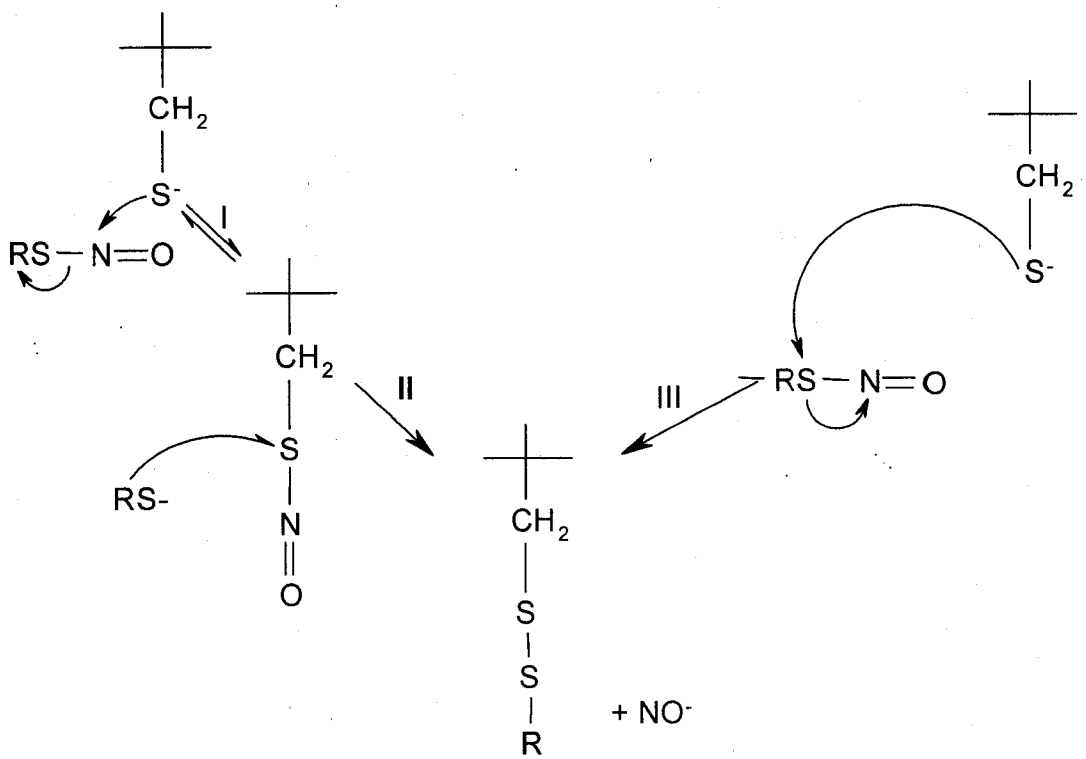


Figure 1.1.6: I: S-transnitrosation reaction II & III: S-thiolation reaction (Konorev et al., 2000)

1.1.4 Biological roles of RSNOs

RSNOs have been suggested to be the most important NO-carrier biomolecules for NO storage and transport. They exhibit NO-like activities through the release of NO such as being potent antiaggretary platelet agents (Radomski et al., 1992) and

vasodilators (Myers et al., 1990; Root et al., 2004). They also have a protective role in atherosclerosis by acting as an antioxidant for LDL peroxidation. Rigobello et al., (2002) reported that lipid peroxidation is remarkably decreased by GSNO, provided that copper ions, kept in reduced forms by ascorbate, are present. GSNO also protects endothelial cells from the cytotoxity exerted by oxLDL (Struck et al., 1995). These protective actions of RSNOs are attributed to the NO released from RSNOs (Rigobello et al., 2002; Struck et al., 1995).

RSNOs can also exert their biological function without the release of NO. They have been suggested to act as modulators of enzyme activity through the modification of protein thiols by S-transnitrosation or *S*-thiolation (Hogg, 2002). For example, Konorev et al (2000) reported that creatine kinase could be inacitivated through both S-nitrosation and *S*-thiolation. S-nitroso-N-acetylpenicillaime (SNAP) predominantly modifies the thiol of creatine kinase by S-transnitrosation to form S-nitrosothiol. However, GSNO predominantly modifies the thiols of creatine kinase by *S*-thiolation. Therefore, the creatine kinase activity can be inhibited by both S-transnitrosation and S-thiolation of the thiol groups. Furthermore, it has been proposed that the relative extent of these reactions in biological systems depends on both the environment of the protein thiol and the chemical nature of the RSNOs.

1.1.6 RSNOs transport across the membrane

RSNOs play an important role in many NO-mediated biological events. But how are RSNOs or their bound S-nitroso group transferred from the extracellular space into

the cytosol or vise versa? To date, several mechanisms responsible for the RSNOs transmembrane transport have been proposed.

1.1.6.1 Cell-surface Protein Disulfide Isomerase (csPDI)-dependent manner

Protein Disulfide Isomerase (PDI) is an enzyme that catalyzes the thiol-disulfide exchange reaction (Voet et al., 1999b). Recently, PDI has been found to exist on the cell surface (Hotchkiss et al., 1998). Zai et al. (1999) proposed that cell surface PDI catalyzes transnitrosation reaction, facilitating NO entry into the cytosol from an extracellular RSNO source, thereby, promoting its intracellular bioactivity. Later, we demonstrated that csPDI catalyzes the NO release from RSNO; released NO then accumulates in the cell surface membrane where it can undergo autooxidation. The resultant intermediate N_2O_3 was then nitrosation of intracellular thiols at the membrane-cytosol interface so as to transport extracellular –SNO functional group into the cytosol (Ramachandran N. et al., 2001) (Figure 1.1.7).

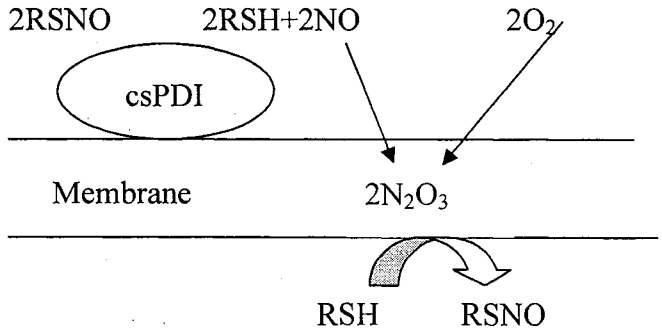


Figure 1.1.7: Mechanism for S-nitrosation of intracellular thiol by csPDI catalyzed NO released from extracellular RSNOs (Ramachandran et al., 2001)

1.1.6.2 System L amino acid transporter-dependent manner

System L amino acid transporter has been demonstrated to be involved in the transportation of functional S-nitroso group from the outside of the cell into the cytosol. For instance, Satoh et al. (1997) reported that S-nitroso-L-cysteine, an endogenous RSNO, is incorporated into rat brain slices via the system L amino acid transporter. Later, Nemoto et al. (2003) provided evidence that, in PC12 cells, S-nitroso-L-cysteine was also incorporated via the system L amino acid transporter and thus regulates cell responses. Recent study demonstrated that L-cystine enhance GSNO-dependent S-nitrosothiol uptake via system L amino acid transporter in RAW 264.7 cells. They proposed that L-cystine firstly was reduced to L-cysteine, and then transnitrosation reaction occured between GSNO and L-cysteine to form S-nitroso-L-cysteine which was taken up by RAW 264.7 cells via amino acid transport system L (Zhang et al., 2004).

1.1.6.3 Anion exchanger-dependent manner

Stamler and coworkers (Pawloski et al., 2001) proposed that export of hemoglobin-derived NO-bioactivity from the red blood cells was mediated by anion exchanger AE1 through the formation of anion exchanger AE1-SNO at the membrane-cytosol interface. Thus, anion exchanger AE1 might play a role in transmembrane translocation of NO bioactivity.

1.1.7 Insulin resistance and endothelial dysfunction

1.1.7.1 Insulin

Insulin is a peptide hormone with the 5,700 molecular mass. It has two polypeptide chains which are joined by two disulfide bridges (Fig. 1.1.8). It is synthesized in the pancreas, it is an important signaling molecule for the maintaining the normal level of blood glucose concentrations, and its rate of secretion is increased in response to the higher levels of blood glucose (Nelson et al., 2000)

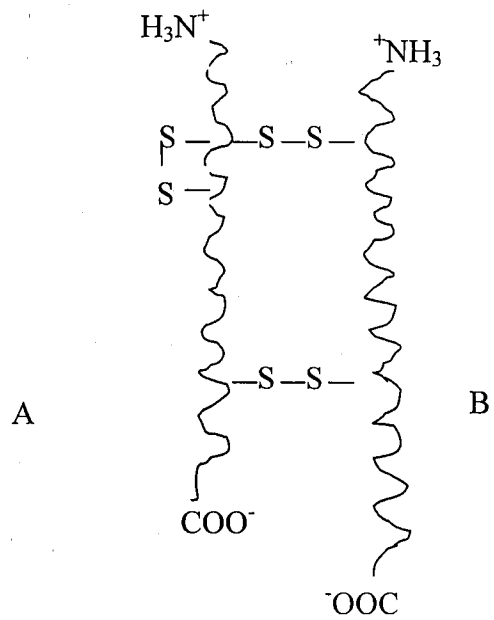


Fig. 1.1.8: Insulin structure

1.1.7.2 Physiological role of insulin in the vascular system

Insulin plays a central role in the regulation of glucose and fatty acid homeostasis. In addition to these traditional roles in energy metabolism, insulin has been suggested to be essential in the regulation of vascular tone. Previous studies have demonstrated that insulin

can modulate vascular tone and tissue blood flow (Baron, 1994). It acts as a vasodilator via the activation of eNOS to produce NO from the endothelium (Scherrer et al., 1994).The mechanism for the activation of eNOS mediated by insulin includes phosphorylation of eNOS and regulation of eNOS cofactor availability such as H_4B (Mather et al., 2001).

1.1.7.3 Insulin resistance

Classical insulin resistance refers to the impairment of insulin's classic action to stimulate glucose uptake in skeletal muscle and fat cells (Kahn et al., 2000). However, it has been suggested that insulin-mediated NO release from the endothelium is also impaired in insulin resistant states such as Obesity and Type II diabetes mellitus (Steinberg et al., 2002).

1.1.7.4 Endothelial dysfunction

The vascular endothelium is a dynamic organ that maintains the homeostasis of vascular system. It exhibits numerous physiological vasoprotective actions such as stimulation of vascular smooth muscle cell relaxation, inhibition of platelet adhesion and aggregation etc. (Celermajer, 1997). These vasoprotective actions are mainly maintained by NO, endothelium-derived relaxing factor (Palmer et al., 1988). Therefore, endothelial dysfunction is characterized by decreased NO bioactivity (Cai et al., 2000). In addition, oxidized LDL has been suggested to be a main factor responsible for the disturbance of these normal endothelial functions (Dinerman et al., 1990). Loss of these physiological endothelial functions is central in the development and progression of atherosclerosis

(Adams et al., 2000). Thereby, oxidized LDL is an essential element in the development of endothelial dysfunction and subsequent atherosclerosis.

1.1.7.5 Interaction of insulin resistance and endothelial dysfunction

A main reason for endothelial dysfunction is due to the loss of NO bioactivity in the vessel wall: insulin resistance often results in the loss of NO bioactivity (Tack et al., 1996). Therefore insulin resistance and endothelial dysfunction frequently coexist. A substantial body of evidence demonstrated that insulin resistance is tightly associated with endothelial dysfunction (Freeman et al., 2001; Baron et al., 1995).

1.1.8 LDL homocysteinylation and its role in vascular diseases

Previous studies demonstrated that elevated homocysteine (Hcy) levels in the plasma are associated with human vascular diseases (Jacobsen, 1998). The mechanisms underlying the pathology of the elevated Hcy in the human vascular diseases are controversial. However, a recent study demonstrated that cytotoxity of Hcy was related to Hcy thiolactone (HcyTL) (Jakubowski, 1997).

HcyTL, a product of Hcy metabolized by methionyl-tRNA synthetase (MetRS) during protein systhesis (Jakubowski, 1995; Jakubowski, 1997), is chemically reactive towards primary amines via nucleophilic addition (Benesch et al., 1956). This reaction can equally occur in biological systems and has been suggested to be impicated in the vascular diseases. Jakubowski (1999) reported that under physiological conditions, proteins including LDL are easily subject to homocysteinylation by HcyTL; side chain amino groups of lysine residues are the major sites of homocysteinylation in proteins; chemical

modification by HcyTL leads to protein damage, such as increased negative charge and electrophoretic mobility; formation of intermolecular disulfide bonds and protein multimers; protein precipitation etc. Thus, protein N-homocysteinylation might account for the damage effect of Hcy in the vascular diseases such as atherosclerosis.

Human plasma LDL, especially oxidized LDL, a key element in atherosclerosis, has also been demonstrated to be subject to homocysteinylation by HcyTL (Ferretti et al., 2003; Jakubowski et al., 2000). The resultant homocysteinylated LDL (LDL-SH) could increase the atherogenicity of LDL. The possible explanations for the atherogenicity of homocysteinylated LDL could be categorized into two classes:

1-Homocysteinylation of LDL could increase the uptake of modified LDL by macrophages with respect to native LDL. Higher uptake of homocysteinylated LDL leads to lipid accumulation in the macrophages, with the resultant formation of foam cells (Naruszewicz et al., 1994); **2**-Homocysteinylation of LDL by HcyTL potentiates its oxidation (McCully, 1993; Olszewski et al., 1993), which is a major factor for the development of atherosclerosis (Yla-Herttuala et al., 1989; Steinberg et al., 1989; Steinberg, 1993). Furthermore, recent a study presented evidence that homocysteinylation of LDL induces a significant increase of lipid peroxidation and oxidative damage on human vascular endothelial cells (EC). The extent of lipid peroxidation is proportional to the levels of SH groups. Thus, LDL-SH exerts a cytotoxic effect that is likely related to an increase in lipid peroxidation and oxidative damage of EC (Ferretti et al., 2004).

1.2 Objectives

Oxidized low density lipoprotein (LDL) is a key element for the development of atherosclerosis. Homocysteinylation of LDL by homocysteine thiolactone occurs under physiological conditions. Homocysteinylation of LDL by HcyTL has been suggested to be atherogenic since homocysteinylated LDL (LDL-SH) increased the susceptibility of LDL to oxidative modification. Furthermore, the extent of oxidation has been shown to relate to the levels of free thiol groups incorportated into the LDL through the interaction with HcyTL. NO plays a protective role in atherosclerosis by serving as a potent inhibitor of LDL oxidation. In addition, thiol groups in the protein have been suggested to be cellular targets for S-nitrosation. S-nitrosation of thiols promotes the formation of S-nitrosothiols (RSNOs). RSNOs have shown NO-like bioactivities. Therefore we wanted to examine whether LDL-SH could be S-nitrosated in vitro to form favorable S-nitroso homocysteinylated LDL (LDL-SNO) so as to block the reactivities of the free thiol groups of LDL-SH. Maintaining the normal levels of human plasma LDL is fundamental to the prevention of the development of vascular diseases such as atherosclerosis. Therefore, whether chemical modifications such as homocysteinylation and S-nitrosation affect the LDL uptake was also examined. Finally, insulin has been suggested to physiologically stimulate LDL uptake. Thus, we also want to investigate whether homocysteinylation and S-nitrosation affect the actions of insulin on LDL uptake.

1.3 Materials and Methods

1.3.1 Materials

3, 3′-dioctadecylindocarbocyanine (DiI) was purchased from Molecular Probes, Inc. (U.S.A.). DL-Homocysteine thiolactone (HcyTL), 5,5'-Dithiobis(2-nitrobenzoic acid (DTNB), 2,3-diaminonaphthalene (DAN), Sodium nitrite, Potassium bromide, Copper sulphate, Sodium hydroxide, Hydrogen chloride, Sodium phosphate, Sodium chloride, Ethylenediaminetetraacetic acid (EDTA), Dimethyl sulfoxide (DMSO), Methanol, Insulin, Cysteine, Sephadex G-25, Fetal bovin serum (FBS) were purchased from Sigma Chemical Co.(St. Louis Mo.). Normal human fibroblasts (NHFs) were obtained from the American Type Culture Collection (Rockville, MD, USA).

1.3.2 Methods

1.3.2.1 Preparation of LDL

Human LDL was prepared from the blood of individual healthy volunteers. The plasma fraction is obtained by low-speed centrifugation of the whole blood for 15min. Lipoproteins were isolated by Sequential Flotation Ultracentrifugation (Verne et al., V128).Briefly, human plasma density (1.006 g/ml) was first adjusted to 1.019g/ml with potassium bromide (KBr). In order to prevent oxidation 0.01% EDTA was present during the isolation procedure. After centrifugation at 40,000rpm for 24h at 10°C using a fixed-angle rotor (Ti60) and Model L8-M ultracentrifuge (Beckman, Palo Alto, CA). The top fraction that contains a mixture of very low-density lipoproteins (VLDL) and intermediate-density lipoprotein (ILDL) was discarded. The bottom fraction (whose

initial density is assumed to be 1.019g/ml) is then adjusted to a final density of 1.063 g/ml with solid KBr and centrifuged again as before, and LDL fraction (1.019-1.060 g/ml) was collected. LDL preparation was extensively dialyzed against 10mM phosphate, 137mM NaCl, 0.01% EDTA (pH7.4) for 36h at 4° C, filter-sterilized (0.45μm, Fisher) and its final protein concentration was determined as described by Bradford (Bradford MM, 1976).

1.3.2.2 Fluorescent Labeling of LDL with 3, 3′-dioctadecylindocarbocyanine (DiI)

LDL was labeled with the fluorescent probe DiI as described by Pitas RE (Pitas et al., 1981).Briefly, 15mg of LDL were added to 30ml of lipoprotein-deficient serum and then filter-sterilized, using 0.45μm filter. To the lipoprotein, 750μl of DiI (3mg/ml) in dimethyl sulfoxide (DMSO) was added, while the solution was gently vortex-mixed, and the sterile mixture was incubated at 37°C for 18 hours. Then the density of the incubation mixture was raised to1.060 g/ml with KBr, and DiI-LDL was isolated as LDL. The DiI labeled LDL was then dialyzed against 10mM phosphate, 137mM NaCl, and 0.01% EDTA (PH7.4) for 36h at 4°C, filter-sterilized (0.45μm, Fisher) and its final protein concentration was determined as described by Bradford (Bradford, 1976).

1.3.2.3 Homocysteinylation of LDL by homocysteine thiolactone

The LDL was homocysteinylated as previously described (Ferguson et al., 1999). 5mM homocysteine thiolactone (HcyTL) was added to 2mg/ml of LDL protein solution. An aliquot of solution mixture was passed through a Sephadex G-25 column equilibrated with 10mM PBS, pH 8.2 every 20 min to separate the unreacted HcyTL. Thiol groups (-

SH) were determined with Ellman's reagent (DTNB) (Packer, V.233). The concentrations of SH groups coupled to LDL are given in terms of moles per mole LDL protein.

1.3.2.4 S-nitrosation of homocysteinylated LDL (LDL-SH)

1.3.2.4.1 Sythesis of S-nitroso-Cysteine

S-nitroso-cysteine (CySNO) was prepared as previously described (DeMaster et al., 1995). Cysteine (Cys) in 2M HCl was incubated with an equimolar concentration of sodium nitrite in the dark for 30 min at 25°C. The solutions were then neutralized with 2M NaOH and 10mM phosphate buffer (pH 7.4).

1.3.2.4.2 Synthesis of S-nitroso homocysteinylated LDL (LDL-SNO)

LDL-SNO was prepared by incubation of 1mg/ml LDL-SH with 6mM CySNO in 10mM PBS containing 500μM EDTA at 37°C for 60 min and protected from light. The solution was eluted on a Sephadex G-25 gel filtration column with 10mM PBS containing 500μM EDTA to separate the LDL-SNO from the excess CysNO.

1.3.2.5 Determination of the concentrations of NO groups coupled to LDL-SH

The concentrations of NO groups coupled to LDL-SH were quantified as previously described (Jourd'heuil D et al., 2000).Briefly, nitrosating agent produced from the Cu^{2+}-catalyzed decomposition of the S-nitrosothiol is trapped by 2,3-diaminonaphthalene(DAN) to form the highly fluorescent derivative 2,3 napthotriazole

(NTA), which allows determination of nitrites (NO_2^-). Because LDL-SNO is degraded in the presence of Cu^{2+} ions, the difference between the NO_2^- level in the presence and absence of Cu^{2+} ions allows the measurement of the NO level of LDL-SNO. 500μl of the sample was added to 1.0ml of redistilled water and 500μl of DAN reagent mixture, which was prepared by the addition of 1.11mM $CuSO_4$ and 15.8mM DAN in 0.62M HCl at 1:4 ratios. The samples were incubated at room temperature in the dark for 10 min. To stop the reaction, the samples were alkalized to pH 12 by the addition of 10M NaOH. Fluorescence measurements were performed at excitation wavelength 362 nm and emission wavelength 406nm.

1.3.2.6 Fluoresence microscopy

To localize the uptake of fluorescent liporoteins, cell cultures were preincubated with medium containing 10% lipoprotein-deficient serum (LPDS) for 24h before the addition of DiI-LDL, DiI-LDL-SH, and DiI-LDL-SNO. Cells were incubated with different concentrations of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO for 30min at 37°C. Cells were then washed three times with PBS containing 0.5% BSA and three times with PBS alone. Cells were then changed back to fresh medium for the determination of intracellular fluorescence intensity. Uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO by cells was observed with the aid of a Zeiss Axiovert 200 fluorescence microscope with a 20 x fluoresence objective. The intracellular fluorescence intensity was measured by computer-assisted image analysis (Northern Eclipse 6.0).

To determine the effects of insulin on the uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO, cells were pretreated with various concentrations of insulin for 24 h

before incubating with DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO. The following experimental steps were performed as described above.

1.3.2.7 Cell culture

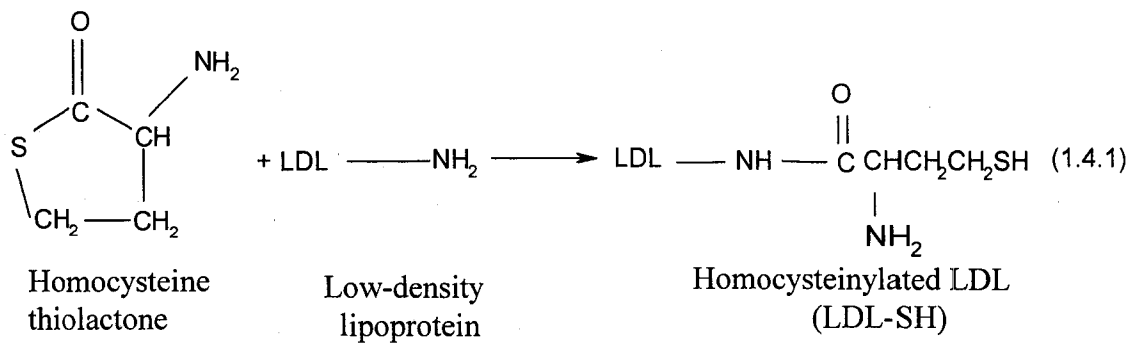
NHFs were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and penicillin streptomycin Solution as antibiotics. Cells were maintained in a humidified incubator at 37°C in 5% CO_2/95% air.

1.4 Results

1.4.1 Kinetics of homocysteinylation of LDL by homocysteine-thiolactone

Previous studies demonstrated that LDL could be homocysteinylated by HcyTL (Ferretti G et al., 2004). In the present study, it was necessary to establish a reproducible homocysteinylation protocol and to determine the degree of LDL homocysteinylation in order to study the extent of S-nitrosation of homocysteinylated LDL as well as the effect of homocysteinylation on the LDL receptor-mediated uptake of LDL by NHFs. Therefore, the kinetics of thiol (SH) groups coupling to human LDL were carried out in the reactions between HcyTL and LDL. Figure 1.4.1 demonstrated that incubation of human LDL with HcyTL resulted in a time-dependent increase in SH groups covently bonded to human LDL.

Homocysteinylation occurs by aminolysis of the thiolactone bond of HcyTL. Thereby, the new homocysteine residue becomes attached through a peptide bond to the ε nitrogen of apo-B lysine residues to form homocysteinylated LDL (LDL-SH) adduct (Eq.1.4.1) (Vidal et al., 1986; Ferguson et al., 1999).



Homocysteine thiolactone

Low-density lipoprotein

Homocysteinylated LDL (LDL-SH)

The SH level was determined by Ellman's reagent (DTNB). As shown in Figure 1.4.1, exposure of LDL to HcyTL for 40min resulted in around 37.5 mol SH/mol LDL protein. In the present study, 18% modification of LDL lysine residues (i.e. 40 min of exposure of LDL to HcyTL) was used in subsequent studies.This is in view of a previous study which demonstrated that lysine residues of apo-B are functional groups for the binding of LDL to the cell surface LDL receptor; furthermore, chemical modification of more than 20% of the lysine residues of LDL prevents the LDL from binding directly to the LDL-specific receptor (Weisgraber et al., 1978).

1.4.2 S-nitrosation of LDL-SH

A recent study demonstrated that LDL-SH was involved in the development of atherosclerosis, probably due to an increase in lipid peroxidation, and that, the extent of lipid oxidation is directly related to the levels of SH groups in LDL-SH (Ferretti et al., 2004). NO is shown to be an effective antioxidant for lipid peroxidation. In addition, RSNOs are potent reservoirs for NO in vivo and potentiate its biological effects. Therefore, we examined whether SH groups in LDL-SH could be S-nitrosated in vitro. Presumably, LDL-SNO possesses NO-like properties such as stimulation of smooth muscle cell relaxation, inhibition of platelet aggregation and adhesion, inhibition of LDL peroxidation, and thus would reduce the possibility of the atherosgenesis induced by LDL-SH.

LDL-SNO was synthesized by the S-transnitrosation of LDL-SH with CySNO, physiological RSNO. LDL-SNO formation was detected based upon the formation of fluorometric compound naphthotriazole (NTA). NTA is formed by the reaction of 2, 3-

diaminonaphthalene (DAN) with NO released from S-nitrosylated proteins in the presence of metal ion such as Cu^{2+}, thus providing a quantitative measure of S-nitrosothiol formation (Jourd'heuil et al., 2000). The result demonstrated that significant LDL-SNO was formed (Figure 1.4.2). Based on the SH level (37.5mol/ mol LDL protein) of LDL-SH, we calculated that approximately 55% of SH groups of LDL-SH were S-nitrosated by CySNO, suggesting that LDL-SH could be S-nirosated in vitro under the present experimental conditions.

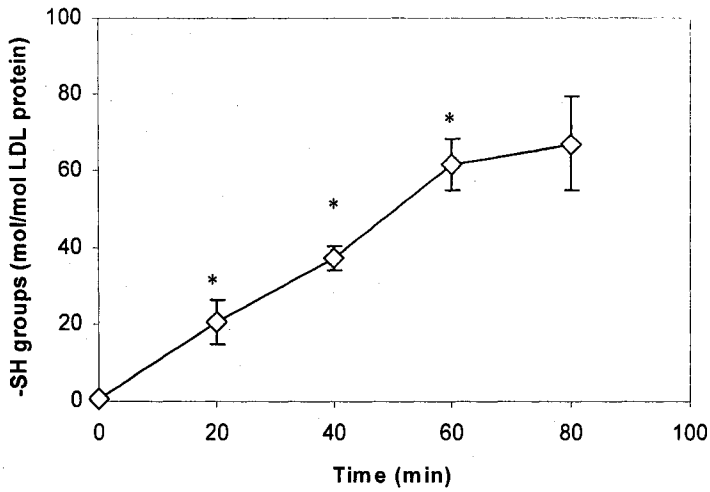


Figure 1.4.1: Kinetics of homocysteinylation of human LDL by HcyTL. The reaction mixtures containing human LDL (2 mg of protein per ml) and 5mM HcyTL were kept at present variable room temperature for the indicated periods. At the indicated times, aliquots of solution were passed through Sephadex G25 to remove excess HcyTL. The levels of the SH groups coupled to human LDL were determined by Ellman's reagent DTNB (ε = 13,600 $M^{-1}m^{-1}$). Those values marked with an asterisk (*) are statistically significant ($p<0.05$).

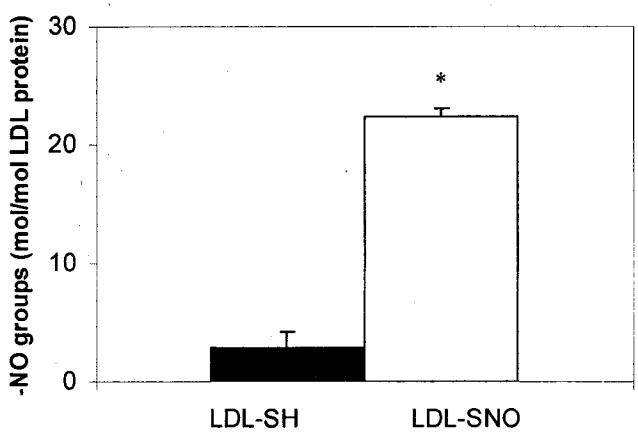


Figure 1.4.2: S-nitrosation of LDL-SH by CySNO. After 40 min homocysteinylation reaction, 6mM CySNO was added into purified LDL-SH solution in the presence of 500μM EDTA, and was stirred for 1h in the dark, then passed through Sephadex G25 column to remove excess CySNO. The level of NO groups coupled to LDL-SH was determined by DAN assay as shown in Methods. Asterisk (*) indicated statistically significant values ($p<0.05$).

1.4.3 Fluorescent labeling of LDL, LDL-SH, and LDL-SNO with DiI

LDL had to be fluorescently labeled in order to monitor its uptake by Normal Human Fibroblasts(NHFs) with the aid of fluorescence microscope. LDL was fluorescently labeled with DiI as previously described (Pitas et al., 1981). Under the present experimental conditions, LDL was successfully labeled by DiI because NHFs have strong fluorescence as shown in Figure 1.4.3 after taking up DiI-LDL in the absence of 100 fold unlabeled LDL, but NHFs almost have no fluorescence after taking up DiI-LDL plus 100 fold unlabeled LDL (at the same settings of fluorescence microscope). This indicated that strong intracellular fluorescence observed after incubation with DiI-LDL alone is the result of specific uptake of DiI-LDL via the LDL receptor pathway. In the present study, DiI-LDL-SH was prepared by homocysteinylation of LDL that had been previously labeled with DiI. DiI-LDL-SH was then subjected to the S-nitrosation reaction to get DiI-LDL-SNO.

1.4.4 Comparison of the uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO

1.4.4.1 Time course of uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO

The effects of homocysteinylation and subsequent S-nitrosation on the uptake of LDL by NHFs were examined. Time course of uptake of DiI-LDL, DiI-HcyLDL and DiI-LDL-SNO by NHFs was first examined. NHFs were incubated at 37°C with DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO at a concentration of 5μg protein /ml for the indicated times as shown in Figure 1.4.4 and then analyzed for fluorescence intensity at indicated times by fluorescence microscope.

As shown in Figure 1.4.4, NHFs became fluorescent after only 10 min of incubation. The uptake of DiI-LDL by NHFs was linear and rapid during the first 30 min of incubation. After that, there was a slower rate of uptake over the next 60 min while the uptake approached saturation. Moreover, the pattern of time course uptake of DiI-LDL-SH and DiI-LDL-SNO was similar to that of uptake of DiI-LDL. 30 min was therefore chosen as the optimal incubation time for DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO in the following experiments.

1.4.4.2 The effect of various concentrations on the uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO by NHFs

NHFs were incubated with 2, 4, 8, 16, 30μg/ml DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO respectively at 37°C for 30min. NHFs were then analyzed for fluorescence intensity to make a comparison among the uptake of DiI-LDL, DiI-HcyLDL and DiI-LDL-SNO by NHFs. As shown in Figure 1.4.5, the uptake of DiI-LDL by NHFs displayed a typical hyperbolic curve. At lower concentrations (<16μg/ml), uptake of DiI-LDL was linearly related to the concentrations of DiI-LDL, Uptake, however, approached saturation at higher concentrations of LDL (>16μg/ml). This was consistent with LDL receptor-mediated process. Furthermore, the pattern of uptake of DiI-LDL-SH and DiI-LDL-SNO was similar to that of DiI-LDL, suggesting that homocysteinylation and subsequent S-nitrosation did not affect the uptake of LDL by NHFs.

In addition, to quantitatively confirm that there is no significant difference among the uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO by NHFs, we calculated the Km for the uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO according to the double-

reciprocol plot of Figure 1.4.5 (Figure 1.4.6). The Km for the uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO was found to be 4.8, 4.9 and 5.0 respectively suggesting that under the present experimental conditions, the uptake of LDL by NHFs has no significant change after chemical modifications (homocysteinylation and subsequent S-nitrosation).

The above results suggested that chemical modification of lysine residues of apolipoprotein (apo)-B_{100} of LDL by HcyTL and further S-nitrosation of LDL-SH by CySNO did not affect the uptake of LDL by NHFs.

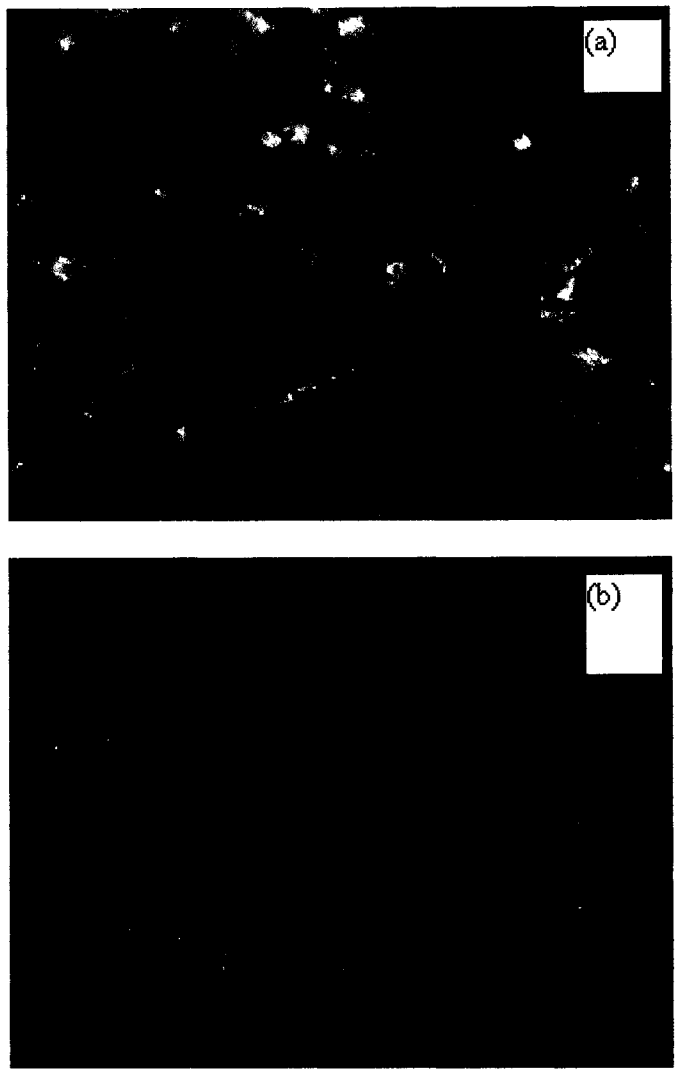


Figure 1.4.3 Visual detection of uptake of DiI-LDL by NHFs. NHFs were preincubated for 24h in DMEM with 10% lipoprotein-deficient serum (LPDS). NHFs were then incubated for 30min with DiI-LDL (4μg/ml) (a) in the absence or (b) in the presence of 100 fold unlabeled LDL in the same medium. NHFs were then washed three times with PBS containing 0.5% BSA and again three times with PBS alone prior to examination by fluorescence microscopy.

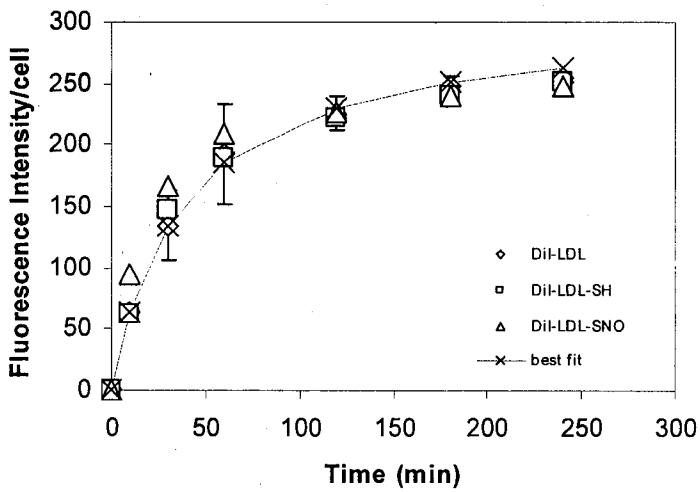


Figure 1.4.4: Time course uptake of DiI-LDL, DiI-LDL-SH, and DiI-LDL-SNO by NHFs. NHFs were preincubated for 24h in DMEM with 10% lipoprotein-deficient serum (LPDS). NHFs were incubated with 5ug/ml DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO at 37°C for 10min, 30min, 1h, 2h, 3h and 4h. NHFs were washed three times with PBS containing 0.5% BSA and again three times with PBS alone prior to examination by fluorescence microscopy.

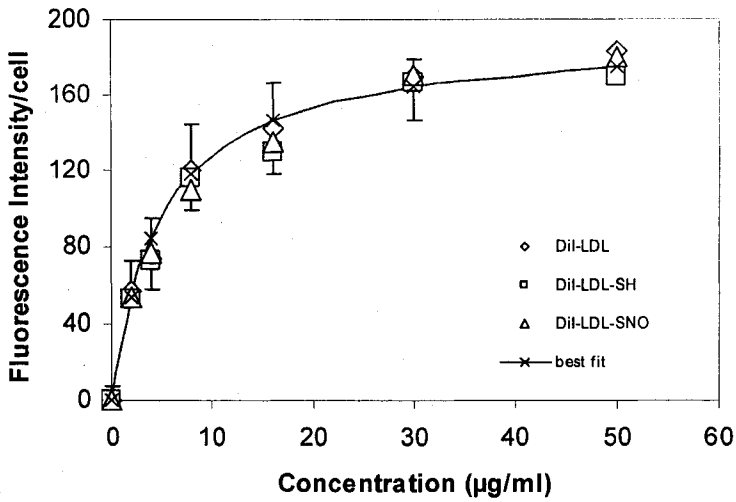


Figure 1.4.5: Comparison of uptake of various concentrations of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO by NHFs. NHFs were preincubated for 24h in DMEM with 10% lipoprotein-deficient serum (LPDS). NHFs were then incubated with DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO at concentrations of 2, 4, 8, 16, 30 µg/ml for 30min at 37°C. NHFs were then washed three times with PBS containing 0.5% BSA and again three times with PBS alone prior to examination by fluorescence microscopy.

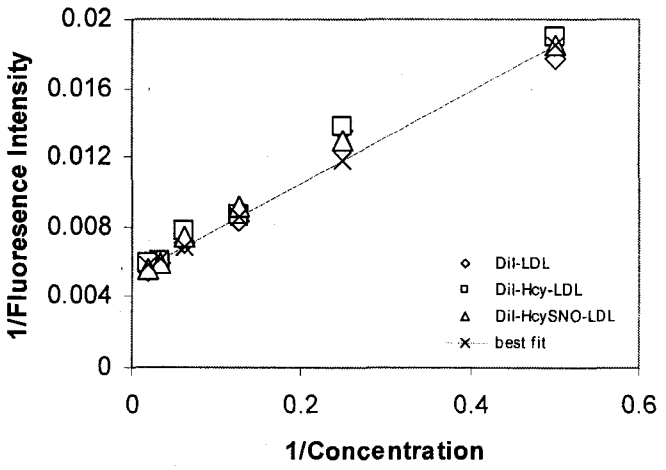


Figure 1.4.6: A double-reciprocal plot of Figure 1.4.5.

1.4.5 Uptake of DiI-LDL-SH and DiI-LDL-SNO by LDL-specific receptor pathway

In order to determine whether the uptake of chemically modified LDL (LDL-SH and LDL-SNO) is specificially through LDL receptor-mediated process, NHFs were coincubated respectively with 4μg/ml DiI-LDL, DiI-LDL-SH, DiI-LDL-SNO and 100 fold unlabeled LDL at 37°C for 30 min, then analyzed for fluorescence intensity of the cells. As shown in Figure 1.4.7, strong fluorescence was observed when NHFs were incubated with DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO without unlabled LDL. Fluorescence, however, could not be detected under the same settings of fluorescence microscope when the NHFs were incubated with DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO plus 100 fold unlabeled LDL (Figure 1.4.8), suggesting that uptake of LDL-SH and LDL-SNO was almost completely blocked by excess native unlabeled LDL; uptake of LDL-SH and LDL-SNO was via LDL-specific receptor pathway.

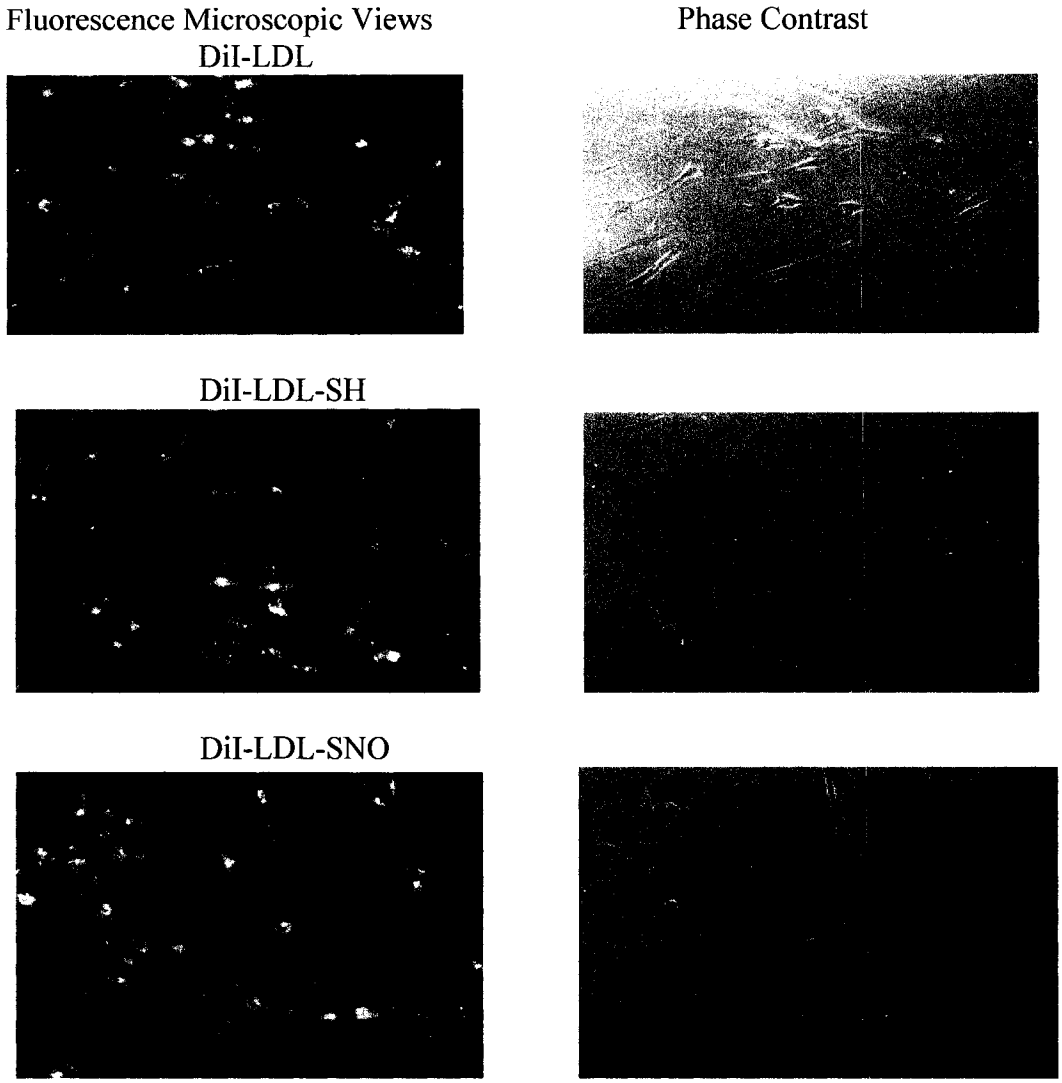


Figure 1.4.7: Uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO by NHFs. NHFs were preincubated for 24h in DMEM with 10% lipoprotein-deficient serum (LPDS). Then NHFs were incubated with DiI-labeled 4μg/ml LDL, LDL-SH and LDL-SNO at 37°C for 30min. NHFs were then washed three times with PBS containing 0.5% BSA and again three times with PBS alone prior to examination by fluorescence microscopy. Phase contrast and fluorescence microscopic views of the same fields are shown.

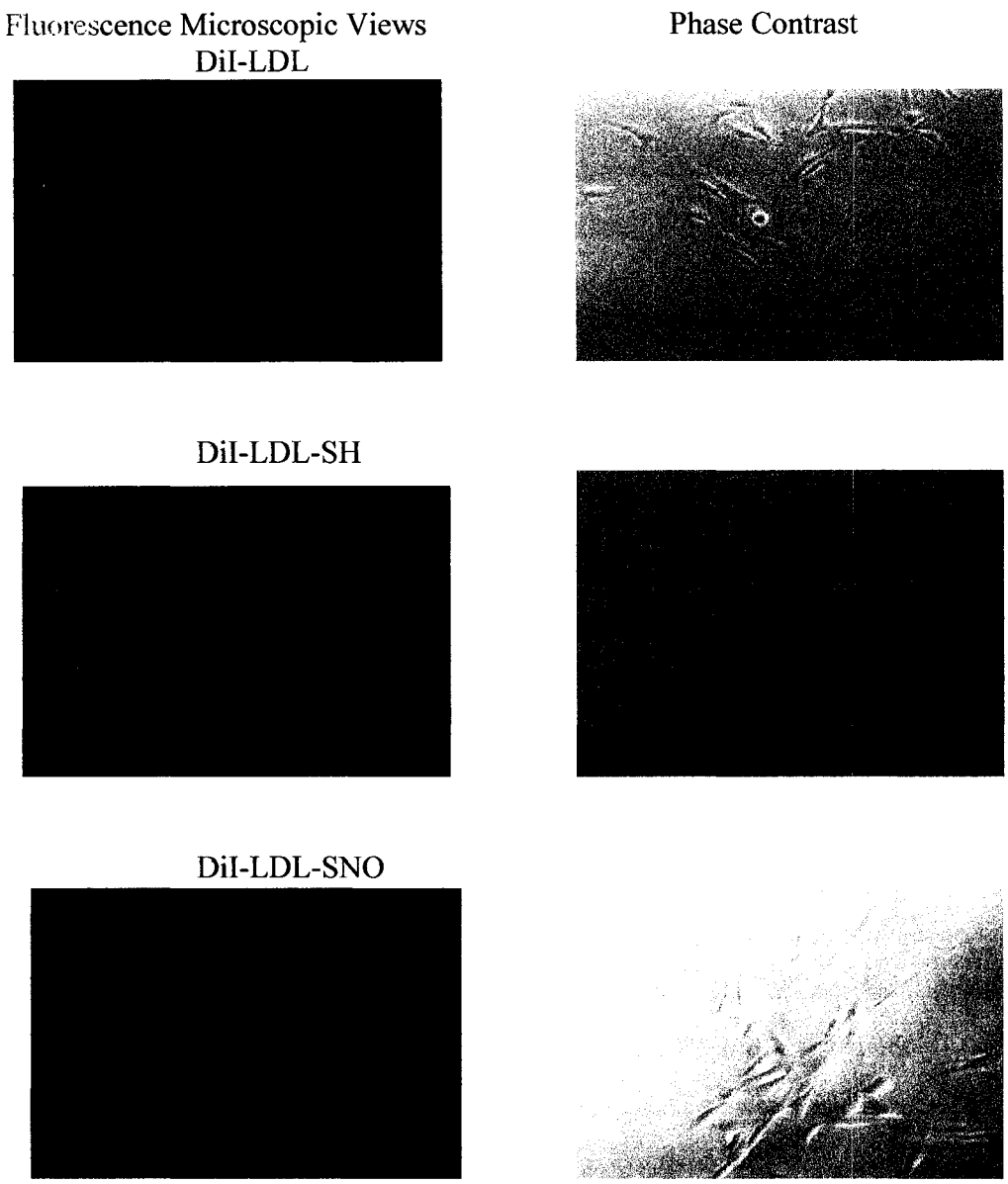


Figure 1.4.8: Competitive inhibition of uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO by unlabeled LDL. NHFs were preincubated for 24h in DMEM with 10% lipoprotein-deficient serum (LPDS). NHFs were coincubated with DiI-labeled 4μg/ml LDL, LDL-SH and LDL-SNO and 100 fold unlabeled LDL at 37°C for 30min. NHFs were then washed three times with PBS containing 0.5% BSA and again with PBS alone for another three times prior to examination by fluorescence microscopy. Phase contrast and fluorescence microscopic views of the same fields are shown.

1.4.6 Comparison of the effects of insulin on the uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO by NHFs

Insulin has been shown to play a regulatory role in maintaining the normal levels of LDL in the plasma. Furthermore, it has been reported that insulin caused the enhancement of uptake of LDL through LDL receptor-mediated endocytosis in NHFs (Chait et al., 1978). In order to determine whether chemical modification of LDL affects the role of insulin in the LDL uptake, the uptakes of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO by NHFs were compared with and without 50nM insulin. As shown in Figure 1.4.9, Figure 1.4.10 and Figure 1.4.11, LDL receptor mediated endocytic uptakes of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO by NHFs were all enhanced in the presence of 50nM insulin. Moreover, the patterns of the increase in the uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO were similar, suggesting that chemical modifications have no significant effect on the role of insulin in the stimulation of uptake of LDL by NHFs.

Dose effects of insulin on the uptake of DiI-LDL, DiI-HcyLDL and DiI-LDL-SNO by NHFs were also examined (Figure 1.4.12). As shown in Figure 1.4.12, the pattern of uptake of native DiI-LDL is quite similar to that of the uptake of DiI-LDL-SH and DiI-LDL-SNO in the presence of various concentrations of insulin. Uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO increased with increased concentration of insulin up to 50nM, and then gradually declined.

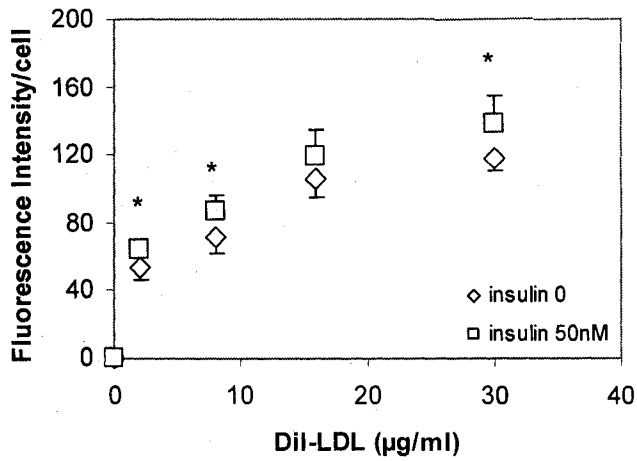


Figure 1.4.9: Comparison of the uptake of DiI-LDL by NHFs with and without 50nM insulin. NHFs were preincubated for 24h in DMEM with 10% lipoprotein-deficient serum (LPDS). NHF cells were then incubated with various concentrations of DiI-LDL with and without 50nM insulin at 37°C for 30min. Then NHFs were washed three times with PBS containing 0.5% BSA and again three times with PBS alone. NHFs were analyzed for fluorescence intensity to compare the difference of uptake of DiI-LDL in the presence and absence of 50nM insulin. Those values marked with an asterisk (*) are statistically significant($p<0.05$).

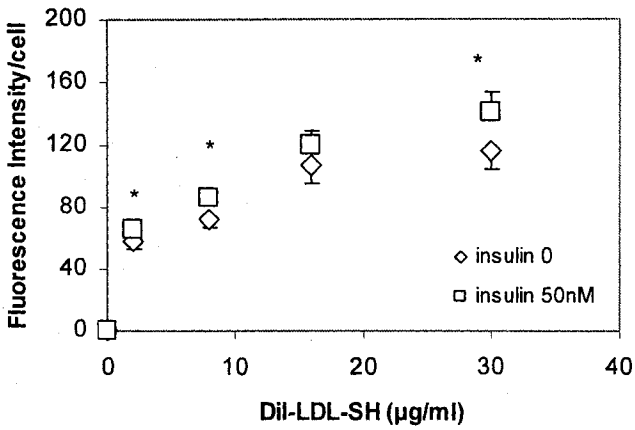


Figure 1.4.10: Comparison of the uptake of DiI-LDL-SH by NHFs with and without 50nM insulin. NHFs were preincubated for 24h in DMEM with 10% lipoprotein-deficient serum (LPDS). NHF cells were then incubated with various concentrations of DiI-Hcy- with and without 50nM insulin at 37°C for 30min. Then NHFs were washed three times with PBS containing 0.5% BSA and again three times with PBS alone. NHFs were analyzed for fluorescence intensity to compare the difference of uptake of DiI-LDL-SH in the presence and absence of 50nM insulin. Those values marked with an asterisk (*) are statistically significant($p<0.05$).

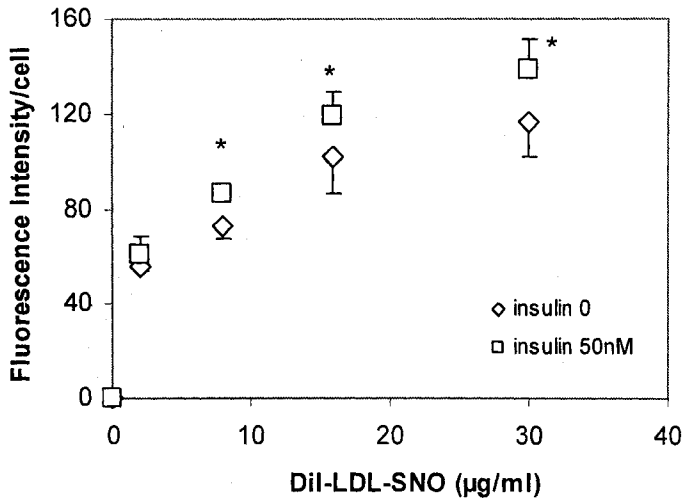


Figure 1.4.11: Comparison of the uptake of DiI-LDL-SNO by NHFs with and without 50nM insulin. NHFs were preincubated for 24h in DMEM with 10% lipoprotein-deficient serum (LPDS). NHFs were then incubated with various concentrations of DiI-LDL-SNO with and without 50nM insulin at 37°C for 30min. Then NHFs were washed three times with PBS containing 0.5% BSA and again three times with PBS alone. NHFs were analyzed for fluorescence intensity to compare the difference of uptake of DiI-LDL-SNO in the presence and absence of 50nM insulin. Those values marked with an asterisk (*) are statistically significant ($p<0.05$).

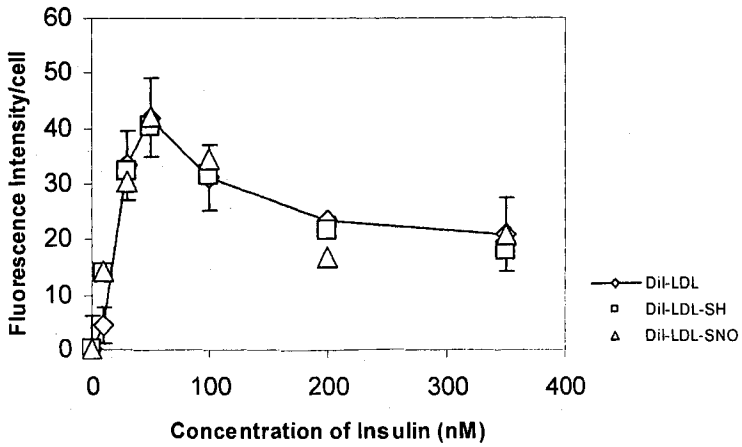


Figure 1.4.12: Comparison of the effects of various concentrations of insulin on the uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO by NHFs. NHFs were preincubated for 24h in DMEM with 10% lipoprotein-deficient serum (LPDS). NHFs were also pretreated with various concentrations of insulin overnight. NHFs were then incubated with 5μg/ml DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO at 37°C for 30min. NHFs were washed three times with PBS containing 0.5% BSA and again three times with PBS alone. NHFs were analyzed for fluorescence intensity to compare the difference of uptake of DiI-LDL, DiI-LDL-SH and DiI-LDL-SNO in the presence of various concentrations of insulin.

1. 5 Discussion

Homocysteinylation of lysine residues of ApoB in LDL is a physiologically relevant modification of LDL by HcyTL. This modification might promote atherogenic ability of LDL because previous studies demonstrated that LDL homocysteinylation increased the uptake of LDL by macrophage in cultures and therefore contributed to the formation of foam cells (Naruszewicz et al., 1994). LDL-SH is more subject to oxidative modification, resulting in a cytotoxic effect on human vascular endothelial cells (Ferretti et al., 2004).These processes can lead to the development of atherosclerosis. In addition, LDL homocysteinylation has been suggested to be immunogenic and specific for HcyTL modified lysine residues (Ferguson et al., 1999). Although the mechanism of how LDL-SH mediates these processes is unclear, the extent of damage to the cells is related to the incorporation of Hcy into the LDL, and more specifically, related to the free -SH groups.

NO has been suggested to be an endothelium-derived relaxing factor. It plays a functional role in the control of vascular tone (Maxwell et al., 1998). NO also plays a protective role in atherosclerosis by serving as a potent inhibitor of LDL oxidation (Rubbo et al., 2002), which is a key step for the development of atherosclerosis (Chisolm et al., 2000). However, the half life of NO has been reported to be only 0.1 second in vivo (Kelm et al., 1990). Therefore, SH groups in amino acids, peptides and proteins in biological systems have been suggested to play a key role in stabilizing shorted lived NO by forming S-nitrosothiols (RSNOs). RSNOs have been suggested to be, in plasma and cellular milieux, stable NO-carrier molecules that preserve NO biological activities such as vasodilation and antiplatelet properties (Stamler et al., 1992a). In addition, Stamler and coworkers (Stamler et al., 1992b) also reported that RSNOs circulate in plasma primarily

as S-nitrosoproteins because they have long half-lives under physiological conditions compared with free nitric oxide or S-nitrosothiol adducts of low-molecular-weight thiols.

As shown in the present results, an increase of the free thiol groups is a characteristic of LDL-SH. LDL-SH was S-nitrosated in vitro through the S-transnitrosation of LDL-SH by CySNO. Here S-transnitrosation not S-nitrosation by NO itself was used because exogenous thiols (homocysteinyl residues) of LDL-SH have been demonstrated to expose to the aqueous phases of LDL (Ferguson et al., 1999); CysNO was hydrophilic. Furthermore, Tsikas et al. (2001) reported that S-transnitrosation of albumin by CysNO could be a more favorable mechanism for the formation of S-nitrosoalbumin in the circulation in vivo than S-nitrosation of albumin by NO itself. Therefore LDL-SH could be S-nitrosated in plasma through the S-transnitrosation to form S-nitrosoprotein, LDL-SNO, under physiological conditions. The resultant LDL-SNO might reverse the detrimental effects of LDL-SH on biological systems. For instance, it could prevent LDL-SH from aggregation because S-nitroso groups block the reactivity of free –SH groups so as not to readily form protein multimers which are responsible for the aggregation of LDL-SH. Blocking the reactivity of free –SH groups might also reduce the immunogenicity of LDL-SH because immune response is specific to Hcy-Lys-epitopes (Ferguson et al., 1998). In addition, it is possible that LDL-SNO could be antiatherogenic by preventing oxidative modification of LDL. Therefore, S-nitrosation of LDL-SH could be physiologically relevant and favorably alter the detrimental effects of LDL-SH on biological systems.

It has been established that LDL is taken up through LDL receptor-mediated endocytosis in human fibroblasts (Brown et al., 1976). Fibroblasts are a common model

used in the study of lipid metabolism (Zimmermann et al., 2001). In addition, HcyTL has been found to be synthesized in human fibroblasts (Jakubowski, 1997). Therefore, we chose normal human fibroblasts (NHFs) as a model to examine whether homocysteinylation and subsequent S-nitrosation affect the uptake of LDL through high affinity LDL receptor-mediated endocytosis by NHFs.

The data presented here demonstrate that time and dose courses of uptake of LDL-SNO and LDL-SH are similar to those of uptake of native LDL. Moreover, uptake of LDL-SH and LDL-SNO was almost completely inhibited by excess unlabeled native LDL. These results suggest that chemical modifications including homocysteinylation and subsequent S-nitrosation do not affect uptake of LDL by NHFs; LDL-SNO and LDL-SH are taken up specifically via LDL receptor pathway by NHFs. Thus, the uptake of LDL-SNO through LDL receptor-mediated enocytosis might have an important biological application for the delivery of the functional group –SNO from the extracellular space into the cytosol. This might also represent a new pathway in vivo for transport of RSNO into the cytosol from extracellular space.

Insulin may stimulate the uptake of plasma LDL. This has been observed in human skin fibroblasts and hepatic cells HepG2 (Chait et al., 1978; Wade et al., 1988). However, it is not known whether chemical modifications (homocysteinylation and subsequent S-nitrosation) of LDL affect the role of insulin in the regulation of LDL uptake. The present data demonstrate that homocysteinylation and S-nitrosation does not change the stimulatory affect of insulin on LDL uptake. However, in a state of insulin resistance, the action of insulin on the stimulation of plasma LDL uptake is impaired (Steinberg et al., 1997). Thus, the LDL plasma levels are elevated; LDL may be

metabolized by vascular endothelial cells. With the consumption of endogenous antioxidants in LDL such as α tocopherol by vascular endothelial cells, oxidative modification of LDL will occur, thus leading to the development of atherosclerosis (Adams et al., 2000). Moreover, LDL-SH potentiates LDL oxidative modification (Ferretti et al., 2004). These adverse effects might be reversed through uptake of LDL-SNO by vascular endothelial cells, because NO is not only an effective EDRF but also a good inhibitor for LDL oxidation. In addition, the stimulation of NO production through the endothelium by insulin is also impaired in insulin-resistance state. Insulin-resistance state often result in the endothelial dysfunction due to the decreased NO bioactivity. Thus, uptake of LDL-SNO by vascular endothelial cells might also improve endothelial dysfunction in insulin-resistance state. Therefore formation of LDL-SNO in vivo might have a significant biological role in insulin resistance state.

1.6 Conclusion

The present results demonstrated that human LDL is homocysteinylated by HcyTL to produce LDL-SH. LDL-SH is S-nitrosated to form LDL-SNO in vitro. Furthermore, homocysteinylation and subsequent S-nitrosation, under the present experimental conditions, did not affect the specific uptake of LDL through LDL receptor mediated endocytosis. In addition, homocysteinylation and subsequent S-nitrosation did not change the effect of insulin in the stimulation of LDL uptake by NHFs. Thus, chemical modification (Homocysteinylation and S-nitrosation) might not affect the LDL homeostasis. S-nitrosation of LDL-SH might reverse the detrimental effect of LDL-SH in biological systems. Uptake of LDL-SNO through LDL receptor-mediated endocytosis might be an alternative pathway for delivery of RSNO into the cells from the extracellular space. Uptake of LDL-SNO might also have an important biological application in insulin resistance state. Of course, whether LDL-SH could be S-nitrosated to form LDL-SNO in vivo and its beneficial effects on biological systems remain to be proven in the future studies.

Part II

Apoptosis induced by S-nitroso-octadecanethiol in SK-MEL-28 human melanoma cells is enhanced by light exposure

2.0 Abstract

S-nitrosooctadecanethiol (ODTSNO), a novel S-nitrosothiolipid, was prepared by the reaction of 1-octadecanethiol with *t*-butylnitrite. The apoptotic role of ODTSNO in SK-MEL-28 human melanoma cells under the dark and light conditions was examined.

The present results demonstrated that ODTSNO could induce apoptosis in SK-MEL-28 human melanoma cells as assessed by typical morphological features of apoptosis such as chromatin condensation and DNA fragmentation. The apoptosis induced by S-nitroso-octadecanethiol involves the release of cytochrome *c* from mitochondria and caspase 3 activation. Furthermore, the apoptotic efficiency is substantially enhanced approximately 30% upon exposure to the light compared to the dark condition. The amount of cytochrome C released from mitochondria in the presence of light was more than that in the dark; the activity of caspase-3 upon exposure to the light is approximately 1.4 fold higher than that of caspase-3 in the dark.

The present study suggests that *S*-nitrosothiollipids have the potential to be used as topical photochemotherapeutic agents for melanoma.

2.1 Introduction

2.1.1 Terminology of apoptosis

Apoptosis, or programmed cell death (PCD), is a highly regulated and energy-dependent process (Hengartner, 2000). Morphologically and biochemically, it is characterized by membrane blebbing, chromatin condensation, cell shrinking, and nucleus fragmentation (Kerr et al., 1972). Caspase, aspartate-directed cysteine protease, has been suggested to be a central executioner for these morphological and biochemical changes (Hengartner MO, 2000). Thus, caspase activation plays a key role in apoptosis. Caspase activation likely initiates by two distinct pathways: an extrinsic/death receptor pathway and an intrinsic/ mitochondrial pathway (Reed et al., 2000; Zimmerman et al., 2001).

2.1.2 Apoptotic pathways

2.1.2.1 Extrinsic/ Death receptor-linked apoptotic pathways

This apoptotic pathway requires the specific binding of a ligand to a cell surface death receptor to initiate caspase activation. Death receptor includes Fas, tumor necrosis factor receptor 1(TNFR 1). The death receptor-linked apoptotic pathway can be exemplified by the interaction of Fas ligand (FasL) and Fas death receptor (Fig 2.1.1). The binding of FasL to Fas death receptor leads to the recruitment of FADD (Fas-associated protein with death domain) to the Fas death receptor via the DD (death domain) interaction. Procaspase-8 is then recruited to the FADD, thus resulting in the

formation of DISC (death-inducing signaling complex). The procaspase 8 is autocatalyzed at DISC to form a mature and active enzyme caspase 8. The apoptotic caspase cascade was then initiated by the active caspase 8 (Reed et al., 2000; Zimmerman et al., 2001).

2.1.2.2 Intrinsic apoptotic pathway

This pathway involves release of multiple polypeptides from mitochondria. The most studied polypeptide is cytochrome *c*, an electron transport chain protein, which accumulates in the cytoplasm when released from mitochondria (Figure 2.1.2). In the cytoplasm, cytochrome *c* binds to the scaffolding protein Apaf-1(apoptotic protease-activating factor-1), leading to the formation of Apaf-1 oligomers. Procaspase-9 then binds to the Apaf-1 oligomers to form apoptosome (high molecular mass complex) (Strasser et al., 2000; Hengartner, 2000). Activated caspase-9 then proteolytically activates caspase-3. Activated caspase-3 then mediates the apoptotic cascades (Kaufmann et al., 2001).

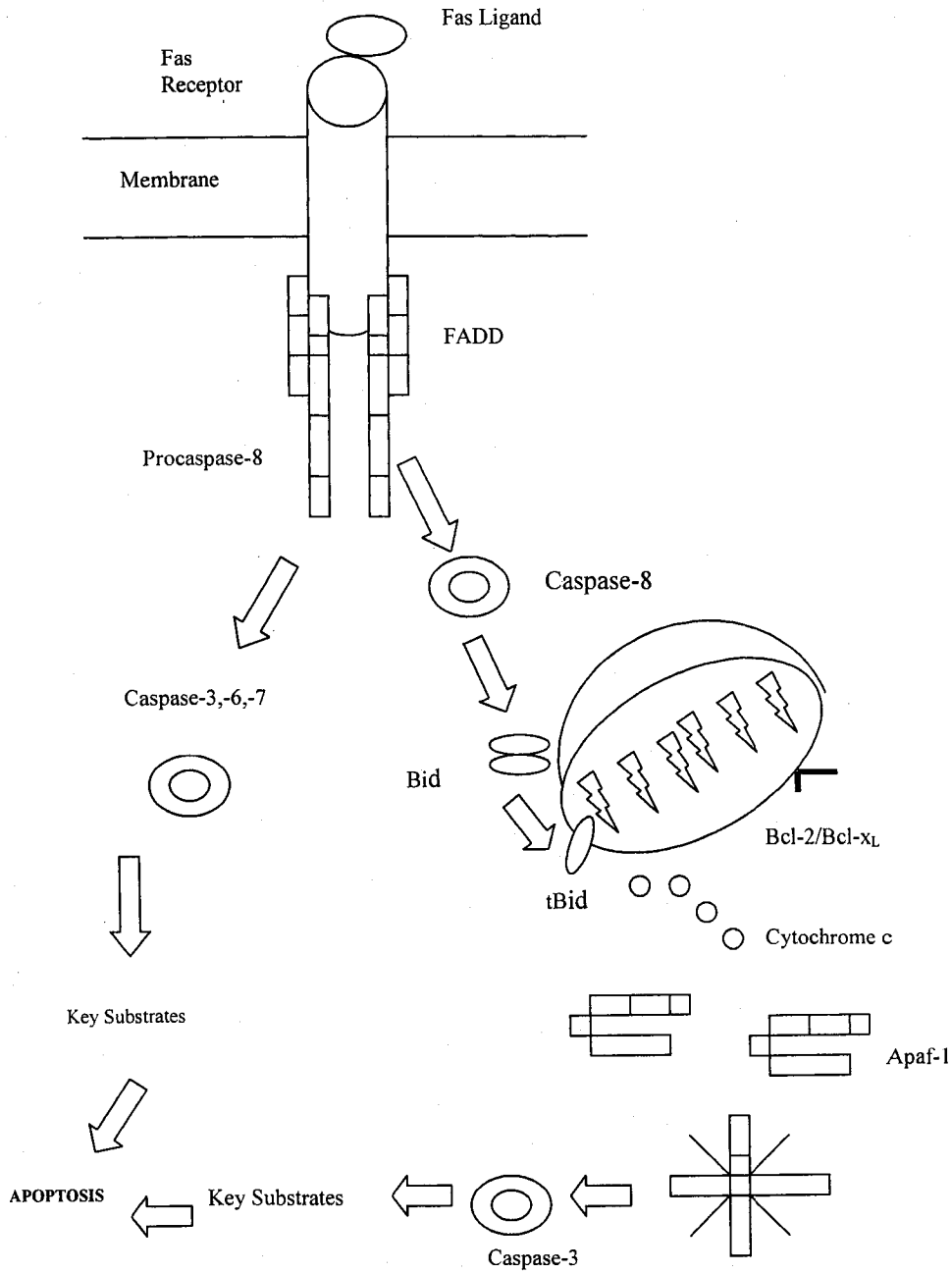


Figure 2.1.1: Induction of apoptosis by the interaction of FasL with Fas receptor. The interaction of FasL with Fas leads to the recruitment of FADD to the death domain of Fas, then procaspase 8 is recruited to FADD, forming DISC (death-inducing signal complex). Procaspase 8 is then autoacitivated to mature and active enzyme caspase-8 to initiate apoptotic caspase cascades (Zimmerman et al., 2001).

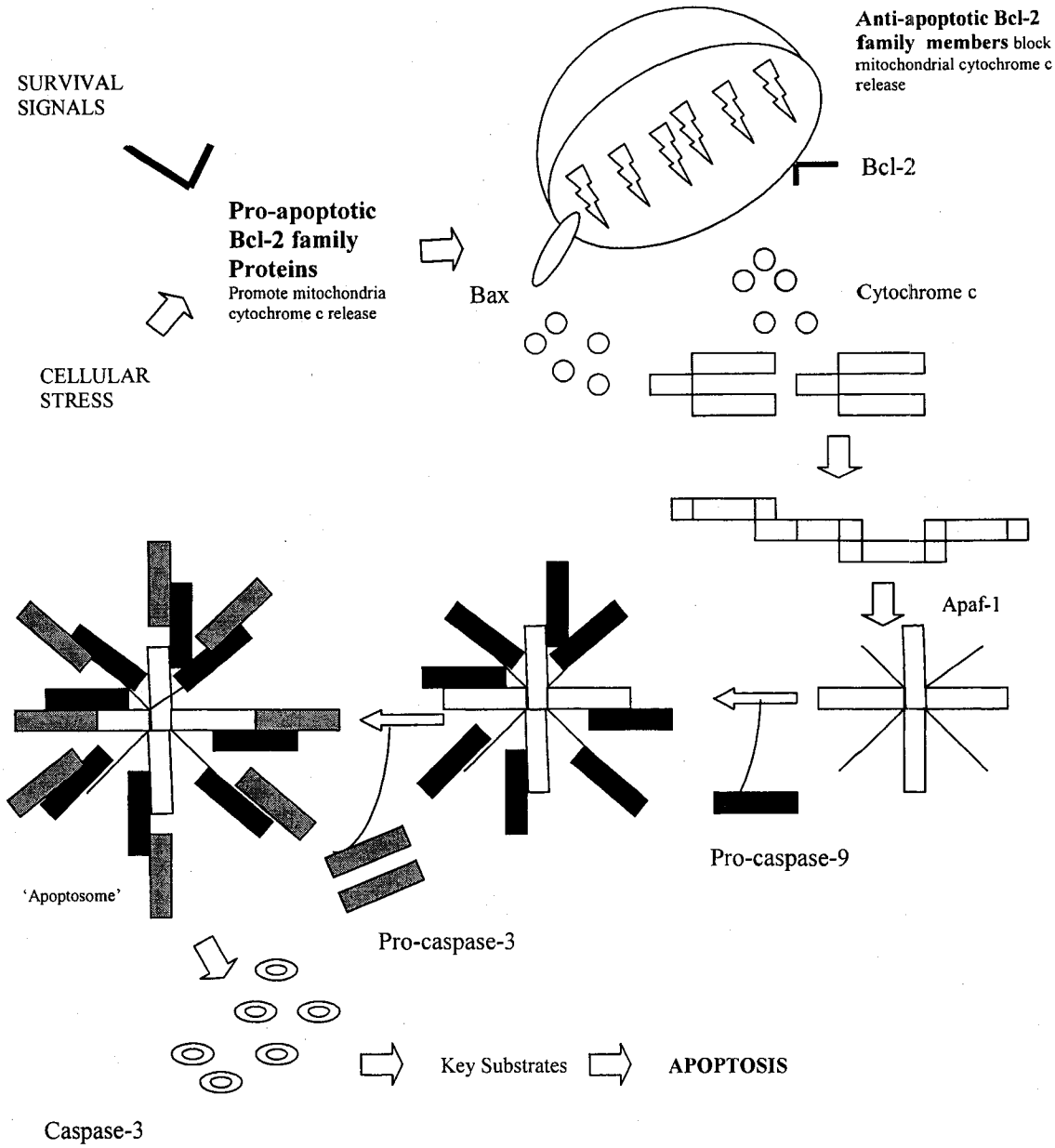


Figure 2.1.2: Induction of apoptosis via the mitochondria pathway. Cellular stress induces pro-apoptotic Bcl-2 family members to translocate from the cytosol to the mitochondria, where they induce the release of cytochrome c. Cytochrome c catalyzes the oligomerization of Apaf-1, which recruits procaspase-9and promotes its activation. This, in turn, activates procaspase-3, leading to apoptosis (Zimmermann et al., 2001)

2.1.3 Apoptotic pathways mediated by NO

NO has been suggested to be an effector molecule for macrophage mediated tumor cell death. Furthermore, NO mediated tumor cell death meets the morphological and molecular criteria of apoptosis (Albina et al., 1998). Therefore, studies have been undertaken to explore the role of NO in apoptosis. For example, many studies demonstrated that NO can induce apoptosis in various cell types such as macrophage cell lines (Messmer et al., 1995), thymocytes (Sandau et al., 1996), chondrocytes (Blanco et al., 1995), mesangial cells (Sandau et al., 1997), neurons (Lipton et al., 1993), vascular endothelial cells (Lopez-C et al., 1997), smooth muscle cells (Nishioet al., 1996), various tumor cells (Lorsbach et al.,1993) etc. Although there are different apoptotic mechanisms in different cell types, several general pathways for apoptosis induced by NO have been established. These pathways are the mitochondria-dependent/intrinsic apoptotic pathways.

2.1.3.1 Direct activation of mitochondria apoptotic pathway

NO can promote apoptosis via a direct effect on mitochondria by the opening of the permeability transition pore (PTP) (Green et al, 1998). Upon the opening of PTP, apoptotic protein cytochrome *c* is released from mitochondria into the cytosol to trigger apoptosis (Liu et al, 1996).

It has been suggested that the mitochondria inner membrane is an important site acted upon by NO (Shiva et al., 2001). In the mitochondria inner membrane, NO can reversibly bind to and inhibit the cytochrome *c* oxidase of the mitochondria respiratory

enzymes, resulting in the generation of reactive oxygen species, such as superoxide anion (O_2^-)

(Poderoso et al., 1996). The near diffusion-controlled reaction between NO and O_2^- produce peroxynitrite ($ONOO^-$), a highly cytotoxic agent (Cadenas et al., 2000). It has been suggested that mitochondria ATP synthase and aconitase can be irreversibly inhibited by peroxynitrite. It also can cause the opening of permeability transition pore, cytochrome *c* release and caspase-3 activation to initiate apoptosis (Brown, 1999).

Indeed, previous studies have shown that $ONOO^-$ can induce apoptosis in various types of cell lines, such as HL-60 cells (Yabuki et al, 1997), canine cerebral vascular muscle cells (Li, 2003) and human colon carcinoma cells (Kim et al, 2004).

2.1.3.2 MAPK (Mitogen-activated protein kinases) signalling pathways

2.1.3.2.1 MAPKs

MAP kinase is an essential part of the cellular phosphorylation/de-phosphorylation signalling cascades. It plays a central role in cell growth, differentiation and programmed cell death (Chang et al., 2001). Mammalian MAPKs can be classified into at least 3 groups, each containing isoenzymes: [1] extracellular signal-related kinases (ERK1/2); [2] c-Jun N-terminal Kinases (JNK1/2/3); [3] p38 kinases (p38δ α/β/ γ) where, JNK and p38 kinases are often described as stress activated protein kinases (SAPKs) because they are usually activated by a number of environmental stressors such as UV irradiation, heat shock and osmotic shock. Therefore, SAPKs are often associated with apoptosis, whereas ERK ½ are typically associated with cell survival (Davis, 2000).

2.1.3.2.2 MAPKs activation by NO

MAPK's activation has been suggested to be involved in mediating NO induced apoptotic signals (Lander, 1996). However, the role of MAPK's activation in the NO induced apoptosis is complex, depending on the types of NO donor and the cells examined. For example, Callsen et al. (1999) reported that GSNO promoted apoptosis in RAW 264.7 macrophages with the activation of ERK1/2, JNK1/2 and p38. However, ERK1/2 activation is anti-apoptotic signal, whereas JNK1/2 activation is a pro-apoptotic signal and p38 activation is independent of GSNO-induced apoptosis in macrophages. While in another study with the same cell type, SNAP was used as NO donor, p38 and JNK1/2 were activated, but ERK1/2 was not activated. P38 activation is responsible for the apoptosis whereas JNK1/2 activation is unrelated to apoptosis (Jun et al., 1999). In yet another study SNAP induced apoptosis in the human Jurkat cells, all of three MAPKs were activated (Lander et al., 1996). These studies suggested that MAPKs activation and the role of MAPKs activation in the NO induced apoptosis are specific to the type of NO donors and cells examined.

How does MAPK's signal pathway stimulate pro-apoptotic protein release from mitochondria and consequently lead to apoptosis? Tournier et al., (2000) reported that activation of JNK, one of the stress-activated protein kinases, is required for mitochondria cytochrome c release and subsequently downstream activation of caspase-9 and -3 to initiate apoptosis. Several studies have demonstrated that the molecular mechanism responsible for cyctochrome *c* release induced by JNK activation may involve regulation of the state of the Bcl-2 protein family such as down-regulation of the

anti-apoptotic Bcl-2 and Bcl-xl proteins and upregulation of the pro-apoptotic Bax and Bad proteins;.or phosphorylation of Bcl-2 and Bcl-xl by JNK (Choi, 2000; Fan, 2000).

2.1.3.3 p53-dependent apoptotic pathway

p53 activation is likely involved in NO-induced apoptosis. p53 activation is the consequence of DNA damage caused by NO (Messmer et al., 1994). p53 activation during NO-induced apoptosis has been observed in various cell types such as RAW 264.7 macrophages (Messmer et al., 1994), RINm5F cells (Messmer et al., 1996), breast cancer MCF-7 cells (Mortensen et al., 1999), astrocytes (Yung et al., 2004) etc. p53 activation is partly due to the DNA damage induced by NO (Messmer et al., 1994), or activation of JNK pathway leading to the phosphorylation of p53, thereby resulting in the stabilization of tumor suppressor p53 protein and subsequent increase of p53 levels (Buschmann et al., 2001).

2.1.4 Antiapoptotic effect of NO

NO has been suggested to be a double-edged sword. In addition to its pro-apoptotic effect, NO can also be anti-apoptotic. One explanation for this is that NO is a potent antioxidant free-radical scavenger (Hogg et al., 1998) and may suppress apoptosis by removing pro-oxidant molecules through radical-radical termination reaction. An alternative explanation for this is that S-nitrosylation of active site cysteine residues of caspase-3, a central executioner for the apoptosis, results in the inhibition of its activity, and consequent inhibition of apoptosis (Mannick et al., 1999).

2.2 Objective

RSNOs may release NO by photochemically homolytic cleavage of the S-NO bond (Singhet al., 1995). The role of photolysis of RSNOs in the cytotoxity of the tumor cells has been explored in our several studies. For example, we have shown that photolysis of GSNO resultes in the enhanced cytotoxity in the HL-60 human leukemia cells. The enhanced cytotoxity results from the NO released from photolysis of GSNO (Sexton et al., 1994). We also demonstrated that photolysis of GSNO is able to induce specific cell death in human Tenon's capsule (TC) fibroblasts (Tannous et al., 2000). Thus, RSNO could be used as a photochemotherapeutic agent in cancer therapy through the delivery of high and toxic concentrations of NO into the site of interest (Hogg, 2000).

Therefore, in the present study, a novel and lipophilic RSNO, S-nitroso-thiollipid was synthesized to explore its apoptotic effect on SK-MEL-28 human melanoma cells upon exposure to the light

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2.3 Materials and Methods

2.3.1 Materials

1-Octadecanethiol, Tert-butyl nitrite, Hoechst 33258, Phenylmethylsulfonyl fluoride, Pepstatin A, Pepstain B, DEVD-AFC and Griess reagent (N-(1-naphthyl)ethylenediamine dihydrochloride and Sulfanilamide), Fetal bovine serum (FBS) and Horseradish peroxidase were purchased from Sigma-Aldrich (St.Louis Mo). SK-MEL-28 human melanoma cells were purchased from ATCC (ATCC number HTB-72).

Monoclonal anti-mouse cytochrome C antibody was from Santa Cruz Biotechnology Inc.

1xPhosphate buffered saline (PBS)-137mM NaCl, 12.7mM KCl, 10mM Na_2HPO_4, and 1.76mM KH_2PO_4, PH 7.4

2.3.2 Methods

2.3.2.1 Synthesis of S-nitrosooctadecane thiol (ODTSNO)

1-octadecanethiol (LSH) was dissolved in chloroform. Equimolar tert-butyl nitrite was added and reaction was carried out in the dark at room temperature for 5 min. The concentration of ODTSNO produced was determined by measuring the absorbance at 340nm (ε_{340nm}=980 $M^{-1}cm^{-1}$) (DeMaster et al., 1995).

2.3.2.2 Preparation of ODTSNO liposomes

50ul ODTSNO solution in chloroform was added into an ependorf centrifuge tube, 1ml 1xPBS was then slowly layered on the top of ODTSNO solution and sonicated

for 5 min at room temperature. The liposome suspension formed in the PBS layer was transferred to another tube and diluted to the desired concentration.

2.3.2.3 Nitrite Assay

NO undergoes a series of reactions with several molecules present in biological fluids. The final products of NO in vivo are nitrite and nitrate. The sum of nitrite and nitrate can be the index of total NO production. Nitrite, a stable NO oxidation product, was determined using the Griess reaction. First, nitrate was converted to nitrite utilizing nitrate reductase. Then 50μl experimental sample was added to wells in triplicate. 50μl of 1% Sulfanilamide in 5% phosphoric acid was first added to the experimental samples and incubated 10min at room temperature in the dark. 50μl of 0.1% NED (*N*-1-napthylethylenediamine dihydrochloride) in water was then added to the experimental samples and incubated for 10min at RT in the dark. Nitrite was thus converted into a deep-purple azo compound. The absorbance of the azo chromophore was measured at 540nm using a microplate reader () to determine nitrite concentrations of the samples against a sodium nitrite standard curve.

2.3.2.4 Western blot analysis for cytochrome *c* released from mitochondria

Cells were scraped and harvested, washed twice with ice-cold PBS. Cells were then resuspended in a lysis buffer (2mM HEPES, 5mM $MgCl_2$, 1mM EGTA and 1% Triton X-100) containing a protease inhibitor mixture (0.1mM phenylmethylsulfonyl fluoride, 5μg/ml pepstatin A, and 1μg/ml pepstain B) and placed on ice for 10min. Cells were then homogenized and centrifuged at 15,000 g for 15 min at 4°C to make cytosolic

fractions for detecting cytochrome c release from mitochondrial. Same amount of Protein samples (20μg) were mixed with an equal volume of 2x SDS sample buffer, boiled for 5 min, and then separated through 14% SDS-PAGE gels. Protein samples were then transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 5% skim milk in TBST (20mM Tris-HCl, 200mM NaCl and 0.2% Tween) for 1h, rinsed, and incubated with anti-cytochrome *c* monoclone primary antibody (1:1000dilution) overnight at 4°C. Primary antibody was removed by 3 x10min with TBST. Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:2000 dilution) was then added and incubated for 1h at RT with gentle shaking. Secondary antibody was removed by repeated washes as described for primary antibody. Blots were developed using the 3, 3'-diaminobenzidine substrate for immunocoupled HRP and bands were visualized with Alpha Innotech Corporation Imaging System. The protein concentration was determined by the Bradford assay.

2.3.2..5 Measurement of Caspase- 3 activity

Caspase-3 activity was determined in the cytosolic extracts by the fluorometric assay. Cytosolic extracts were prepared by the centrifugation of the cell lysates at 15,000g for 15min. The supernatants were incubated in caspase assay buffer [100mM Hepes (pH 7.5), 10% (w/v) sucrose, 0.1% CHAPS, 1mM EDTA, 10mM dithiothreitol in the presence of fluorochromic caspase substrate DEVD-AFC for 1h at 37°C. The increase in fluorescence as a result of AFC release was monitored at 505nm and caspase activity was expressed in fluorescence intensity/per mg of protein/min.

2.3.2.6 Cell culture

SK-MEL-28 human melanoma cells were purchased from ATCC (ATCC number HTB-72). The SK-MEL-28 melanoma cells were cultured in ATCC medium: Minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.4 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 90% of 1.0 mM sodium pyruvate,; 10% of fetal bovine serum and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Exponentially growing skin cancer cells were seeded at $1x10^6$ per six-well plate, and then the cells were exposed to various agents indicated in the results part.

2.4.7 Hoechst staining

Cells were stained with Hoechst dye (5μg/ml) for 5min, washed and examined under fluorescence microscopy at 365nm excitation wavelength and 465nm emission wavelength. The percentage of apoptotic cells was determined by counting the number of nuclei showing chromatin condensation and fragmentation characteristic of apoptosis.

2.4.8 Light source

Overhead projector with the power of 300W was used as visible light source for treating the cells throughout the experiments.

2.4 Results

2.4.1 Instability of ODTSNO liposome upon exposure to the light

ODTSNO was synthesized as described in Methods. To determine the optimum duration of photolysis of ODTSNO liposome, the absorbance of ODTSNO at 340nm before and after exposure to the light was measured at the indicated exposure time as shown in Figure 2.4.1. ODTSNO liposome is relatively stable in the absence of light within 30min. In contrast, ODTSNO liposome is substantially decomposed in the presence of visible light within 30min. For example, about 80% of ODTSNO liposome was decomposed upon exposure to the light for 15 min. Therefore, 15min was used as exposure time in the present study in order to avoid potentially adverse effect of longer exposure to the light on the cells.

2.4.2 Uptake of ODTSNO liposomes

To determine the required duration for the SK-MEL-28 human melanoma cells to take up ODTSNO liposome, 1mM ODTSNO liposome was added to the cells and incubated for various durations, then nitrite production in the cytosolic extraction was measured. As shown in Figure 2.4.3, the more nitrite, the longer the incubation time. However, uptake of ODTSNO liposome by Sk-MEL-28 human melanoma cells reached saturation after 30min incubation. In contrast, intracellular [NO_2^-] of cell incubated with ODT remained constant for the duration of the experiment. Thus, 30 min was used as incubation time of ODTSNO liposome for the SK-MEL-28 human melanoma cells in the following experiments.

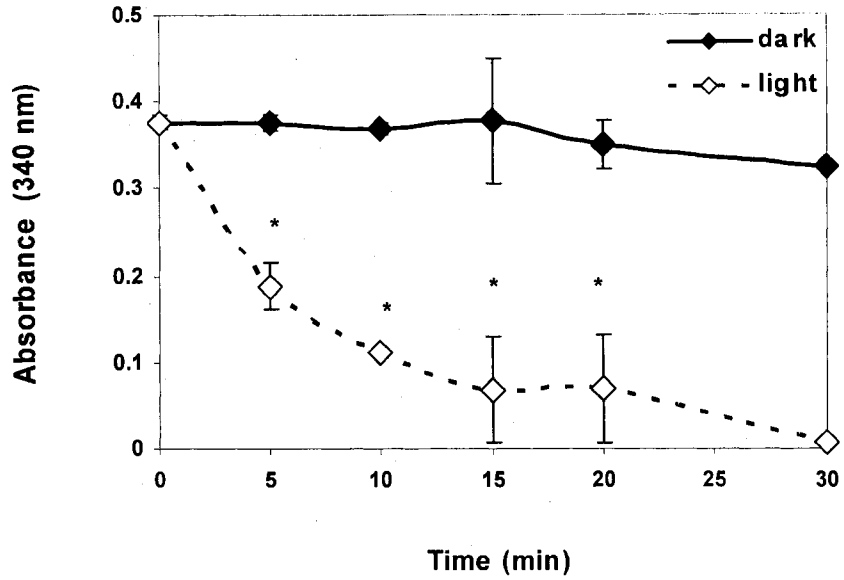


Figure 2.4.1: Photolysis of ODTSNO liposome. 500uL of 780μM ODTSNO liposome and 500 uL PBS were combined in each of ten vials. Five vials were exposed to light, the other five vials were kept in the dark. At the indicated exposure times, absorbance at 340nm was measured by extraction of ODTSNO liposome with 1ml chloroform to determine the stability of ODTSNO liposome upon exposure to the light. Those values marked with an asterisk (*) are different from controls with statistical significance ($p<0.05$).

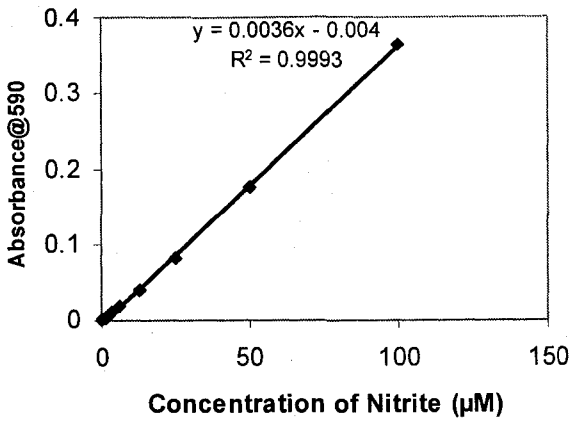


Figure 2.4.2: Standard curve for Griess reagent assay

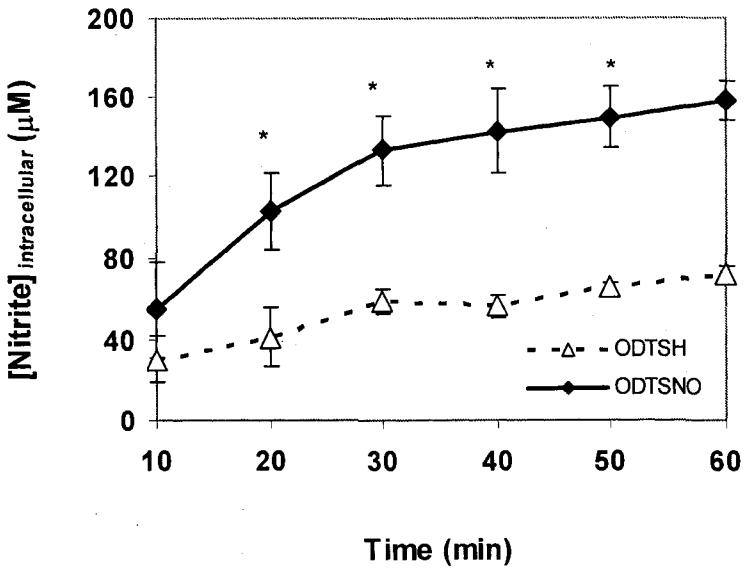


Figure 2.4.3: Nitrite production from ODTSNO liposome. The SK-MEL-28 human melanoma cells were incubated with 1mM ODTSNO liposome for the different lengths of time; excess liposomes were then removed. Cells were changed back to fresh medium. The cytosolic nitrite production in the medium was assayed as described in Methods. Those values marked with an asterisk (*) are different from controls with statistical significance ($p<0.05$).

2.4.3 Comparison of apoptosis induced by ODTSNO in the presence and absence of light in SK-MEL-28 human melanoma cells

NO is potently cytotoxic to tumor cells in vitro (Hibbs et al., 1988). Furthermore, previous studies have shown that NO can induce apoptosis in various types of cells including tumor cells. Much of this work has been performed with chemical NO donors, such as RSNOs. RSNOs as an NO donor have a biological relevance since they represent a storage form of NO in biological systems (Kolb, 2000). RSNOs have been suggested to be potential photochemotherapeutic agents for cancer. For example, we have shown that photolysis of GSNO resulted in an enhanced cytotoxic effect of GSNO on HL-60 leukemia cells (Sexton et al., 1994).

In the present study, we synthesized a novel and hydrophobic RSNO, S-nitroso-octadecanethiol (ODTSNO), to explore its apoptotic effect on SK-MEL-28 human melanoma cells upon exposure to the light.

SK-MEL-28 human melanoma cells were exposed to 2mM ODTSNO liposome for 30min. Excess ODTSNO liposome was then removed and the cells were changed back to fresh medium. One plate of SK-MEL-28 human melanoma cells in the fresh medium was exposed to the overhead projector for 15min; another plate of cells was kept in the dark. Both plates of cells were then put back to incubator for 24 hours before the examination of the apoptotic effect of ODTSNO on SK-MEL-28 human melanoma cells. As shown in Figure 2.4.4 (A), SK-MEL-28 human melanoma cells treated by ODTSNO with and without light exhibit morphological apoptotic features such as membrane blebbing, chromatin condensation, and DNA fragmentation, compared with untreated and

1-octadecanethiol (ODTSH) treated cells. In addition, as shown in figure 2.4.4 (B) the percentage of apoptosis induced by ODTSNO in the presence of light in SK-MEL-28 human melanoma cells was approximately 65%, compared to approximately 30% in the absence of light.

In order to confirm that the observed apoptotic effects were due to NO and not to the chemical carrier moiety ODTSH, we repeated the experiments using an equimolar concentration of ODTSH. The results demonstrated that ODTSH had no effect on the apoptosis of SK-MEL-28 human melanoma cells under dark and light conditions, thereby confirming the apoptotic role of NO released from ODTSNO in SK-MEL-28 human melanoma cells. In addition, in order to rule out the possibility that the apoptotic effect is partly due to the light itself, we also controlled the experiments using cells treated only with light. As shown in Figure 2.4.4 (B), light does not account for the apoptosis of SK-MEL-28 human melanoma cells, suggesting that the increase of apoptotic efficiency in the presence of the light is attributed to the higher concentrations of NO released from ODTSNO with respect to the cells treated by ODTSNO without light.

Treatment of SK-MEL-28 human melanoma cells with various concentrations of ODTSNO resulted in a concentration-dependent induction of apoptosis. Furthermore, apoptotic efficiency is higher in the presence of light than in the absence of the light at all the concentrations examined (Figure 2.4.5). In addition, SK-MEL-28 human melanoma cells apoptosis induced by ODTSNO was time-dependent, with apoptotic percentage much higher in the presence of the light than in the absence of the light at all the times examined (Figure 2.4.6).

These results demonstrated that ODTSNO, a novel NO-donor compound of RSNOs, could induce apoptosis in SK-MEL-28 human melanoma cells as do other RSNOs such as GSNO. Furthermore, the apoptotic efficiency was greatly enhanced in the presence of light, suggesting that higher concentrations of NO were released by photolysis of ODTSNO with respect to the dark conditions; ODTSNO might have a potential to act as topical photochemotherapeutic agents for melanoma.

Figure 2.4.4: Apoptosis induced by ODTSNO in the presence and absence of light in SK-MEL-28 human melanoma cells

A-Legend

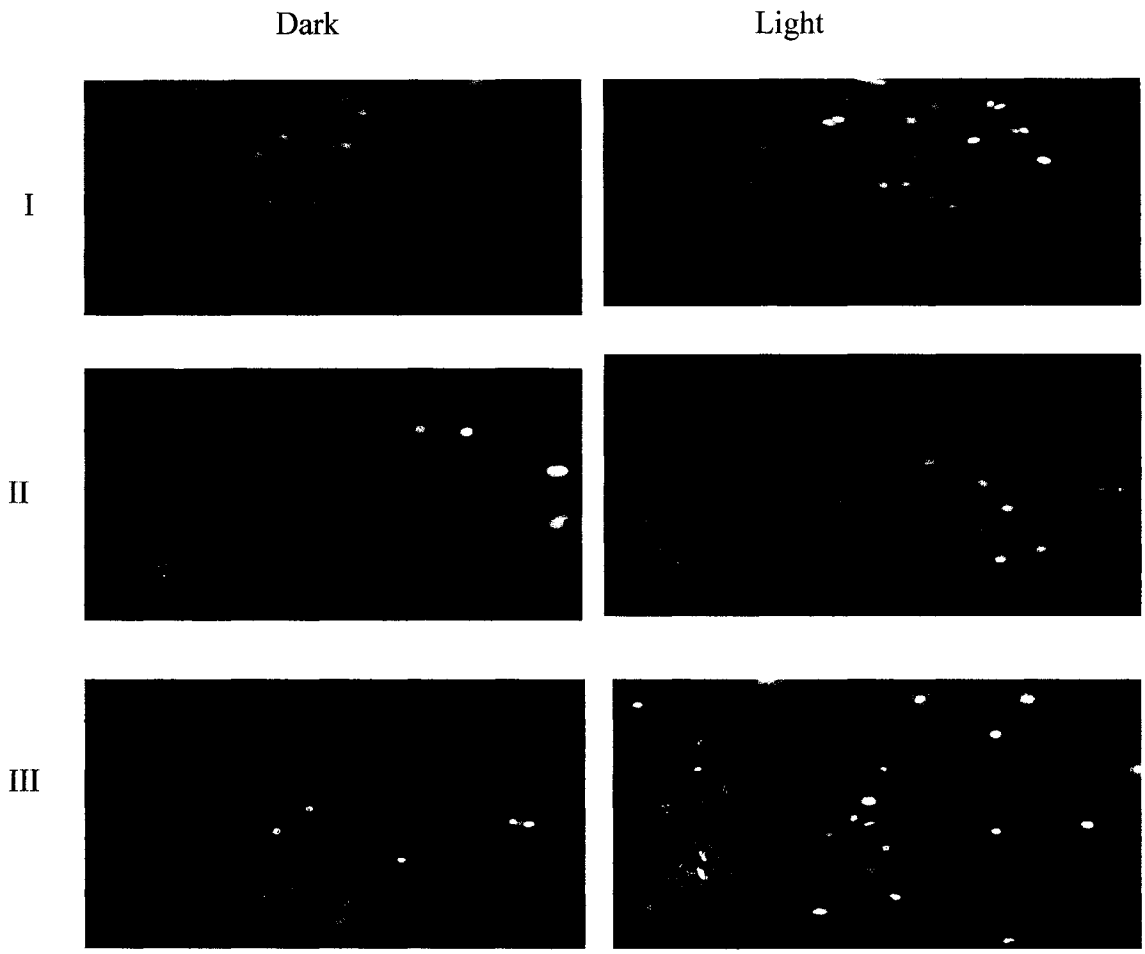
(I) SK-MEL-28 human melanoma cells were incubated with medium alone (II) SK-MEL-28 human melanoma cells were treated with LSH. (III) SK-MEL-28 human melanoma cells were treated with ODTSNO. All the cells were stained with Hoechst dye 33258, and examined by a fluorescence microscope.

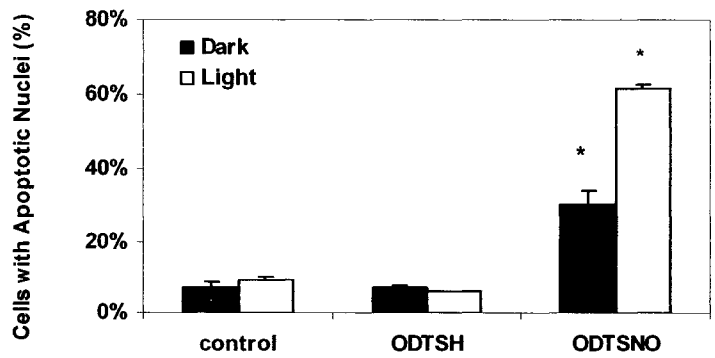
B-Legend

SK-MEL-28 human melanoma cells were grown in 6-well plates and treated with 2mM ODTSNO liposome for 30min; excess ODTSNO liposome was removed. The cells were then exposed to the light for 15min and put back to the incubator for 24h. Apoptosis was determined by hoechst stain assay. Those values marked with an asterisk (*) are different from controls with statistical significance ($p<0.05$).

Figure 2.4.4

A





B

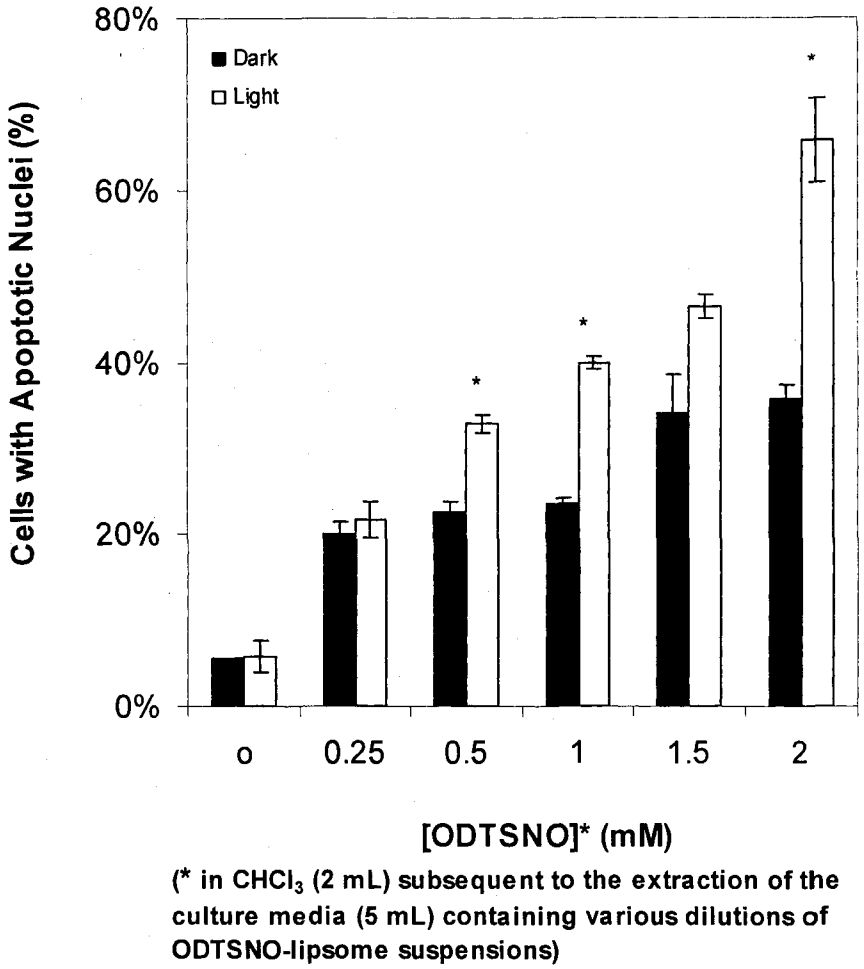


Figure 2.4.5: Dose-dependent apoptosis induced by ODTSNO under dark and light conditions. Cells were treated with various concentrations of ODTSNO liposome for 30min, and then exposed to light for 15min. The cells were changed back to fresh medium and put back to incubator for 24h. Apoptosis was determined by Hoechst stain assay as described in Methods. Those values in the presence of light that are different from those in the dark with statistical significance ($p<0.05$) are marked with an asterisk (*).

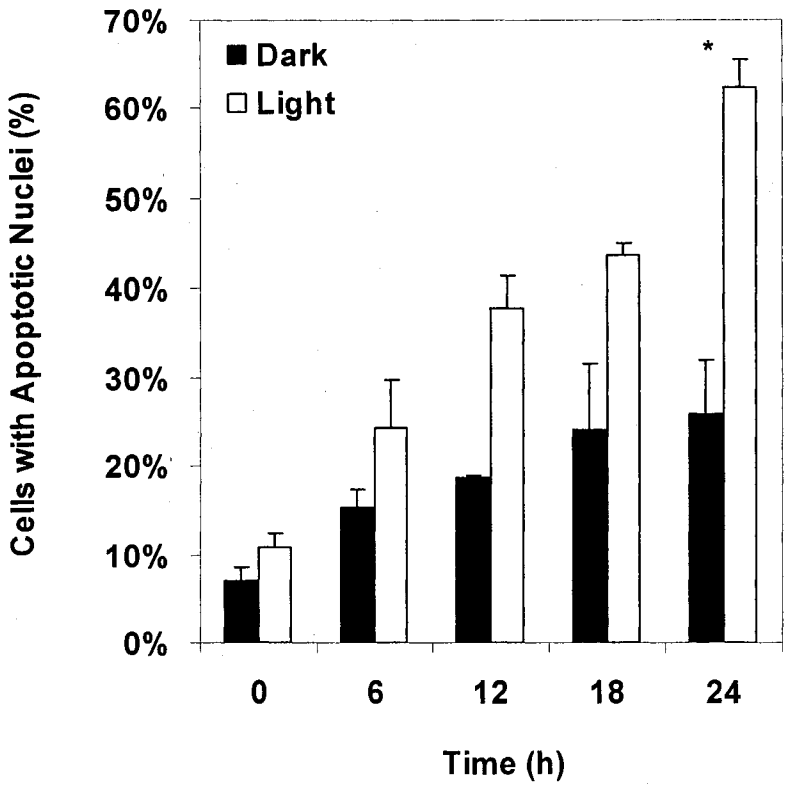
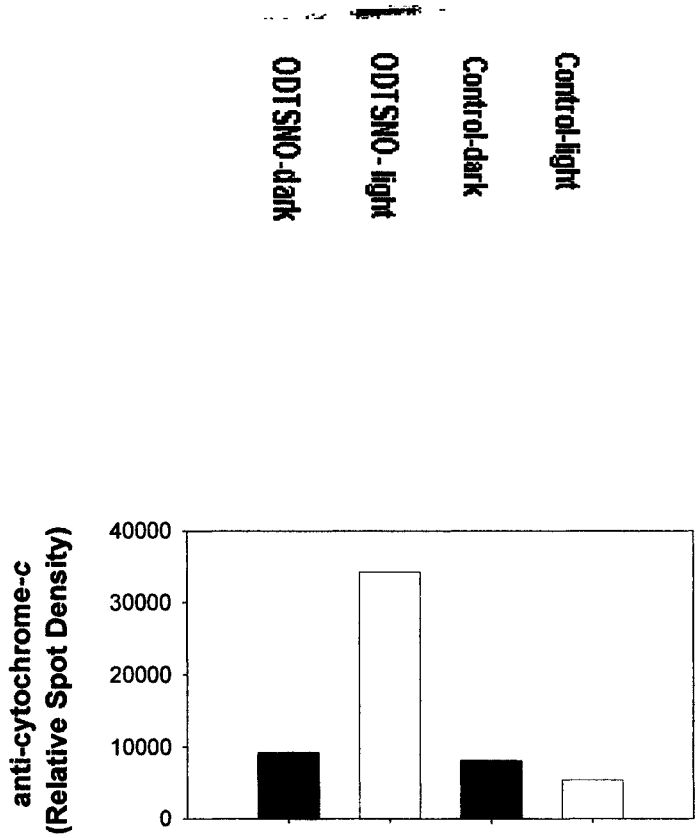


Figure 2.4.6: Time course of the ODTSNO-induced apoptosis in SK-MEL-28 human melanoma cells under dark and light conditions. SK-MEL-28 human melanoma cells were treated by 1mM of ODTSNO liposome for 30min, and then exposed to light for 15min. The cells were changed back to fresh medium and put back to incubator. Apoptosis was then determined at the indicated times by Hoechst stain assay as described in Methods. Those values in the presence of light that are different from those in the dark with statistical significance ($p<0.05$) are marked with an asterisk (*).

(A) Cytochrome *c* 13kDa



(B)

Figure 2.4.7: (A) The release of cytochrome *c* from mitochondria during ODTSNO-induced SK-MEL-28 Human melanoma cells apoptosis under dark and light conditions. SK-MEL-28 Human melanoma cells were treated by 1mM ODTSNO for 30 min; excess ODTSNO was then removed and changed back to fresh media. The cells were exposed to the light for 15 min and put back incubator for 24h; cytochrome *c* release was assayed by Western blot. (B) The corresponding band densities of anti-cytotchtome c western blots.

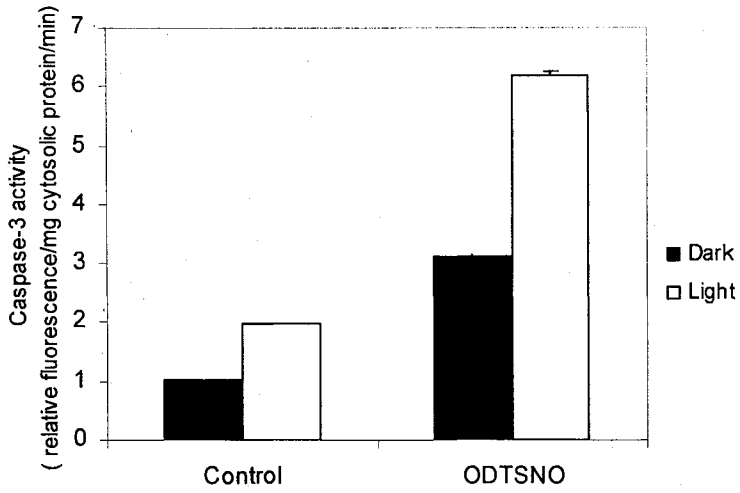


Figure 2.4.8: Activation of caspase-3 during ODTSNO-induced apoptosis. SK-MEL-28 human melanoma cells were treated with 1mM of ODTSNO liposome for 30min, Excess ODTSNO liposome was removed and cells were then exposed to light for 15min. The cells were then changed back to fresh medium and put back to incubator for 24h. Cells were harvested and cell extracts were obtained as described in Methods. Cytosolic extracts were incubated with the fluorogenic substrate Ac-DEVD-AFC, and the fluorescence of AFC released on proteolytic cleavage by active caspase-3 was measured.

2.5 Discussion

It has been suggested that NO has an important physiological role as an effector molecule in mediating macrophage-induced tumor cell cytotoxicity (Hibbs Jr., et al., 1988; Stuehr et al., 1989). Cytotoxic tumor cells exhibit morphological features of apoptosis, such as membrane blebbing, chromatin condensation and membrane-bound apoptotic bodies. Therefore, in this regard, macrophage-derived NO inhibits neoplastic cell growth by leading to the apoptosis in target tumor cells (Albina et al., 1998). NO has been suggested to play an essential role in mediating photo-induced cell death in human maligant cells (Ali et al., 2003). Many of the studies have demonstrated that NO can induce apoptosis in various tumor cell lines such as murine mastocytoma cells (Kitajima et al., 1994), leukemia cells (Shami et al., 1998) etc.

It has been reported that one NO source for the induction of apoptosis in tumor cell lines results from the endogenous NO produced from L-arginine via the NOS-catalyzed pathway or exogenous NO such as authentic NO gas and chemical NO-donor compounds. Most studies are performed with chemical NO donor compounds, especially RSNOs, since RSNOs are relatively stable storage forms of NO in biological systems (Kolb, 2000, Stamler et al., 1992a). RSNOs possess NO-like activities such as vasodilation (Myers et al., 1990) and antiplatelet properties (Radomski et al., 1992). It has been suggested that RSNOs can exert their biological functions through the release of NO produced by photochemical cleavage of the S-NO bond. For example, we have shown that photolysis of GSNO with visible light resulted in enhanced cytotoxity to HL-60 leukemia cells (Sexton et al., 1994), suggesting that RSNOs could be used as photochemotherapeutic agents.

In the present study, ODTSNO, a novel lipophilic RSNO, was synthesized to explore its apoptotic role in SK-MEL-28 human melanoma cells upon exposure to the light. The present results demonstrated that ODTSNO could result in cell death of SK-MEL-28 human melanoma cells in the dark by inducing the apoptosis, suggesting that ODTSNO was also subject to spontaneous release of NO in the dark, probably due to the buffer contaminated with metal ions such as Cu^{2+} (Williams et al., 1996a) or thiol groups (Scorza et al., 1997) in the medium or cell surface PDI present in the cells (Ramachandran et al., 2001). However, apoptotic efficiency was substantially enhanced in the presence of light, suggesting that ODTSNO could undergo photochemical cleavage to release higher concentrations of NO in the desired sites so as to increase the apoptotic efficiency, consistent with early conclusion that high levels of NO could cause self-destruction of tumor cells (Xie et al., 1997).

But how does ODTSNO induce apoptosis in SK-MEL-28 human melanoma cells? Two pathways have been suggested to be responsible for the apoptosis (Zimmerman et al., 2002; Reed et al., 2000). One is death receptor-mediated apoptotic pathway; another is mitochondria-dependent apoptotic pathway. Apoptotic signalling pathways initiated by NO have been suggested to belong to a mitochondria-dependent apoptotic pathway. One typical feature of the mitochondria-dependent apoptotic pathway is the release of cytochrome *c* from mitochondria, which leads to caspase activation through the apoptotic protease-activating factor-1 (Apaf-1). Therefore, we want to examine whether apoptosis induced by ODTSNO in SK-MEL-28 human melanoma cells involves the release of cytochrome *c*. The present data demonstrated that cytochrome *c* was released during the apoptosis induced by ODTSNO under the dark and light conditions.

Caspases, aspartate-directed cysteine proteases, are central to apoptosis (Hengartner, 2000). Caspase-3 is a well-characterized member of the effector caspases. Activation of caspase-3 has been proposed to be a key step in initiating the execution of apoptosis (Lin et al., 1998). Therefore, Caspase-3 activity was also examined in the present study during the apoptosis induced by ODTSNO. As a result, caspase-3 was activated, but the activity of caspase-3 is much higher in the presence of the light than in the absence of the light.

Therefore, apoptosis induced by ODTSNO in SK-MEL-28 human melanoma cells involved the release of cytochrome *c* from mitochondria and caspase-3 activation.

In addition, compared with the known water-soluble RSNOs, ODTSNO is lipophilic. Thus, the effective concentration of NO released from ODTSNO could be enhanced with respect to the classical hydrophilic RSNO, because NO concentration in a hydrophobic compartment is approximately 9 times higher than that in aqueous solution (Liu et al., 1998). The short half-life of NO in aqueous solution can be attributed to the presence of an active intermediate, N_2O_3, product of NO autooxidation, that is hydrolyzed to nitrite (Wink et al., 1998). However, lipophilic ODTSNO could rapidly accumulate in the membrane fraction. Thereby, higher concentrations of NO should remain in the membrane fraction after photolysis of ODTSNO. Higher concentrations of N_2O_3 should form in the membrane so as to initiate NO-mediated apoptotic signalling. Thus, the effective and actual concentration of ODTSNO in apoptosis should be less than that of classical and hydrophilic RSNO such as GSNO. In this regard, lipophilic ODTSNO might be an effective anti-tumor photochemotherapeutic agent compared to the classical RSNOs.

2.6 Conclusion

The present results demonstrated that novel and lipophilic ODTSNO could induce apoptosis in SK-MEL-28 human melanoma cells. The release of cytochrome *c* and caspase-3 activation was observed during the apoptosis induced by ODTSNO in SK-MEl-28 human melanoma cells. The apoptosis initiated by ODTSNO attributed to the NO released from ODTSNO. Furthermore, apoptotic efficiency is substantially enhanced approximately 30% upon exposure to the light. More cytochrome *c* was released in the presence of the light than in the absence of the light; the activity of caspase-3 increased approximately 1.4 fold in the presence of light relative to dark condition. Therefore, the present study suggested that S-nitrosothiollipids might have the potential to be used as topical photochemotherapeutic agents for melanoma.

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