Chapter 2

Review of Literature

This chapter provides any information related to other previous works and theoretical concepts support our research studies as we described in chapter 1. Some research data are pointed out and remain obscure.

Our hypothesis focuses on in which species of ROS/RNS influence upon vascular dysfunction through redox-sensitive signaling. We anticipate greater expression of LOX-1 activated by various degree of oxidized LDL represents intracellular ROS and RNS activation. Consequently, the redox signals might overwhelm redox-sensitive enzymes, protein, lipid and DNA damage.

Topics reviewed are listed as follows:

1). Atherosclerosis and modified oxidative hypothesis

2). Impact of oxidized LDL

3). The crucial role of LOX-1

4). Vascular reactive oxygen species (ROS) and reactive nitrogen species (RNS)

5). Impact of endothelial dysfunction

2.1. Atherosclerosis and modified oxidative hypothesis

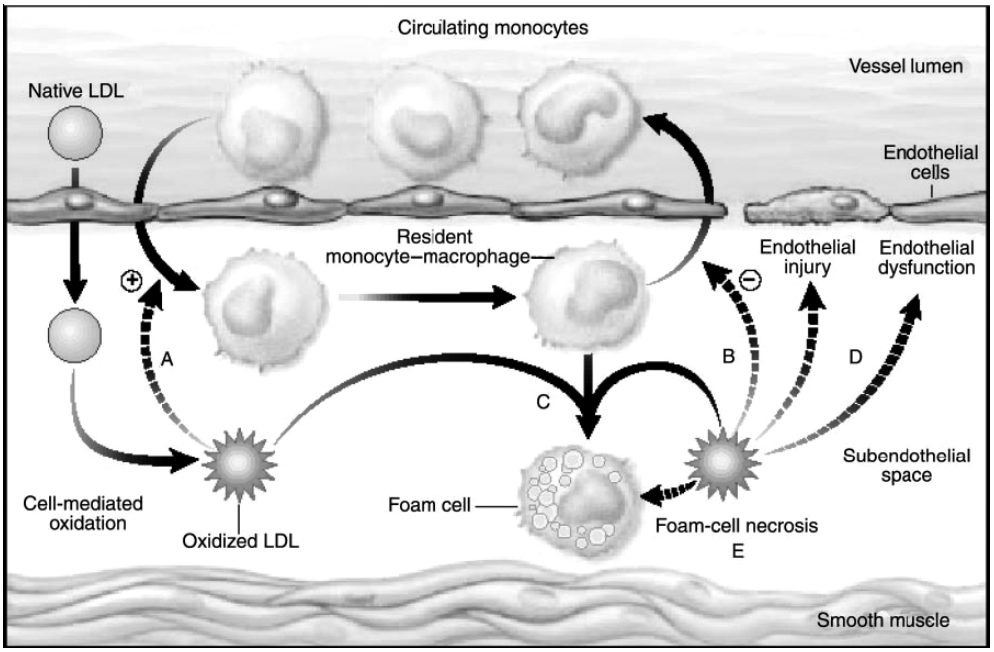
Atherosclerosis is a disease of large and medium-sized elastic and muscular arteries and results in the progressive accumulation of inflammatory cells, smooth muscle cells, lipids and connective tissue within the intima layer. The characteristic lesion of atherosclerosis is the lipid plaque. The area between the lumen and the necrotic core contains smooth muscle cells, macrophages, lymphocytes, foam cells and connective tissue components. Plaques may progress with time from a simple fibro-fatty lesion to a complicated plaque with erosion, surface ulceration, plaque hemorrhage, thrombosis, calcification and aneurysm. Atheromas usually form where arteries branch, possibly because the constant turbulent blood flow, injury the arterial wall. Long term injury to the arterial wall causes endothelial dysfunction and

an inflammatory response with the release of mediator factors promoting development of atherosclerosis.

There have been numerous efforts to explain the complex events associated with the development of atherosclerosis and proposed three distinct hypotheses: the response-to-injury; the response-to-retention and finally, the oxidative modification of low density lipoprotein (LDL). We focus on the oxidative modification of LDL theory as described in chapter 1 for our research questions and studies. In the past decade, we believed that circulating LDL in its native state was not atherogenic but when it was entrapped in the subendothelial space, it was subjected to oxidative modification by resident vascular cells such as endothelial cells, smooth muscle cells, and macrophages that we called “cell-mediated oxidation” (figure 2.1). LDL modified chemically was readily internalized by macrophages through scavenger receptor pathway. Oxidized LDL stimulated monocyte chemotaxis and supported foam cell formation. Once formed, oxLDL also resulted in endothelial dysfunction and injury. Foam cells became necrotic due to the accumulation of LDL, meanwhile, smooth muscle cell was proliferated and increasing growth leading to narrow lumen of vessel.

Figure 2.1

Oxidative modification hypothesis model



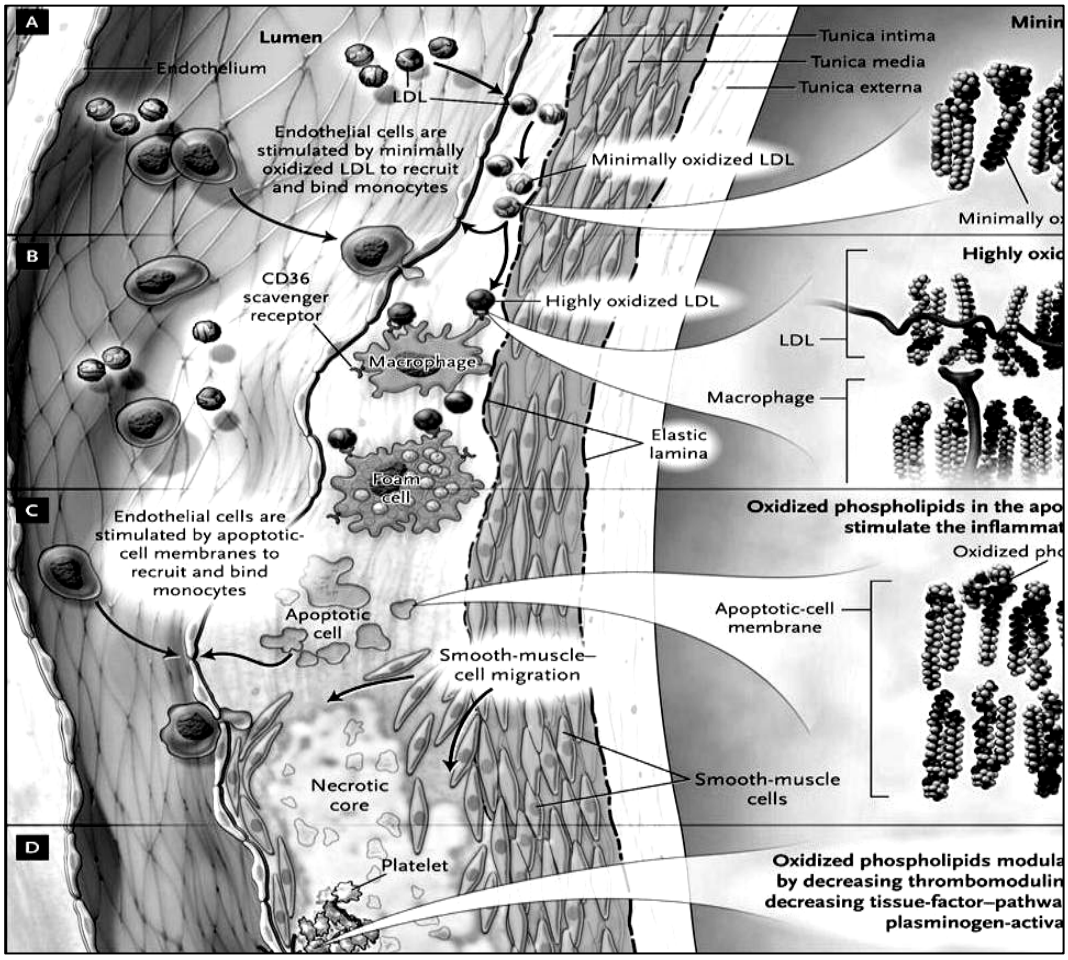
(Diaz, Frei, Vita, & Keaney, 1997)

Based on the concept of oxidative modification hypothesis as proposed by Steinberg et al in 1989, several new receptors for oxLDL in macrophage such as CD36, LOX-1 and SR-PSOX etc have been discovered but not found at endothelium and cause the endothelial dysfunction to initiate atherosclerosis (Steinberg, et al., 1989). In 1997, Sawamura et al. found a new receptor for oxLDL in endothelial cells and named lectin-like oxidized LDL or LOX-1 receptor (Sawamura, et al., 1997). Until now, the concept of modified oxidation theory has been changed, As shown in figure 2.2, LDL becomes minimally oxidized in circulation and then is taken up through LOX-1 receptor at endothelium. Activated endothelium recruits and binds monocytes to subendothelial space where they become macrophage with developing CD36 scavenger receptors for foam cell progression (Tsimikas, et al., 2005). Interestingly, oxidative stress is implemented to the circulation causing modified oxidation of LDL and also to the vasculature leading to further oxidation and subsequently progression of atherosclerosis. As we known, oxidative stress is defined as imbalance of oxidant and antioxidant systems in which occurs in compartment and

leads to pathophysiology of disease. Therefore, the pathophysiological mechanisms of atherosclerosis are complicating, indicated complex events concerning both of oxidant and antioxidant balance.

Figure 2.2

Purposed new concept of oxidized LDL



(Tsimikas, et al., 2005)

2.2 Impact of oxidized LDL

2.2.1 Lipid peroxidation and protein oxidation

LDL is a heterogeneous class of lipoprotein particles consisting of a hydrophobic core containing triglycerides and cholesterol esters in a hydrophilic shell

of phospholipids, free cholesterol and apolipoproteins, predominantly B-100, the latter acting as ligands for lipoprotein receptors. The density of LDL ranges from 1.019 to 1.063 g/ml and its diameter is between 20 and 25 nm (Smith, Pownall, & Gotto, 1978). The chemical composition of LDL is shown in table 2.1.

Table2.1

The chemical composition of LDL

Component	Weight, %	Mol/LDL	LH/LDL*
	Protein		
Apolipoprotein B-100	22.0±1.9	1	
	Lipids		
Cholesterol esters	42.3±3.8	1,600	1,165
Phospholipids	22.3±3.9	700	375
Cholesterol	9.6±0.7	600	0
Triacylglycerols	5.9±2.7	180	50
	Antioxidants		
α-TOH		6-12	
$CoQ_{10}H_2$		0.5-1.0	

(Smith, et al., 1978)

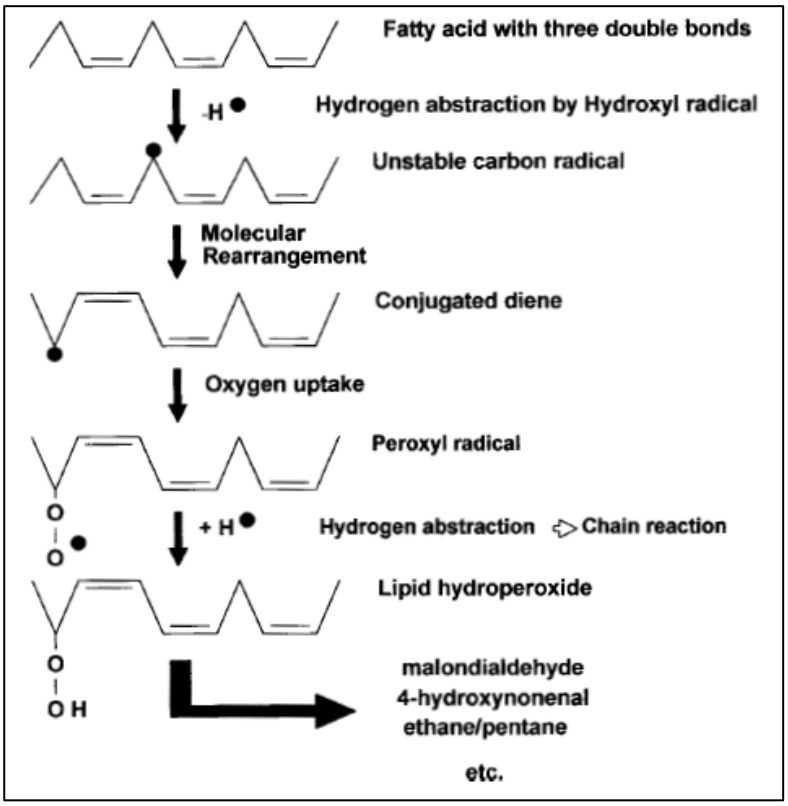
* LH refers to lipid containing bisallylic hydrogen atoms

However, the present concept is that atherosclerosis represents a state of heightened oxidative stress characterized by lipid and protein oxidation in the vascular wall. Once initiated, oxidation of LDL is a free radical-driven lipid peroxidation chain reaction. Lipid peroxidation is initiated by free radical attacks on a double bond associated with a polyunsaturated fatty acid (PUFA). This result in the removal of a hydrogen atom a methylene (CH_2) group, the rate of which determines the rate of key step initiation. Molecular rearrangement of the resulting unstable carbon radical results in numerous stable configuration “conjugated diene”. After that, the conjugated diene reacts very quickly with molecular oxygen resulting the peroxyl radical as a crucial intermediate. A PUFA peroxyl radical in LDL may abstract a

hydrogen atom from an adjacent PUFA to a hydroperoxide and another lipid radical, a reaction which results in chain propagation. Removal of hydrogen atoms by the peroxyl radical from other lipids including cholesterol eventually yields oxysterols. Lipid hydroperoxides fragment is shorter chain aldehydes including malondialdehyde and 4-hydroxynonenal. These reactive aldehydes in turn may bind to ε-amino group of apo B-100, giving an increased net negative charge of protein. The classical LDL receptor recognizes a specific domain of positive charges from lysine, arginine and histidine residues on apo B. Alteration of this domain results in failure of binding by the apo B/E receptor and an increase in negative surface charge on apo B-100 results in increased recognition by the scavenger receptor (Figure 2.3)(Young & McEneny, 2001).

Figure 2.3

Basic reaction sequence of lipid peroxidation

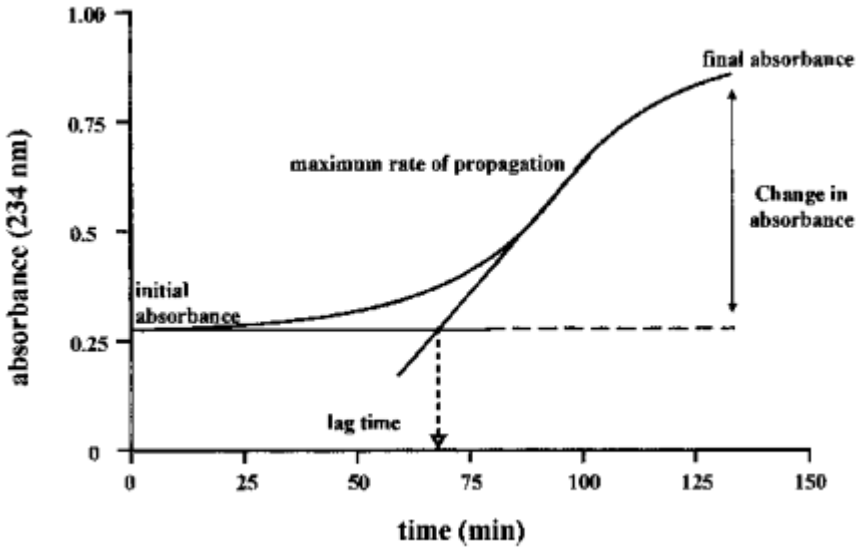


(Young & McEneny, 2001)

In the presence of a lipid phase chain-breaking antioxidant such as α-tocopherol, the peroxyl radical should be scavenged. The toco-pheoxyl radical, once formed, has very low reactivity and will generally result in chain termination. Thus, LDL exposed to oxidative stress *in vitro* will not form significant amounts of hydroperoxides until it becomes deplete of chain-breaking antioxidants. *In vitro* study of LDL oxidation using $CuSO_4$, the existence of lag, propagation and decomposition phases is presented as conjugated diene formation recorded by U/V spectrophotometer at λ 234. Lag phase indicates the resistance of oxidation represents to the endogenous antioxidant available and then is consumed (by $CuSO_4$) completely reaching the plateau phase or decomposition phase (Figure 2.4) (Esterbauer, Striegl, Puhl, & Rotheneder, 1989; Esterbauer & Zollner, 1989).

Figure 2.4

Typical absorption profile at 234 nm of conjugated diene formation produced during oxidation of isolated LDL by $CuSO_4$ *in vitro*



(Young & McEneny, 2001)

Several lines of evidence support the occurrence of LDL oxidation *in vivo*. Recently, oxidized apo B-100 epitopes and increased levels of lipid peroxidation products can be detected in LDL gently extracted from both rabbit and human

atherosclerotic lesions (Yla-Herttuala, et al., 1989). Circulating anti-oxLDL antibodies have been demonstrated in serum and titres correlate with the progression of atherosclerotic lesion (Palinski, et al., 1989). Summarized data in animal model treated with antioxidant are reviewed (Steinberg, 1997) that "the effect depends on antioxidant activity comes from the rough parallelism observed between the effectiveness of these compounds in protecting circulating LDL from oxidation in an ex vivo test system and their effectiveness in inhibiting atherogenesis". *In vivo* study, many cell types are capable of oxidizing LDL including monocytes, macrophages, neutrophils, endothelium, smooth muscle cell and fibroblasts. It seems likely that LDL is oxidized in microenvironment of vascular wall where there is oxidative stress at that site. Transition-metal-mediated oxidation such as iron and copper seems only to occur in advanced lesion while oxidation mediated by myeloperoxidase, or reactive nitrogen species (RNS) occurs throughout plaque development. However, it is still unclear which oxidative mechanisms or radical species are involved exactly especially oxidant and antioxidant enzymes. Impact role of oxLDL is summarized in table 2.2.

Table 2.2

Impact role of proatherosclerogenic and proinflammatory properties of oxLDL *in vitro* studies

1.oxLDL supports macrophage foam cell formation
2.oxLDL is chemotactic for monocytes, T cells and tissue macrophage
3.oxLDL enhances the expression of adhesion molecules on monocyte and endothelium
4.oxLDL alters inflammatory gene expression in vascular cells through a redox-sensitive mechanism
5. oxLDL induces tissue factor, prothrombogenic molecules expression and platelet aggregation
6. oxLDL impairs endothelium derived releaseing factor by altering NO activity
7. oxLDL is cytotoxic and could induce apoptosis in endothelial and smooth muscle cells

(Nakajima, Nakano, & Tanaka, 2006)

2.3 The crucial role of LOX-1

LOX-1 was first identified and cloned by Sawamura et al in 1997 as a mammalian endothelial receptor for oxLDL. This class E scavenger receptor has subsequently been found on other cell types including macrophage, vascular smooth muscle cell and platelets (Murphy, Tedbury, Homer-Vanniasinkam, Walker, & Ponnambalam, 2005; Murphy, et al., 2008). The human LOX-1 protein is a 273-residue protein with a short cytoplasmic N-terminus (36 residues), a single transmembrane domain, and an extracellular domain comprising a potential helical neck region (80 residues) and a C-type lectin-like domain (130 residues). LOX-1 is a 50 KDa type II membrane protein that structurally belongs to the C-type lectin family (Sawamura, et al., 1997). Notably, the lectin-like domain is highly conserved among species, especially at the positions of the six cysteine residues. This is consistent with its function as a ligand-binding domain and initiator of the processes of internalization and phagocytosis (M. Chen, Keene, Costa, Tahk, & Woodley, 2001). LOX-1 gene is located on human chromosome 12 within a cluster of lectin-like natural killer genes involved in immune recognition (Sobanov, et al., 2001). LOX-1 has high affinity (Kd ≅ 0.9 pmol/L) for protherogenic oxLDL (Moriwaki, et al., 1998) and is also activated by various stimuli as shown in table 2.3.

Table 2.3

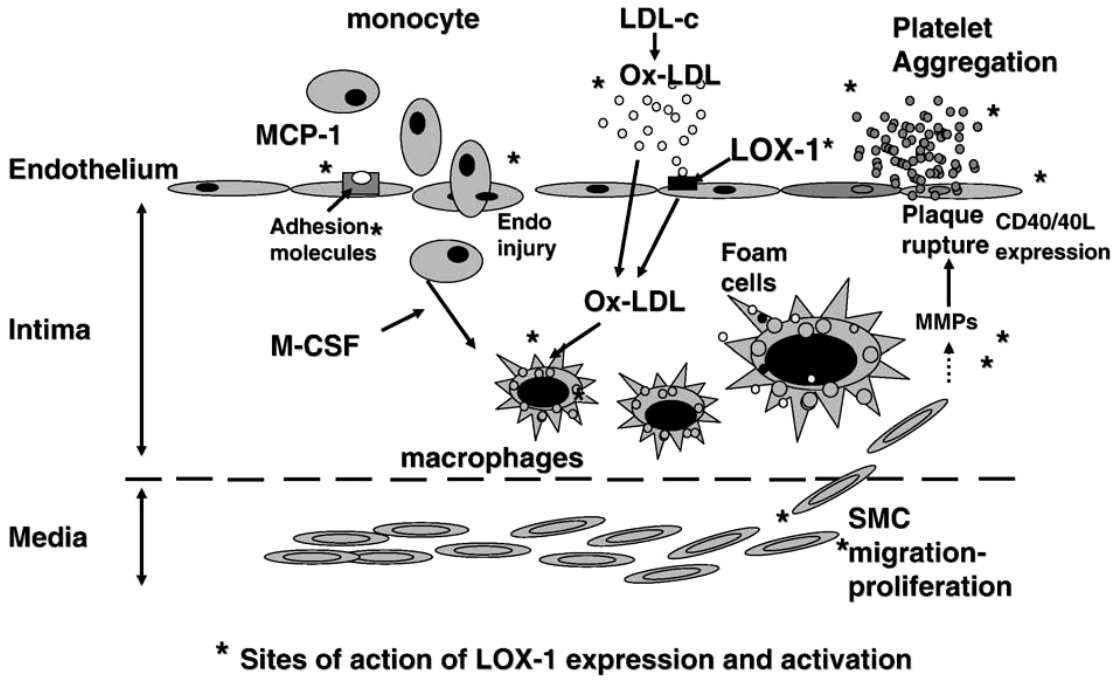
Elevation of LOX-1 receptor levels *in vitro* and *in vivo* studies

In Vitro	***In Vivo***
- oxLDL (Kataoka et al., 2001)	Hypertension (Nagase et al., 1997)
- Fluid shear stress (Murase et al., 1998)	Hyperlipidemia (Ando and Fujita 2004)
- Angiotensin II (Ando and Fujita 2004)	Diabetes mellitus (Ando and Fujita 2004)
- Proinflammatory cytokines	Atherosclerosis (Kataoka et al., 1999)
(Murase et al., 2000)	Ischemia-reperfusion injury
- Free radicals (Cominacini et al., 2003)	(Li et al., 2003)
- Glucose (Li et al., 2004)	

LOX-1 has been postulated to be an oxLDL endothelial sensor that regulates vascular function. Stimulation of the endothelium by binding of oxLDL to LOX-1 produces additional ROS (Cominacini, et al., 2000) and generating a positive feed back loop for further LDL oxidation. ROS induces activation of key transcription factors including nuclear factor κB which regulates gene expression for proinflammatory and adhesion molecules such as tumor necrosis factor (TNF), ICAM-1 (Cominacini, et al., 2000). Recently, LOX-1 transgenic mice have raised further question about LOX-1 because of overexpression of LOX-1 but lacking apoE and shown increasing LOX-1 for 10-fold in intramyocardial vasculopathy (Inoue, Arai, Kurihara, Kita, & Sawamura, 2005). Immunostaining studies on human and WHHL rabbit atherosclerotic tissues have shown a characteristic pattern of LOX-1 expression ((H. Chen, Li, Sawamura, Inoue, & Mehta, 2000). Moreover, LOX-1 was most prominently stained in the endothelial cells of early atherosclerotic lesions suggests a potential role for LOX-1 in the initiation of atherosclerosis. The persistent accumulation of LOX-1 protein in the atherosclerotic lesion implies long term effects such as plaque rupture and thrombosis formation. Depicted concept of activated LOX-1 and oxLDL during atherosclerosis is purposed by Metha JL and other (2006) as shown in figure 2.5.

Figure 2.5

Depicted concept of oxLDL-mediated LOX-1 in atherosclerosis



(Mehta, Chen, Hermonat, Romeo, & Novelli, 2006)

2.4 Vascular reactive oxygen species (ROS) and reactive nitrogen species (RNS)

2.4.1 What is the oxidative stress?

"Physiological function" is referred to the balance between ROS rates of production and their rates of clearance by various antioxidant compound and enzymes to reset the original state of "redox homeostasis". Cell in a stable state, a major mechanism of redox homeostasis is based on ROS-mediated induction of redox sensitive signal cascade terms "redox signaling". However, redox signaling balance is disturbed, either by an increase in ROS concentration or a decrease in the activity of one or more antioxidant systems; it may induce cell to oxidative stress. Numerous physiological functions are controlled by redox–responsive signaling pathways. NO is also a strong indication for the physiological relevance of redox signaling in

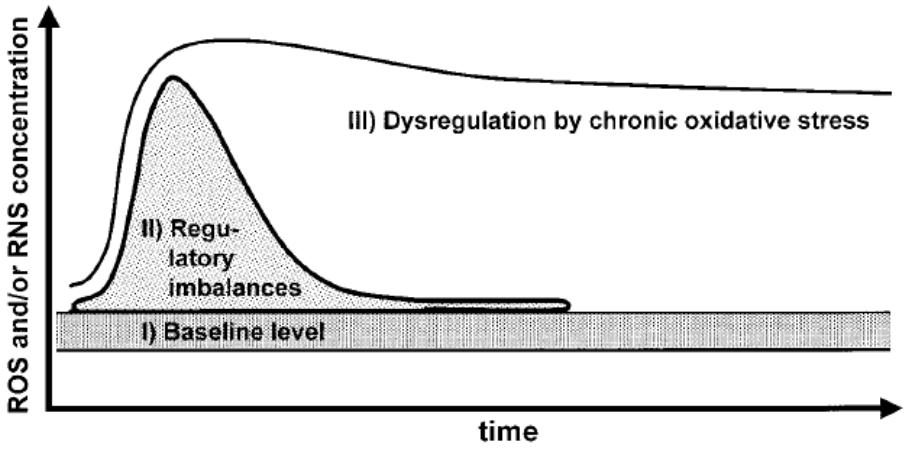
biological regulation. Physiological roles for ROS/RNS are involved to transmit biological information such as oxygen sensing function, regulation of vascular tone, during development, and leukocyte adhesion for inflammatory response to pathogens. The regulation of vascular tone by cGMP is special case. The enzyme guanylate cyclase was found to be activated by both NO and H_2O_2 (Wolin, Xie, & Hintze, 1999). Moreover, ROS have been demonstrated that a major dilator factor released from the vascular endothelium is H_2O_2 but other ROS ($O_2^{\bullet-}$ and $^{\bullet}OH$), directly effect of vascular tone is not known (Stahl, Halliwell, & Longhurst, 1992).

Term of "Oxidative stress" is defined as the imbalanced redox state in which pro-oxidants overwhelm antioxidant capacity, resulting in increased production of ROS, implicated in the pathogenesis of every stage of vascular lesion formation in atherosclerosis (Madamanchi, Vendrov, & Runge, 2005). Increased rates of vascular production of $O_2^{\bullet-}$ contribute to initiation of pro-inflammatory events, transcriptional regulation of various gene expressions that sensitive to changes in cellular oxidant production as well as to modulation of cell-signaling events (Ushio-Fukai, et al., 1999). $O_2^{\bullet-}$ is subsequently catalyzed by iron and endogenous SOD to be H_2O_2 which reacts with Fe^{2+} generating $^{\bullet}OH$. $O_2^{\bullet-}$and H_2O_2 may act as a regulatory in signaling processes leading to altered gene transcription, protein and enzyme activities, rapidly inactive NO, thereby causing endothelial dysfunction (Li & Shah, 2004).

An increase in superoxide or nitric oxide production leads to temporary imbalance that forms the basis of redox regulation. The persistent production of abnormally large amounts of ROS or RNS, however, may lead to persistent changes in signal transduction and gene expression, which, in turn, may give rise to pathological conditions (Figure 2.6) (Droge, 2001).

Figure 2.6

Regulatory events and their dysregulation depend on the duration of the change in ROS or RNS concentration. ROS and RNS normally occur in living tissues at relatively low steady-state levels.



(Droge, 2001)

2.4.2 Free radicals: ROS and RNS in vascular cells

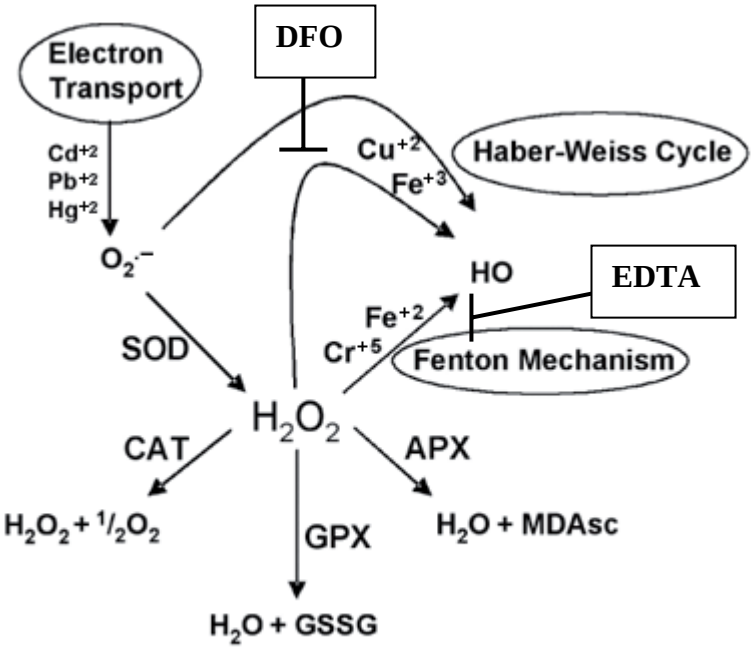
Basal free radical has necessary in physiology, but excessive of free radical, generally superoxide anion ($O_2^{\bullet -}$), causes implicated in the pathogenesis of atherosclerosis. Both of ROS and RNS are free radicals and also oxidants. ROS are the following: superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), lipid peroxyl radicals ($LOO^{\bullet}$), lipid hydroperoxide (LOOH), and aldehydes, RNS: nitric oxide radical (NO), peroxynitrite ($ONOO^-$) etc. The radicals establish "cross talk" between reaction ROS and RNS is $ONOO^-$ that formed by the combination of the two radicals $O_2^{\bullet -}$ and NO. Peroxynitrite mediates toxicity of NO and by oxidation and nitration that leads to altered protein function. Moreover, extracellular ROS/RNS can generate intracellular ROS/RNS through induction of various intracellular oxidase and stimulation of redox-sensitive signaling pathways. ROS act as secondary messengers, and has importance in vascular function ranging from physiological responses to the alterations observed in vascular diseases.

However, the mechanisms of how individual ROS interact with signaling systems are rather poorly understood.

All vascular cell types produce ROS, including endothelial, smooth muscle and adventitial cells. The most relevant sources of ROS with respect to vascular disease appear to be xanthine oxidase, uncoupled endothelial nitric oxide synthase (uncouple eNOS) and NAD(P)H oxidase. Many studies have shown that the major source of ROS in the vascular wall is NAD(P)H oxidase, which utilizes NADH/NADPH as the electron donor to reduce molecular O_2 and produce $O_2^{\bullet -}$. The negatively charged $O_2^{\bullet -}$ radical is unstable in aqueous solution (half-life of a few second) and is rapidly dismutase to H_2O_2 by superoxide dismutase (SOD) (rate constant $\sim 2\times10^9$ mol/l/second), which subsequently converted to water by catalase and glutathione peroxidase (Nagano & Fridovich, 1985). The hydroxyl radical has a high reactivity, making it a very dangerous radical with a very short *in vivo* half-life of approx. 10^{-9} s (Pastor & Hadengue, 2000). Under oxidative stress conditions, an excess of superoxide releases "free iron" from iron-containing molecules that we called "metal transition-driven ROS". The released Fe^{2+} participates in the Fenton reaction, generating highly reactive $^{\bullet}OH$ (Valko, Morris, & Cronin, 2005). However, vascular tissue contains various endogenous antioxidant enzymes, especially three forms of superoxide dismutase (SOD); a cytosolic copper-zinc form of SOD (CuZn-SOD), a mitochondria manganese form of SOD (Mn-SOD), and an extracellular CuZn-SOD (Ec-SOD). The presence of SOD is able to reduce $O_2^{\bullet -}$ concentration but these low levels of $O_2^{\bullet -}$ can be reacts with NO with rate constant of 7×10^9 mol/l/second to generate $ONOO^-$, which is three times the rate of its reaction with SOD. $ONOO^-$ is able to oxidize protein, lipid and nucleic acid, causing cell damage (Carr, McCall, & Frei, 2000).

Figure 2.7

ROS generated by heavy metal and scavenged by endogenous antioxidant enzymes



(Benavides, Gallego & Tomaro, 2005)

GSH, glutathione; GSSG, glutathione disulfide; GRed, glutathione reductase; GPx, glutathione peroxidae; DFO, desferoxamine; EDTA, ethylenediaminetetraacetic acid; APX, ascorbate peroxidase

Overproduction of RNS is called nitrosative stress (Klatt & Lamas, 2000; Ridnour, et al., 2004). Nitrosative stress may lead to nitrosylation reaction that can alter the structure of proteins and so inhibit their normal function. NO can react with free radicals quickly, large amounts of NO as generated by inducible NOS (iNOS) in inflammatory setting, interaction of NO and $O_2^{\bullet-}$ are give rise to the formation of nitrosating agent (N_2O_3) and peroxynitrite ($ONOO^-$), respectively (Pfeilschifter, Eberhardt, & Huwiler, 2001). The half-life of $ONOO^-$ is short (~10-20 ms), but sufficient to cross biological membranes, and allow significant interaction with most critical biomolecules directly (Denicola, Souza, & Radi, 1998). $ONOO^-$ can react with tyrosine to form nitrotyrosine. This reaction, known as nitration, has also been shown to affect protein function.

Summary, both of ROS/RNS products are important for normal cellular metabolism and playing a dual role as both harmful and beneficial to living systems. The delicate balance between beneficial and harmful effects is achieved by mechanisms called “redox regulation”. The process of “redox regulation” protects living organisms from oxidative stress and maintains “redox homeostasis” by controlling the redox status in cell (Droge, 2002). Redox-sensitive signaling pathways is also involved the processes of vascular pathogenesis (Kovacic & Jacintho, 2001). This occurs when there is an overproduction of ROS and/or a deficiency of antioxidants. Oxidative stress causes damage to cellular lipid, protein and DNA. At basal low/moderate concentration, NO, $O_2^{\bullet-}$ and related ROS, play an important role as regulatory mediators in signaling processes maintaining the redox homeostasis (Figure 2.6). Overproduction of ROS usually result cellular damage through redox signaling pathway (Irani, 2000).

2.4.3 ROS and RNS signaling pathway: p38 mitogen activated protein kinase (p38 MAPK) and role of oxLDL

Molecular targets of ROS/RNS and the exact physiological process involved remain incompletely understood. Common mechanisms involve redox-dependent covalent modification of specific cysteine residues in the thiolate form that are found in some proteins can be specific targets for reaction with H_2O_2 (Finkel, 1999). Previouly, ROS have been shown to alter numerous signaling pathways. Sublethal levels of oxidative stress inactivate tyrosine and serine/throeine protein phosphatase, contributing to increase activities of protein kinase in several signaling pathways. For example, molecular targets of ROS including all of the mitogen-activated protein kinase (MAPK) (i.e, extracellular signal-regulate kinase;ERK, c-jun NH_2 –terminal kinase; JNK, p38MAPK), are activated through specific and separate kinase cascades, protein tyrosine kinase, protein tyrosine phosphatase, gene expression, cell cycle protein and ion channels (Milliat, et al., 2006). Thus, intracellular levels of ROS are modulation of protein kinase activity and redox-sensitive gene expression in both of vascular physiology and pathology condition.

There are reported that MAPK mediated vascular smooth muscle cell (VSMC) proliferation and increasing LOX-1 (J. C. Chen, Huang, Wingerd, Wu, & Lin, 2004; Kyaw, et al., 2002). The mechanisms generated oxidized lipids *in vivo* are numerous include metal-dependent Fenton oxidation, enzyme-catalyzed oxidation, cell-dependent oxidation via a diversity of $O_2^{\bullet -}$ and H_2O_2-generating oxidase, and oxidation by NO-derived reaction species (Daugherty, 1994; Panasenko, Briviba, Klotz, & Sies, 1997; Radi, Beckman, Bush, & Freeman, 1991). As we known that NO can undergo reaction with $O_2^{\bullet -}$ to yield oxidizing and nitration species such as $ONOO^-$ mediated peroxidation of unsaturated fatty acids in the absence of transition metal catalyst, thus $ONOO^-$ may readily interacted with vascular signaling systems. $ONOO^-$ promotes the activation and/or inhibition signaling processes on tyrosine phosphorylation. In addition, $ONOO^-$ is able to activate the mitogen-activated protein kinase (MAPKs) family (ERK, JNK, and p38) pathway and its transcription factors during the process of apoptosis, cell proliferation, cell growth, and inflammatory gene expression in many cell type including vascular endothelial and smooth muscle cells (Gow, Duran, Malcolm, & Ischiropoulos, 1996; Upmacis, et al., 2004). The activation of redox-sensitive transcription factors are mediated via different signaling pathways includes p38 MAPK by LOX-1 activation (Mehta, 2004). LOX-1 activated through apoptotic pathway, promotes the overexpression of Bax, reduces the expression of Bcl-2 and caspase 9, thereby promoting susceptibility to apoptosis (Kataoka, et al., 2001; Okura, et al., 2000). OxLDL triggers the overexpression of the pro-apoptotic protein p53 (Napoli, de Nigris, & Palinski, 2001). Moreover, oxLDL alters the activity of the transcription factor, NF-κB, that is involved in the expression of immune and inflammatory genes. The oxLDL-induced NF-κB activation may also be mediated, and inactivation of NO, at least in part, by intracellular $O_2^{\bullet -}$, possibly generated subsequently to the binding of oxLDL to LOX-1 (Cominacini, et al., 2001). Data indicate that both native LDL and minimally oxLDL uncouple endothelial nitric oxide synthase (eNOS) activity and those minimally oxLDL can also activate xanthine oxidase and NADPH oxidoreductase inducing greater increases in $O_2^{\bullet -}$generation. OxLDL also activates the three main MAPK pathways, but their role is apparently different since p38 MAPK and SAP/JNK play a role in the oxLDL

induce toxicity (Napoli, et al., 2000), whereas ERK1/2 is rather involved in the mitogenic response (Metzler, Hu, Dietrich, & Xu, 2000).

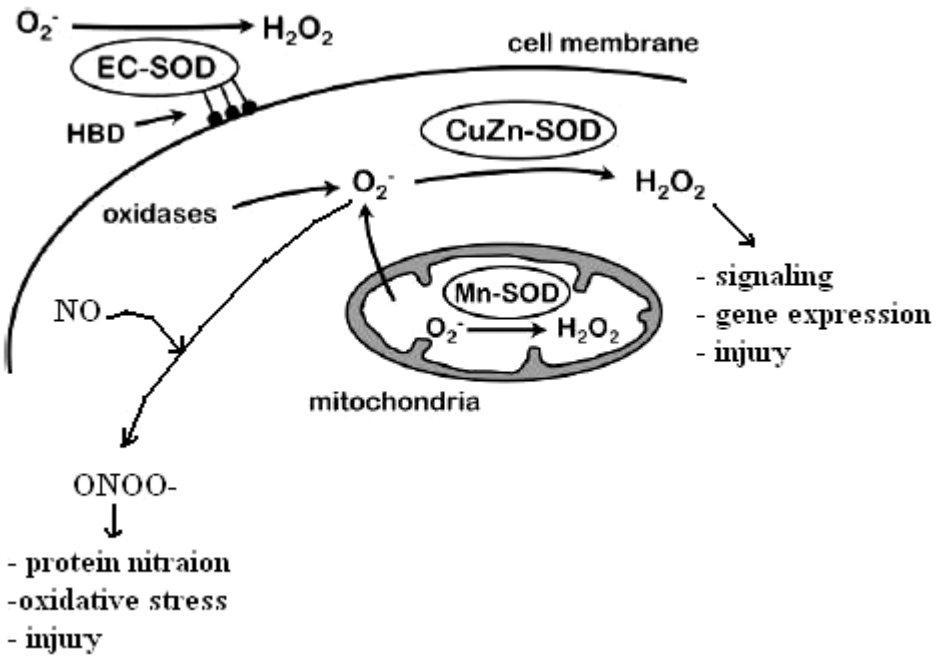
Interestingly, a signal transduction pathway that mediates cell functions is the MAPK cascade, and the mechanisms by which oxLDL alters function of cells in the vessel wall remain undefined. Recently, oxLDL increases phosphorylation and activation of p38 MAPK in vascular cells growth in concentration and time-dependent manners and may induce cytotoxicity in VSMC (Coppock, et al., 1999).

2.4.4 Antioxidant enzyme: superoxide dismutase (SOD) activity in vascular cells

Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms (Cadenas, Barja, Poulsen, & Loft, 1997). One of defense mechanisms, an antioxidant, is against free radical induced oxidative stress. Superoxide dismutase (SOD), an endogenous antioxidant enzyme, is one of antioxidant defense in the endothelium (Muzykantov, 2001). There are three isoforms of SODs, cytosolic or copper-zinc SOD (Cu-Zn SOD or SOD1), manganese SOD (Mn-SOD or SOD2) localized in mitochondria, and extracellular form of Cu-Zn SOD (Ec-SOD or SOD3) (Figure 2.8). SOD dismutase $O_2^{\bullet -}$ into H_2O_2 and O_2. Low levels of ROS activate the expression of several gene products involved in the antioxidant defense including Mn-SOD, and glutathione reductase (Hassan & Fridovich, 1977).

Figure 2.8

The subcellular localization of three SOD isoforms: CuZn-SOD located primary in cytosol, Mn-SOD localized in mitochondria, and extracellular CuZn-SOD (Ec-SOD) located at cell membrane.



(Faraci, 2003)

All three isoforms of SOD catalyze the same reaction that producing H_2O_2 from $O_2^{\bullet-}$. H_2O_2 is an important signaling molecule, but in combination with Fe^{2+}, H_2O_2 can also produce injury by forming hydroxyl radical ($^{\bullet}OH$). Superoxide ($O_2^{\bullet-}$) can react with NO to form peroxynitrite ($ONOO^-$) (Figure 2.8) (Faraci, 2003). Several functions of SOD have been reported such as against $O_2^{\bullet-}$- mediated cytotoxicity, prevent vascular endothelial cell-mediated LDL oxidation (Rosen & Freeman, 1984) , protected NO and reducing formation of $ONOO^-$ (Packer, 2002).In summary, SOD reduces (1) nitration of tyrosine residues, (2) activation of poly (ADP-ribose) polymerase (PARP) and expression of inducible NOS (iNOS), (3) oxidation of tetrahydrobiopterin (BH_4), resulting eNOS "uncoupling" (Zou, Shi, & Cohen, 2002). Consequence of SOD activity is formation of H_2O_2, a signaling molecule and

regulator of gene expression, and is mediator of hypertrophy of vascular smooth muscle in response to various stimuli such as angiotensin II (Blanc, Pandey, & Srivastava, 2003; Griendling, Sorescu, Lassegue, & Ushio-Fukai, 2000). Any evidences have shown that addition of SOD does not inhibit endothelial cell-induced LDL oxidation (van Hinsbergh, Scheffer, Havekes, & Kempen, 1986). In contrast, transient overexpression of Cu-Zn SOD was highly effective in reducing endothelial cell-induced LDL oxidation (Fang, Sun, Tian, & Cong, 1998).

The functions of individual SOD isoforms have been difficult to define. The activity of CuZn SOD may be necessary to limit an increase in $O_2^{\bullet-}$. Since the activity of CuZn SOD accounts for 50% to 80% of total SOD activity (Stralin, Karlsson, Johansson, & Marklund, 1995), deficiency in Cu-Zn SOD results in highly $O_2^{\bullet-}$and $ONOO^-$ (Baumbach, Sigmund, & Faraci, 2003; Landmesser, et al., 2000). However, Mn-SOD activity is considered to be a first line of defense against oxidative stress. The promoter region of the Mn-SOD gene contains response elements for the redox-sensitive transcription factors such as activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) that critical in regulation of many inflammatory related genes (Macmillan-Crow & Cruthirds, 2001). Ec-SOD has found at extracellular but the expression or activity is still uncertain. At least one major function of Ec-SOD is protecting NO as it diffuses from endothelium to its major target, soluble guanylate cyclase (sGC) in vascular smooth muscle cell (Oury, Day, & Crapo, 1996). Thus, SOD is necessary to protect vascular cells from oxidative stress and maintaining endothelial function through redox-sensitive signaling pathway.

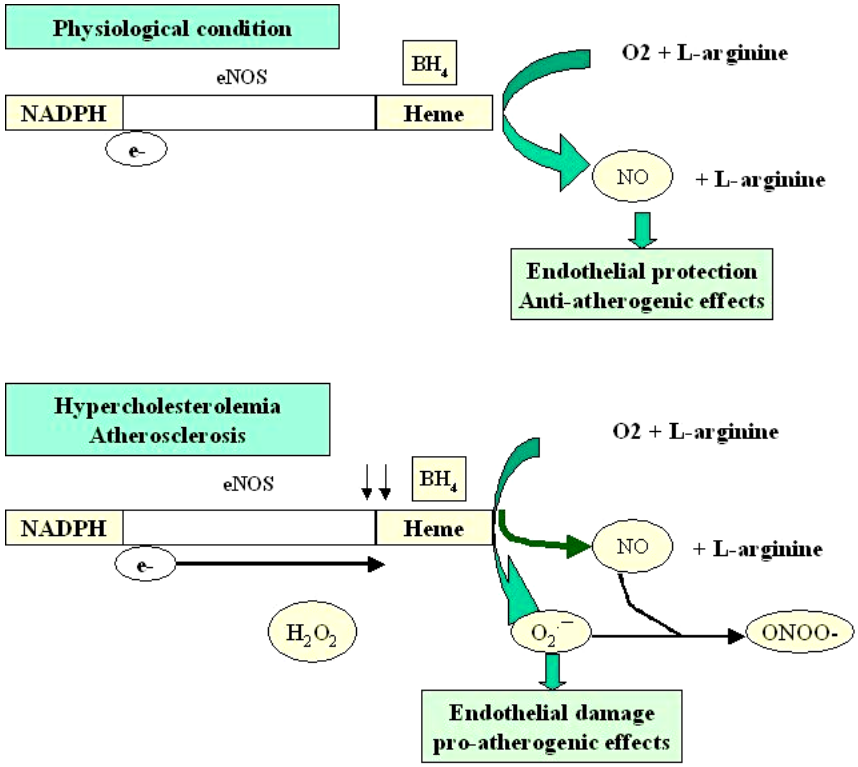
2.4.5 Nitric oxide (NO) and endothelial nitric oxide synthase (eNOS)

Physiologically, NO is generally less reactive than molecular oxygen. In particular, NO has only on unpaired electron, which allows it to bind strongly to the iron in heme groups, which is crucial to its biological activity of activating soluble guanalate cyclase (sGC) and subsequent production of cGMP as a principle-signaling messenger that modulates intracellular calcium levels to modulate many diverse activities of cell responses. Although the biological half-life of NO is only a few seconds *in vivo*, but it is freely permeable to membrane and only 5-10 nm NO is

necessary to activate cGC. The other action of NO is scavenging $O_2^{\bullet-}$ and other free radicals, inhibiting $O_2^{\bullet-}$-driven Fenton reaction, and lipid peroxidation. A defect in NO production causes oxidative stress leads to uncoupling eNOS resulting from inhibiting BH_4. (d'Uscio, Milstien, Richardson, Smith, & Katusic, 2003). Under physiological conditions, tissue levels of BH_4 are optimal for eNOS catalytic activity, and activation of eNOS generates NO and L-citrulline. Thus, NO is generated by eNOS serves as an anti-atherogenic molecule. During oxidative stress in hypercholesterolemia and atherosclerosis, tissue levels of BH_4 are reduced and when activation of eNOS, it leads to "uncoupling of eNOS" with subsequent generation of $O_2^{\bullet-}$ rather than NO (Figure 2.9). $O_2^{\bullet-}$, $ONOO^-$, and H_2O_2 serve to damage endothelial cells and thus they promote atherosclerosis (Moore & Freeman, 2006). Despite, NO has a half-life of only a few seconds in an aqueous environment but it has greater stability in an environment with a lower oxygen concentration (half-life >15 s). However, since it is soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes. In the extracellular, NO reacts with oxygen and water to form nitrate and nitrite anions (Chiueh, 1999).

Figure 2.9

Hypothetical scheme illustrating the possibility of divergent roles of eNOS in atherosclerosis



(Moore & Freeman, 2006)

Excess of ROS, the most important cause of endothelial dysfunction is from derangement of the eNOS/NO pathway, which includes the reduced activity and expression of eNOS, decreased sensitivity to NO, and increased degradation of NO by reaction with $O_2^{\bullet-}$(Harrison, 1997). Generally, there are two endothelial forms of NOS: constitute NOS; eNOS or type III and inducible NOS; iNOS or type II. Under basal condition in blood vessel, NO is generated in endothelial cells from its precursor L-arginine via an enzymatic action of endothelial nitric oxide synthase (eNOS). Cofactors such as tetrahydrobiopterin (BH_4) and nicotinamide adenine dinucleotide phosphate (NADPH) are also involved in NO production (Behrendt & Ganz, 2002). Endogenous NOS inhibitors such as asymmetric dimethylarginine (ADMA) and N-monomethylarginine (NMA) are also revealed to be involved in mechanism of

reduced NO in atherosclerosis (Miyazaki H et al., 1999). Various stimuli such as pro-atherogenic lipids and oxLDL inhibit signal transduction from receptor activation to eNOS activation (Hirata K et al., 1991). Hypercholesterolemia and oxLDL upregulate caveolin that augments caveolin-eNOS heterocomplex, and thereby attenuates NO production from endothelial cells (Feron & Kelly, 2001).

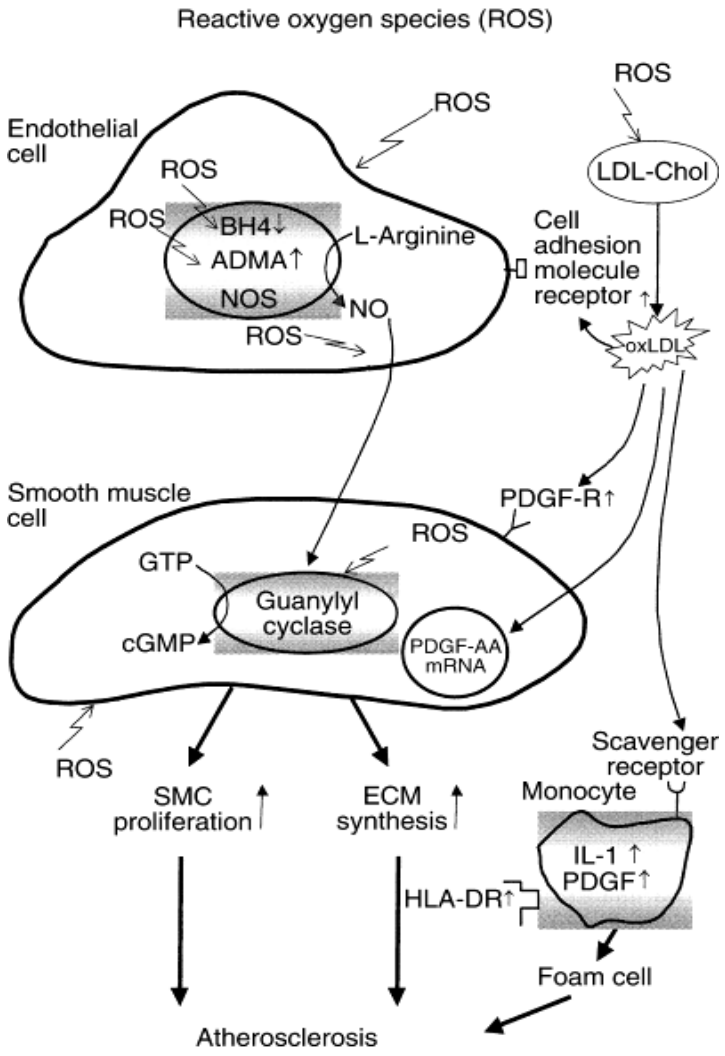
In hyperlipidemia and atherosclerosis, eNOS is dysfunction and can produce $O_2^{\bullet-}$ and impaired endothelium-derive relaxing factors, NO. NO acts as anti-atherogenic molecule including inhibits the migration, proliferation of vascular smooth muscle cells and also platelet aggregation, inhibits leukocyte-endothelial adhesion and reduces endothelial expression of adhesion molecules and proinflammatory cytokines (De Caterina, et al., 1995). Thus, balance of NO and ROS is important in redox-signaling. ROS production is increased, tetrahydrobiopterin (BH_4) generation is inhibited, and endothelial NO synthase (eNOS) produces superoxide ($O_2^{\bullet-}$). Excess generation of $O_2^{\bullet-}$ by different sources such as NADPH oxidase, uncoupled eNOS, xanthine oxidase, myeloperoxidase, cyclooxygenase, mitochondria will reduce NO bioavailability and convert NO into peroxynitrite ($ONOO^-$), which has deleterious effects. Recently, reduced activity of superoxide dismutase (SOD) will also result in enhanced ROS accumulation in the vascular wall and uncoupling eNOS generation.

2.4.6 Effects of ROS on vascular cell functions

ROS impair vascular functions by injury to endothelial and vascular smooth muscle cell membranes as the following: reduced nitric oxide (NO) and inactivation of tetrahydrobiopterin (BH_4) as cofactor of nitric oxide synthase (NOS); induced peroxidation of native low-density lipoprotein (LDL) to oxidized LDL (oxLDL); stimulation of synthesis of asymmetric dimethylarginine (ADMA)-yielding NOS inhibition; inhibition of guanylyl cyclase, which leads to decreased production of cyclic guanosine monophosphate (cGMP), activation of platelet aggregation and adhesion and upregulation of LOX-1 by ROS induced oxLDL (Figure 2.10).

Figure 2.10

ROS impair vascular functions



(Annuk, Zilmer, Lind, Linde, & Fellstrom, 2001)

ECM, extracellular matrix; IL-1, interleukin 1; GTP, guanosine triphosphate; mRNA, messenger RNA.

2.5 Impact of endothelial dysfunction

Growing evidences indicate that oxLDL was seen in early to late stages of atherosclerotic lesions of hyperlipidemia patients and impaired NO production (Casino, Kilcoyne, Quyyumi, Hoeg, & Panza, 1994). Normally, endothelial cell

maintains vascular homeostasis through multiple complex interactions with vascular cells and regulates vascular tone by balance of vasodilators as NO, and vasoconstrictors as endothelin. Furthermore, the endothelium controls blood fluidity and coagulation through mediators that regulate platelet activity, clotting cascade, and fibrinolysis (Libby, 2002).

It is a widely purpose that impaired endothelial function is the initial step in atherogenesis with reduced NO bioavailability and increased oxidative stress. It contributes not only to initiation but also to progression of atherosclerotic plaque formation and triggering of cardiovascular events. Vascular endothelium is a major target of oxidant stress in the circulation, thus impairment of endothelium will promote leukocyte adhesion and increasing inflammatory cells such as adhesion molecules involve in smooth muscle cells migration. In addition, ROS also act as mediators of angiogenic vascular endothelial growth factor (VEGF) which induces vascular cells migration, proliferation, and formation that is essential events in the process of angiogenesis as well (Kuroki, et al., 1996) (Figure 2.11).

Figure 2.11

Showing normal endothelium functions compared with those of endothelial dysfunction