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Atherosclerosis is not merely a disease in its own right, but a process that is the principal contributor to the pathogenesis of myocardial and cerebral infarction, gangrene, and loss of function in the extremities. The process, in normal circumstances a protective response to insults to the endothelium and smooth muscle cells (SMCs) of the wall of the artery, consists of the formation of fibrofatty and fibrous lesions, preceded and accompanied by inflammation. The advanced lesions of atherosclerosis result from excessive inflammatory-fibroproliferative response to different forms of insult. These lesions when excessive, become the disease and may occlude the concerned artery.

ATHEROSCLEROSIS- THE BACKGROUND

Lobstein coined the generic term 'arteriosclerosis' in 1829. In 1904, Marchand used the term 'atherosclerosis' for the first time. Since then, cellular and molecular approaches to the study of cells of the vascular wall as well as new methods of understanding the abnormalities of lipid metabolism have provided new insights into the pathobiology of atherogenesis. Atherosclerosis has been linked to inflammation since 1823 by Rayer. Since then, many investigations have added to our understanding of the pathogenesis of the disease (Getz et al., 1969; Ross and Glomset, 1973 and 1976; Ross 1988; Orekhov et al., 1990; Stary et al., 1995). It is now clear that atherosclerosis is not simply an inevitable degenerative consequence of aging, but rather a chronic inflammatory condition that can
be converted into an acute clinical event by plaque rupture and thrombosis (Lusis, 2000).

Atherogenesis is a complex process. While genetic factors determine the limits under which atherosclerosis develops, environmental factors help to determine a person's risk within these limits. Hypercholesterolemia has long been accepted as a high risk factor for the development of atherosclerosis since high fat diet feeding results in cholesterol deposition in the arterial wall (Brown and Goldstein, 1984). Cholesterol feeding is being used as a means of developing atherosclerosis experimentally since the work of Anitschkow and Chalatow (1913). The other classical risk factors for atherosclerosis include male sex, advancing age, cigarette smoking, high blood pressure, diabetes and abnormal plasma lipids.

Atherosclerosis has a multifactorial etiology and various hypotheses have been put forward to explain the lesion formation. However, Badimon et al., (1993) have suggested the most likely sequence of events as follows: vascular injury, monocyte recruitment, macrophages formation and lysis, lipid deposition, platelet and growth factors, role of vascular smooth muscle cells and synthesis of extracellular matrix. Hyperlipidemia has been associated with each step of this sequence of events.

PATHOLOGIC FEATURES OF ATHEROSCLEROSIS

The lesions of atherosclerosis represent a protective, inflammatory-fibroproliferative response against different agents that can cause the
disease. The disease proceeds through a series of pathological stages: (a) intimal medial thickening; (b) fatty streaks; (c) intermediate lesions or atheromatous plaques; (d) fibrous plaques; (e) complicated plaques.

The early lesions consist of subendothelial accumulations of cholesterol-loaded macrophages, called 'foam cells'. These macrophage-rich lesions called fatty streaks, though not clinically significant, are precursors of more advanced lesions characterized by the accumulation of lipid-rich necrotic debris and smooth muscle cells. While fatty streaks consist of a single layer of foam cells, these are followed by atheromatous plaques consisting of layers of foam cells in the subendothelial space. Fibrous lesions typically have a 'fibrous cap' consisting of SMCs and extracellular matrix that encloses a lipid-rich 'necrotic core'. Plaques may become complex with calcification, vascularization and ulceration at luminal surface. Qualitative changes in these fibrous plaques, at some later stage, may result in hemorrhage and/or thrombosis leading to arterial occlusions.

The events of atherosclerosis have been greatly clarified by studies in animal models, including rabbits, pigs, non-human primates and rodents. The earliest event following the feeding of high-fat, high-cholesterol diet is the accumulation of lipoprotein particles and their aggregates in the intima. Within days or weeks, monocytes adhere to the endothelium. Monocytes then transmigrate across the endothelial
monolayer into the intima, where they proliferate, differentiate into macrophages and take up lipoproteins, forming foam cells (Ross, 1993). With time foam cells die, contributing their lipid-filled contents to the necrotic core of the lesion. Growth factors released by platelets at the site of injury stimulate the proliferation of SMCs and their migration to subintimal region. With secretion of fibrous elements by the SMCs, occlusive fibrous plaques develop and increase in size. Atherogenesis can, thus be viewed as a 'response to injury', with lipoproteins or other risk factors as the injurious agents (Ross, 1993; Libby, 1999).

CELLULAR COMPOSITION OF THE ATHEROSCLEROTIC LESIONS

Several cell types have been recognized in the lesions of human atherosclerosis. Earlier it was generally accepted that the major cell found within the advanced lesion of human atherosclerosis, the fibrous plaque, is the smooth muscle cell, derived from the medial smooth muscle cells (Ross, 1986). However, through histochemical and electron-microscopic studies, it is now clear that in many lesions there is a significant component of monocytes and/or macrophages, depending, in part, on the stage of the process studied (fibrous plaque or the fatty streak) (Geer, 1965; Schaffner et al., 1979).

According to Capron (1993), atherosclerotic plaques harbor three main cell types: monocyte-macrophages, T lymphocytes and arterial
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smooth muscle cells, thus, giving support to the view that plaques are foci of chronic inflammation. Evidence exists that fibrofatty lesions are composed almost exclusively of macrophages and lymphocytes and are devoid of SMCs. SMCs, on the other hand are the predominant cell type of fibrous plaques (Vedeler et al., 1984; Aqel et al., 1985; Gown et al., 1986). Advanced plaques are heterogeneous but contain macrophages too.

ROLE OF MACROPHAGES IN THE PATHOGENESIS OF ATHEROSCLEROSIS

In lesions of atherosclerosis at all stages of lesion development, the presence of monocytes and macrophages is a common feature. Various investigators (Schaffner et al., 1979; Lewis et al., 1983; Faggiotto et al., 1984 a, b) have implicated the blood monocyte as the major contributor to the intimal foam cell accumulation that characterizes the fatty streak. One of the earliest events in the development of atherosclerotic lesions is the adherence of blood monocytes to arterial endothelium. Animal models with diet-induced hypercholesterolemia (Gerrity, 1981; Joris et al., 1983) and Watanabe rabbits having a defective LDL-receptor gene (Rosenfeld and Ross, 1985) have been used to demonstrate this.

Monocyte adhesion is followed by their migration to the intima and modulation into lipid-laden macrophages. Multiple factors such as uptake of oxidized lipids can contribute to initiating the inflammatory response of macrophages. Macrophages in early lesions of both humans and rabbits
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express major histocompatibility complex, CD antigens, and a variety of cytokines, and growth regulatory molecules like PDGF, TNF, IL-1, MCP-1 and M-CSF (Stary et al., 1994; Ranies et al., 1995). Also, the activation state of monocyte/macrophage within a developing lesion depends both on the differentiation state of the cell and the local environment. Evidence suggests a gradual shift towards a more differentiated phenotype with presumably longer residence time within the lesion (Roessner et al., 1987; van der Waal et al., 1992; Poston and Hussain, 1993).

Replicating macrophages constitute a sizeable percentage of the proliferating cells in experimental and human lesions of atherosclerosis (Chang et al., 1995; Rekhter and Gordon, 1995). Macrophage- colony stimulating factor (M-CSF) responsible for monocyte proliferation, has been demonstrated in experimental and human atherosclerosis (Clinton et al., 1992; Rosenfeld et al., 1992). Macrophages in the core region of the lesion die, and high rates of DNA fragmentation characteristic of apoptotic cells are observed in macrophages situated within and adjacent to the necrotic core (Geng and Libby, 1995; Han et al., 1995).

The normal role of macrophage is to act not only as an antigen-presenting cell to T lymphocytes, but also as a scavenger cell to remove noxious materials and as a source of growth-regulatory molecules and cytokines. The macrophage is, thus the principal, inflammatory mediator of cells in the atheromatous plaque microenvironment. Within the lesions,
there may be autoamplification of the inflammatory response of macrophages. Further, as in wound healing, the macrophages are critical to the fibroproliferative response (Leibovich and Ross, 1975) which includes stimulation of SMC migration into the intima, proliferation and connective tissue synthesis. This fibroproliferative response contributes significantly to the lesion mass and may prevent underlying macrophages from leaving the lesion. The prolonged retention of macrophages within the lesion may contribute to the formation of the necrotic core and destabilization of the plaque.

Activated macrophages release matrix metalloproteinases (MMPs), a family of proteolytic enzymes, that mediate the catabolism of basement membranes and extracellular matrix allowing cells to migrate through the matrix. MMPs produced by inflammatory cells within or adjacent to the fibrous cap destroy the matrix proteins of the cap, thereby increasing the likelihood of rupture (Libby, 1995). Cytokines produced by macrophages – IL-1β and TNF-α as well as macrophages themselves by direct cell-cell contact have been shown to induce apoptosis in SMCs (Weissberg, 1999).

LIPOPROTEINS AND THEIR INTERACTIONS

A primary initiating event in atherosclerosis is the accumulation of low density lipoproteins (LDL) in the subendothelial matrix. Accumulation is greater when levels of circulating LDL are raised, and both the transport and retention of LDL are increased in the preferred sites for lesion
formation. LDL diffuses passively through endothelial cell junctions, and its retention in the vessel wall seems to involve interactions between the LDL constituent apolipoproteinB (apoB) and matrix proteoglycans (Borén et al., 1998).

An increased concentration of plasma low density lipoprotein (LDL) cholesterol constitutes a major risk factor for atherosclerosis as is demonstrated by various clinical, epidemiological and genetic studies (Jialal and Devaraj, 1996). Both diet-induced hypercholesterolemia and LDL-receptor defective models are characterized by alteration in the level and composition of plasma lipoproteins. Lipid-rich LDL and β-VLDL, both have been shown to induce a dose-dependent increase in monocyte adhesion to endothelial cells (Alderson et al., 1986; Endemann et al., 1987). Hypercholesterolemia has been shown to augment several parameters of monocyte/macrophage function that may contribute to the development of atherosclerotic lesions including adhesion, production of vascular SMC mitogens and chemotactic agents (Rogers et al., 1986).

OXIDIZED LIPOPROTEINS AND THE 'OXIDATION HYPOTHESIS' OF ATHEROSCLEROSIS

'Oxidation hypothesis' (Steinbrecher et al., 1990; Ross, 1993) states that the oxidative modification of LDL (or other lipoproteins) is important and possibly obligatory in the pathogenesis of the atherosclerotic lesion. Oxidative modification of LDL is a prerequisite for macrophage uptake
and cellular accumulation of cholesterol since native LDL is not taken up by macrophages rapidly enough to generate foam cells (Steinberg et al., 1989).

LDL is a complex of a large-molecular-weight protein, apolipoprotein B (apoB), neutral and polar lipids, and lipophilic antioxidants, mainly vitamin E and β-carotene. The oxidation of LDL occurs in two stages. The first stage occurs before the monocytes are recruited and starts in the polyunsaturated fatty acids (PUFAs) in LDL-surface phospholipids, with little change in apoB. The second stage begins when monocytes recruited to the lesion convert into macrophages and contribute their enormous oxidative capacity. The oxidation then propagates to core lipids, resulting in oxidative modification, not only of the PUFAs, but also of the cholesterol moiety itself and of phospholipids, and modification and degradation of apoB (Steinbrecher et al., 1990; Witztum, 1993). This modification presumably involves reactive oxygen species (ROS) produced by endothelial cells and macrophages. Several enzymes are also thought to be involved including myeloperoxidase, sphingomyelinase and a secretory phospholipase, all of which occur in human atherosclerotic lesions (Lusis, 2000).

The modification of the protein portion of LDL leads to a loss of recognition by the LDL receptor and a shift to recognition by the scavenger receptors and/or the oxidized LDL receptor (Brown and
Goldstein, 1990; Freeman et al., 1990). This shift in the receptor recognition leads to cellular uptake of oxidized LDL by receptors that are not regulated by the cholesterol content of the cell. The result is a massive accumulation of cholesterol leading to the formation of foam cells. Two such scavenger receptors- SR-A and CD36 have been identified (Suzuki et al., 1997; Febbraio et al., 2000). The expression of scavenger receptors is regulated by peroxisome proliferator-activated receptor-γ, a transcription factor whose ligands include oxidized fatty acids, and by cytokines such as TNF-α and interferon-γ (IFN-γ) (Tontonoz et al., 1998).

Oxidized LDL has been shown in the arteriosclerotic plaques of animals and humans (Ylä-Hertuala et al., 1989; Rosenfeld et al., 1991). Oxidative modification of LDL increases its atherogenicity (Steinberg et al., 1989; Steinberg and Witztum, 1990). Oxidized LDL is a potent chemoattractant for monocytes, is an inhibitor of macrophages mobility and can promote endothelial dysfunction and atherogenesis by altering gene expression in the arterial wall (Jialal and Devaraj, 1996). It can stimulate interleukin-1β (IL-1β) secretion from macrophages, which promotes SMC proliferation and endothelial adhesiveness to leukocytes (Libby and Hansson, 1991).

Oxidized LDL can also initiate an immune response involving T cells and macrophages (Stemme et al., 1995). Immune complexes
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Consisting of oxidized LDL and antibodies to oxidized LDL are found in atherosclerotic lesions of rabbits and humans (Ylä-Herttuala et al., 1994). Oxidized LDL is also toxic to many cell types including macrophages (Reid and Mitchinson, 1993; Marchant et al., 1995; Hardwick et al., 1996).

As a result of oxidation of LDL, a wide variety of biologically active molecules are formed like oxidized sterols, oxidized fatty acids and phospholipid and protein derivatives generated by adduct formation with breakdown products of oxidized fatty acids. Products such as lysophosphatidylcholine, oxidized sterols or modified phospholipids can leave the modified LDL and cause diverse biological effects in the arterial wall.

OXYSTEROLS AND ATHEROSCLEROSIS

Among the principal components of oxidized LDL responsible for cellular injury, oxysterols play a critical role both in vivo and in vitro (Imai et al., 1980; Smith and Johnson, 1989), particularly those oxidized in C7 such as 7β-hydroxycholesterol and 7-ketocholesterol (Hughes et al., 1994; Lizard et al., 1996).

Increased levels of oxysterols have been found in the hypercholesterolemic human plasma (Brooks et al., 1983; Addis et al., 1989), in the arterial wall of hypercholesterolemic rabbits (Hodis et al., 1991), and in atheromatous plaques from hypercholesterolemic patients (Carpenter et al., 1993). Sterols oxygenated in the 7-position predominate
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in copper-oxidized LDL (Carpenter et al., 1994; Breuer et al., 1996; Brown et al., 1996 and 1997) and these appear to be concentrated in foam cells isolated from human atherosclerotic plaque relative to whole plaque material mass (Mattson-Hultén et al., 1996). A significantly higher concentration of oxysterols has also been observed in the plasma of patients with angina (Zhou et al., 2000).

Oxysterols are 27-carbon product of enzymatic or non-enzymatic oxidation of cholesterol having diverse biological effects (Brown and Jessup, 1999). 7β-hydroxycholesterol (Cholest-5-en-3β,7β-diol; 7β-OH) is formed by non-enzymic oxidation in vivo (Breuer and Björkhem, 1995). Higher plasma 7β-OH levels are associated with an increased risk of atherosclerosis (Salonen et al., 1997; Ziedén et al., 1999). Macrophage-rich fatty streaks show the highest concentration of 7β-OH relative to cholesterol (Carpenter et al., 1993).

BIOLOGICAL EFFECTS OF OXYSTEROLS

A number of studies have suggested a determinant role of oxysterols in inducing cytotoxicity in the vascular wall (Boissonneault et al., 1991; Ramasamy et al., 1992; Zwijsen et al., 1992). Cytotoxicity of oxysterols to many cell types has been widely reported such as endothelial cells (Lemaire et al., 1998; Lizard et al., 1999), macrophages (Aupeix et al., 1995; Clare et al., 1995), SMCs (Nishio et al., 1996; Nishio and Watanabe,
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1996; Lizard et al., 1999), fibroblasts (Lizard et al., 1999) and lymphocytes (Christ et al., 1993).

Oxysterols are very potent regulators of cell sterol levels, downregulating both the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Kandutsch and Chen, 1973) and that of the number of LDL receptors on cells at both transcriptional and post-transcriptional levels and enhancing cellular cholesterol esterification by activation of acyl-CoA:cholesterol acyltransferase (Goldstein and Brown, 1990). By affecting the cholesterol metabolism of macrophages, the oxysterols thus contribute towards foam cell formation.

Recently, oxysterols (7\beta-OH and 7-KC) have been associated with induction of IL-1\beta and IL-8 secretion in endothelial cells and macrophages (Lizard et al., 1997). 7-oxygenated sterols have been shown to induce the expression of adhesion molecules on endothelial cells and suggested to favor adhesion and extravasation of monocytes and lymphocytes (Lemaire et al., 1998). Oxysterols like 7\beta-OH and 25-hydroxycholesterol are suggested to contribute to cell death in atherosclerotic plaques due to their inability to induce hsp70 expression in endothelial cells (Pirillo et al., 1999).

The diverse biological effects of oxysterols are attributed to their modulation of the structure and function of membrane-associated proteins
as these get inserted into and affect the physical properties of cell membranes (Brown and Jessup, 1999).

**THE STRESS RESPONSE AND STRESS PROTEINS**

The arterial wall is continuously subjected to various types of stresses- mechanical stress from blood pressure, acute or chronic injury from toxins such as lipopolysaccharide and oxidized LDL and from hyperlipidemia. To maintain the homeostasis of the vessel wall, the vascular cells produce a high level of stress proteins or heat shock proteins, which protect against damage.

Stress proteins or heat shock proteins (hsp's) are a family of approximately two dozen proteins characterized by a sequence which is highly conserved along the evolutionary scale (Lindquist, 1986). From prokaryotes to humans, production of hsp's is central to the generalized response of organisms to environmental and metabolic stressors (Young and Elliot, 1989; Morimoto, 1993). Their functions are essential to cell survival not only in the presence of environmental stressors but also under physiologic conditions.

Hsp's act as molecular chaperones (Georgopulos and Welch, 1993; Hendrick and Hartl, 1993) by helping in the correct folding and unfolding of nascent polypeptides to achieve a functional conformation (Beckmann et al., 1990; Ang et al., 1991); assist the translocation and/or delivery of newly synthesized proteins to the proper intracellular target or along
secretory pathways (Chirico et al., 1988; Haas, 1995); interact with proteins of the cytoskeleton (Koyasu et al., 1986) and modulate the binding of steroid hormones to their receptors (Pratt, 1993).

In situations of stress-temperature shock (Ang et al., 1991), ischemia (Knowlton et al., 1991), infections (Garry et al., 1983), hemodynamic overload (Deleayre et al., 1988), exposure to oxygen radicals or cytokines (Donati et al., 1990; Fincato et al., 1991)- when the constitutive cellular pools of hsps becomes inadequate, synthesis of hsps is readily induced to increase cellular defenses. Genes responsible for this induction of hsp synthesis are different from the constitutively expressed stress genes. Hsp73 and hsp72 (the inducible form and its constitutive homologue), for example, are products of two different genes.

The expression of heat shock genes can be regulated at the transcriptional or the translational levels, depending upon the organism and the type of cell within the same organism (Lindquist, 1986). Activation of gene transcription is mediated by one or more of a family of heat shock transcription factors (HSFs) (Lis and Wu, 1993). While HSF1 is activated by heat shock, oxidative stress, aminoacid analogues and heavy metals, HSF2 activation is linked to transcription of genes in differentiation processes (Morimoto, 1993). Induction of hsp70 in the arterial wall is shown to be mediated through the activation of HSF1 in response to high blood pressure (Xu et al., 1996). The expression of heat shock genes is
also regulated by hsps themselves through a negative feedback mechanism (Lindquist, 1986; Morimoto, 1993).

HEAT SHOCK PROTEINS AND ATHEROSCLEROSIS

There is a growing evidence for involvement of hsps in atherosclerosis. Several factors that contribute to the etiology of the disease can also activate a stress response, for example, inflammation, immune response or endothelial injury caused by mechanical or chemical stress.

Wick et al. (1995) proposed a 'two-stage' model for the formation of atherosclerotic lesions. According to their hypothesis, the first stage, essentially mediated by the immune system would lead to the formation of reversible lesions, containing monocytes, lymphocytes and smooth muscle cells. The appearance of foam cells and the development of persistent lesions characterize the second stage. Various stimuli potentially associated with the induction of a stress response might be involved in the initiation of the first stage by activating an immune response and by causing endothelial dysfunction. Lipoprotein overload and/or calcification of the vessel wall would lead to the second stage of the process.

The hsps are subdivided into multimember families based on the molecular weights of the proteins encoded- the hsp90, hsp70, hsp60 and small hsp families. In the field of atherosclerosis, hsp60 and hsp70 are the most widely investigated hsps.
ROLE OF HSP60

A number of studies suggest that hsp60 may act as an autoantigen. Xu et al. (1992) have shown that normocholesterolemic rabbits immunized with recombinant hsp65 or with *Mycobacterium tuberculosis* (MT; an hsp65-rich bacterium) developed atherosclerosis when fed a normal chow diet. Epidemiological studies have shown that serum antibodies against hsp65 were significantly increased in subjects with carotid atherosclerosis and coronary heart disease (Xu et al., 1993b; Hoppichler et al., 1996). Very recently, Xu et al. (2000) have proposed a strong correlation between serum soluble hsp60 and atherosclerosis suggesting that serum hsp60 may play important roles in activating vascular cells and the immune system during the development of atherosclerosis.

Further, immune reaction after acute arterial injury is suggested to be associated with binding and recognition of hsp65 by dendritic γδ T cells (Heng and Heng, 1994). More recently, George et al. (1999) have shown that mice fed high-cholesterol diet develop significantly enhanced fatty streak accumulation when immunized with recombinant hsp65 or with MT containing the hsp65.

Schett et al. (1997) have shown that anti-hsp65/60 antibodies positively stain the surface of heat-stressed macrophages and mediate macrophage lysis in the presence of complement and effector cells thus, suggesting that cytotoxicity of these antibodies could lead to areas of
severe cell death and necrosis, especially at macrophage-rich areas of atherosclerotic lesions. Hsp60 has been demonstrated to be coexpressed with adhesion molecules in arterial and venous endothelial cells in response to cytokines and LPS, though oxidized LDL failed to induce hsp60 expression (Amberger et al., 1997). Apart from being involved in the induction/progression of atherosclerosis, very recently, hsp60 has been suggested to be marker for early cardiovascular disease (Pockley et al., 2000).

**ROLE OF HSP70**

The most frequently studied subgroup of hsps is that of hsp70. Hsp70 has been shown to be present in normal and atherosclerotic human arteries (Berberian et al., 1990). Johnson et al. (1993) suggested that hsp70 localization changes in aortae during atherosclerotic evolution without affecting overall aortic hsp70 content. Studies have shown induction of hsp70 expression in cultured human endothelial cells and SMCs in response to oxidized LDL (Zhu et al., 1994 and 1995). Low molecular weight stress proteins are also induced in mouse peritoneal macrophages exposed to oxidized LDL (Yamaguchi et al., 1993). On the other hand, oxysterols from oxidized LDL failed to induce hsp70 expression in endothelial cells (Pirillo et al., 1999).

The protective role of hsp70 during cell stress is well documented. Exogenous hsp70 in low concentration (10μg/ml) increased survival of
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arterial SMCs from both normal and atherosclerotic cynomolgus macaques (Johnson et al., 1990). Further, it has been shown that exogenous hsp70 protects viability of stressed SMCs through interactions with the cell surface rather than via internalization (Johnson and Tytell, 1993). Hsp70 is reportedly induced in the myocardium (or cardiac myocytes) under various kinds of stress, thus improving cardioprotection *in vivo* (Yellon et al., 1992) and *in vitro* (Iwaki et al., 1993).

The inducible form of hsp70, ihsp70 is shown to be cytoprotective in a number of studies using various models and different stressors (Heads et al., 1995; Simon et al., 1995; Chi and Mestril, 1996; Radford et al., 1996; Samali and Cotter, 1996). Constitutive hsp70 overexpression is shown to confer oxidative protection to myocytes (Chong et al., 1998). Various studies in different cell lines (U937 and PEER T cell lines) and in mouse embryo fibroblasts have indicated that the protective effect of hsp70 is due to its inhibitory effect on the activation of stress kinases (Gabai et al., 1997; Mosser et al., 1997; Buzzard et al., 1998; Meriin et al., 1998).

On the other hand, while heat shock-induced hsp70 had no protective effect on apoptosis or necrosis induced by tobacco smoke in human premonocytic cell line U-937, hsp70 overexpression was shown to prevent tobacco smoke-induced necrosis and consequently increase apoptosis (Vayssier et al., 1998). Recently, apoptosis induced by TNF-α/cycloheximide, LPS and verocytotoxin has been shown to be increased
by hsp72 expression in human umbilical vein endothelial cells (Lucas et al., 2000). Also, hsp70 response in COS-1 cells induced by oxidized LDL failed to protect cells from lipoprotein toxicity (Pirillo et al., 2001).

There is recent evidence that antibodies to hsp70 are present in vascular patients suggesting that hsp70 might be involved in the pathogenesis and propagation of atherosclerosis (Chan et al., 1999). In fact, interaction of hsp70 and hsp90 with antigen presenting cells is shown to involve specific receptors, two of which have been identified recently (Binder et al., 2000).

**ROLE OF HSP90**

Hsp90 is primarily a cytosolic protein. Although the exact function of hsp90 under stress is not known, under physiological conditions, it has been shown to regulate the function of several proteins that are important in cell activation, such as protein kinases (Galea-Lauri et al., 1996) and steroid hormone receptors (Pratt et al., 1989; Demarco et al., 1991).

Elevated levels of hsp90 expression have also been reported in the autoimmune disease systemic lupus erythematosus and excess of hsp90 is associated with increased apoptosis and thus suggested to play a role in controlling the part played by mononuclear phagocytes in immunopathology (Galea-Lauri et al., 1996). Recently, Liao et al. (2000) have shown that brief oxidative stress causes sustained release of hsp90 among other proteins from vascular SMCs that can stimulate extracellular
signal-regulated kinases, indicating these to be important mediators for the effects of ROS on vascular function.

**OXIDATIVE STRESS AND ATHEROSCLEROSIS**

Oxidative stress is a cellular or physiological condition of elevated concentrations of reactive oxygen species that cause molecular damage to vital structures and functions. Oxidative stress occurs when redox homeostasis within the cell is altered. This imbalance may be due to either an overproduction of reactive oxygen species (ROS) or a deficiency in an antioxidant system.

Various factors like energetic radiation, hyperoxia, quinone compounds and molecules containing a metallic cation can generate ROS. The stimulation of immune system may lead to massive local production of ROS and hypochlorous acid because of the activities of an NADPH oxidase and a myeloperoxidase in the phagocytes (Hampton et al., 1998b). Compounds that inhibit key enzymes involved in the synthesis of glutathione or of other ROS-scavenging enzymes cause a sustained oxidative stress (Michiels and Remacle, 1988) e.g. buthionine-S,R-sulphoximine, which inhibits γ-glutamyl-cysteine synthase.

Accumulating evidence points to oxidative stress as an important trigger in the complex chain of events leading to atherosclerosis. Increases in oxidant stress are involved in the pathophysiology of endothelial dysfunction, the first step in the pathogenesis of atherosclerosis (Zalba et
Oxidative stress and the production of reactive oxygen species are suggested to function as physiological regulators of vascular gene expression mediated via specific redox-sensitive signal transduction pathways and transcriptional regulatory networks (Kunsch and Medford, 1999). Oxidants are suggested to exert mitogenic effects through generation of growth factors. Further, oxidants are implicated in creating imbalance in the coagulation system thus contributing towards platelet aggregation and thrombus formation (Ruef et al., 1999).

It is currently thought that ROS and thus, oxidative stress plays a critical role in vascular SMC proliferation (Griendling and Ushio-Fukai, 1998). Very recently, H₂O₂ is shown to induce hsp70 expression in vascular SMCs by activating hsp70 promoter (Madamanchi et al., 2001). Also, a role for ROS in the activity and expression of macrophage scavenger receptor (MSR) has been suggested as oxidant stress induced by the uptake of acetylated LDL was shown to cause increased activity of MSR by stabilizing MSR-I mRNA (De Kimpe et al., 1998). Oxidative stress may lead to the formation of oxidized lipids in cell membranes (Halliwell and Gutteridge, 1990). ROS readily react with the polyunsaturated fatty acids and cholesterol present in the cell membranes such that the direct addition of the resultant oxidized lipids can induce apoptosis (Christ et al., 1993).
While mild oxidative stress induces antioxidant defense enzymes, severe stress, on the other hand, has been shown to cause oxidative damage to lipids, proteins and DNA within the cells, leading to DNA strand breakage and disruption of calcium ion metabolism (Halliwell and Cross, 1994; Dhalla et al., 2000) even at the subcellular level causing mitochondrial dysfunction (Kowaltowski and Vercesi, 1999). In fact, oxidative stress has been suggested to be a mediator of apoptosis (Buttke and Sandstorm, 1994).

Oxidative stress has also been shown to activate several stress-activated protein kinases or phosphatases (Clerk et al., 1998; Ushio-Fukai et al., 1998; Wang et al., 1998). However, ROS production can also downregulate the expression of various genes. Oxidative stress has been shown to inhibit the T cell proliferative response. Also oxidative stress has been implicated in specific degradation of mitochondrial RNA (Morel and Barouki, 1999) and inhibition of protein synthesis in endothelial cells at the translational level, specifically at the level of chain initiation (Jornot et al., 1991).

GLUTATHIONE- THE CELLULAR DEFENSE MECHANISM

One of the cellular protective mechanisms that serves to limit oxidative damage due to free radicals and ROS is glutathione. Glutathione is an abundant intracellular non-protein thiol that is synthesized primarily by the enzymes γ-glutamylcysteine synthetase and GSH synthase and is
present in millimolar concentrations in mammalian cells (Meister and Anderson, 1983).

Glutathione regulates several cell functions including reduction of disulfide linkages of proteins and other molecules (Meister, 1983), amino acid transport (Vina and Vina, 1983), enzyme activities, thiol-disulfide balance (Ziegler, 1985), synthesis of DNA precursors (Holmgren, 1979) and also cell proliferation (Kavanagh et al., 1990). Reduced glutathione (GSH) can effectively detoxify ROS in the presence of superoxide dismutase (SOD) (Meister and Anderson, 1983; Pichoner et al., 1995). It is also required for the preservation of glutathione peroxidase (GSH-Px) in its reduced active form.

The GSH-Px is a cytosolic, tetrameric selenoenzyme that catalyzes the reduction of organic hydroperoxides to the corresponding non-toxic alcohols (Flohe et al., 1979). The high ratio (about 100:1) of GSH to GSSG (oxidized glutathione) found intracellularly is maintained by the activity of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent GSSG reductase, a widely distributed flavoprotein.

GLUTATHIONE AND HEAT SHOCK PROTEINS

Apart from GSH and the related metabolism, another system that participates in the protection of cells under stress is the group of heat shock proteins. Many of the reagents/conditions that induce the heat shock response are capable of either generating oxidizing species (like reactive
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oxygen intermediates) in cells or depleting cellular sulfhydryl function; these include reoxygenation following anoxia, oxidizing agents, uncouplers of oxidative phosphorylation, heavy metal ions and SH-reactive agents (Huang et al., 1994).

There are several reports that have addressed the relationship between intracellular GSH and hsp70 synthesis. Cellular glutathione depletion has been implicated in decreased total protein as well as specific heat stress proteins synthesis in various cell models (Mitchell and Russo, 1983; Russo et al., 1984; Huang et al., 1994) by impairing transcriptional activation of heat shock genes by inhibiting the nuclear translocation of HSF1 (Rokutan et al., 1996). Higher GSH concentration in cisplatin resistant cells is suggested to play an important role in promoting hsp72 gene expression through processes downstream of activation of HSF-DNA binding (Abe et al., 1999).

On the other hand, synthesis of hsp70 is shown to be enhanced in glutathione-depleted Hep G2 and CHO cells (Freeman et al., 1993; Sierra-Rivera et al., 1994). Oxidative injury to coronary venular endothelial cells has been shown to deplete intracellular glutathione and induce hsp70 mRNA (Aucoin et al., 1995). Oxidation of protein thiols, protein denaturation and aggregation are implicated in the initiation of stress responses by glutathione depletion (Freeman et al., 1997).
GLUTATHIONE, OXIDATIVE STRESS AND ATHEROSCLEROSIS

Oxidative stress in endothelial cells, macrophages and SMCs occurs as a result of the depletion of intracellular reduced glutathione content. Glutathione becomes oxidized in response to the accumulation of oxidized lipids.

Glutathione and glutathione-dependent enzymes represent a coordinately regulated defense against oxidative stress. Coordination of this response involving glutathione synthesis, GSH-Px, glutathione S-transferases and glutathione S-conjugate efflux pumps, is achieved in part through the antioxidant responsive element (ARE) which is found in the promoters of many of the genes that are inducible by chemical and oxidative stress (Hayes and McLellan, 1999). Modulation of redox state by glutathione depletion is important in the regulation of various transcription factors that contain redox-sensitive cysteine residues, whose oxidation can affect DNA binding activity. These factors include AP-1 (Abate et al., 1990), NF-κB/Rel (Kumar et al., 1992), Myb (Guehmann et al., 1992) and NF1 (Novak et al., 1992).

Glutathione depletion is shown to lead to lipid peroxidation and thus, severe cellular damage (Comporti, 1987). A weak glutathione-related enzymatic antioxidant shield is present in human atherosclerotic lesions (Lapenna et al., 1998). It has been suggested that GSH-Px may be inactivated during oxidative stress (Condell and Tappel, 1983) which...
makes the cells prone to oxidative damage. Both GSH and GSH-Px were found to protect macrophages and endothelial cells from the toxic effects of oxidized LDL (Kuzuya et al., 1989; Thomas et al., 1993; Gotoh et al., 1993) and macrophages and endothelial cells exposed to oxidized LDL are shown to have elevated levels of glutathione (Gotoh et al., 1993; Cho et al., 1999).

**NITRIC OXIDE SYNTHASE**

Nitric oxide synthases (NOS), the enzymes that produce nitric oxide, have received considerable attention as potential candidates for vascular gene therapy (Channon et al., 2000) as NO has pleiotropic antiatherogenic properties and abnormalities in NO biology are apparent very early in the atherogenic process.

Nitric oxide (NO) an important biological product of mammalian cells, is synthesized by a family of three NO synthases (NOS). The three mammalian enzyme isoforms are the constitutive neuronal NOS (nNOS), endothelial NOS (eNOS) and the inducible NOS (iNOS). NOS catalyzes the oxidation of terminal guanidino nitrogen of L-arginine to yield NO and citrulline (Palmer et al., 1987; Ignarro, 1990; Moncada et al., 1991). NO is subsequently converted to nitrite and nitrate in oxygenated solutions (Marletta et al., 1988; Ignarro, 1990).
NITRIC OXIDE AND ATHEROSCLEROSIS

Evidence has accumulated that NO activity is an important determinant in normal vessel wall homeostasis (Palmer et al., 1987; Anggard, 1994). In vitro, NO inhibits all key processes involved in the early pathogenesis of atherosclerosis.

Major risk factors for atherosclerosis such as smoking (Celermajer et al., 1996; Heitzer et al., 1996), dyslipidemia (Creager et al., 1992; Chowienczyk et al., 1992), diabetes (Clarkson et al., 1996; Giugliano et al., 1997), hypertension (Egashira et al., 1995; Quyyumi et al., 1997) and hyperhomocysteinemia (Lentz et al., 1996) have been associated with impaired NO activity in vivo. The impairment of NO activity has been shown to be an early event in the development of atherosclerosis (Sorenson et al., 1994; Kari et al., 1997), preceding the advent of structural alterations (Reddy et al., 1994).

NO is shown to interfere with the elaboration of superoxide anion and may reduce the oxidative modification of LDL by macrophages (Clancy et al., 1992; Mao et al., 1992). NO release by iNOS action induced by cytokines in vascular SMCs may play a protective role in oxidative modification of LDL during the atherosclerotic process (Rikitake et al., 1998).

On the other hand, there are reports of NO acting as a proatherogenic molecule. The direct reaction of NO with free radicals is
suggested to have either pro- or antioxidant effects (Patel et al., 2000).
Production of NO has been shown to be enhanced rather than impaired in hypercholesterolemic and atherosclerotic rabbit aorta (Minor et al., 1990).
iNOS expression has been demonstrated in T lymphocytes and macrophages in atheromatous plaques of cholesterol-fed rabbits (Esaki et al., 1997).

It has been suggested that iNOS is present in monocyte/macrophage foam cells in human atherosclerotic lesions and that oxidized LDL inhibits this iNOS of macrophages (Yang et al., 1994). Oxidized cholesterol in oxidized LDL is suggested to be responsible for the inhibition of LPS-induced NO production in macrophages (Liu et al., 1998). Further, cholesterol oxides including 7-KC and 7β-OH, have been shown to inhibit release of NO from human vascular endothelial cells (Deckert et al., 1998). Also, cholesterol derivatives oxidized in position 7, i.e., 7-KC, 7α-OH and 7β-OH, can reduce maximal arterial relaxation (Deckert et al., 1997).

Several mechanisms have been proposed which may contribute to impaired NO production during hypercholesterolemia - impairment of expression of G-proteins and disruption of receptor G-protein interactions (Davies et al., 1994; Shibano et al., 1994), decreased L-arginine availability (Clarkson et al., 1996; Wang et al., 1996; Aji et al., 1997; Boger et al., 1997), reduction in NOS protein (Liao et al., 1995; Jessup,
1996) and increased NO degradation by oxygen radicals (Minor et al., 1990; Harrison and Ohara, 1995).

**NITRIC OXIDE AND GLUTATHIONE**

The relationship between NO and cellular glutathione levels has attracted attention of many investigators recently and the effects of NO on the regulation of antioxidant enzymes are now emerging. GSH protects against oxidized LDL by detoxifying the lipid intermediates formed during oxidation and also protects against the loss of NO production from endothelium.

A mechanistic link between intracellular glutathione levels, the loss of NO formation in hypercholesterolemia and modified lipoproteins has been suggested (Cox and Cohen, 1996). Nanomolar concentrations of NO have been demonstrated to induce GSH synthesis in bovine aortic endothelial cells through a mechanism involving \( \gamma \)-glutamylcysteine synthase and \( \gamma \)-glutamyl transpeptidase (Moellering et al., 1999).

Also, NO has been shown to stimulate cystine uptake and elevate intracellular GSH levels (Li et al., 1999). A short exposure to NO has been reported to increase cellular GSH levels (Luperchio et al., 1996). Rosenberg et al. (1999), on the other hand, suggested that NO protects against injury induced by GSH depletion though it does not induces GSH synthesis in rat oligodendrocytes.
SELENIUM AND CELLULAR ANTIOXIDANT DEFENSE SYSTEM

Selenium is an essential trace element for higher eukaryotes and many bacteria. In the elemental form, it is biologically inactive and is available to the biosphere after its conversion to selenides, selenites, selenates and organic selenium. Selenium has been shown to be distributed in all the cells and tissues of the body at the levels that vary with the tissue as well as the amount of selenium in the diet.

Selenium is essential in the activity of two antioxidant enzymes: glutathione peroxidase (GSH-Px) and phospholipid hydroperoxide glutathione peroxidase (PLGSH-Px). GSH-Px is one of the constituents of the cellular antioxidant defense system localized in the cytoplasm and mitochondria. It acts by reducing organic hydroperoxides into alcohols and hydrogen peroxide into water.

During the reduction of $O_2$ to water, electrons are transferred and through electron reductions, finally hydrogen peroxide ($H_2O_2$) is formed, which is removed either by catalase localized in the peroxisomes or by GSH-Px localized in both mitochondrial and cytosolic compartments.

$$\text{GSH-Px} \quad 2\text{GSH} + H_2O_2 \quad \rightarrow \quad \text{GSSG} + 2 \text{H}_2\text{O}$$

$$\text{GSH-Px} \quad 2\text{GSH} + \text{ROOH} \quad \rightarrow \quad \text{GSSG} + \text{ROH} + 2 \text{H}_2\text{O}$$
Selenium is an integral structural part of the enzyme, GSH-Px containing selenium in the form of selenocysteine in its peptide backbone (Forstrom et al., 1978). Selenium is added into selenocysteine in a regulated fashion in the presence of a hairpin structure (Secis element) in the 3'UTR of selenoprotein genes. Secis element directs the insertion of selenocysteine at UGA codons, which function as opal stop codons in the absence of suitable Secis element and in selenium deficiency (Ebert-Dumiq et al., 1999).

GSH-Px has been shown to be selenium dependent in cell culture systems as well as in animals and in humans (Sandström et al., 1987). Cellular GSH-Px status has been shown to be increased by selenium supplementation to the cells (Sandström et al., 1987; Baker et al., 1993). Selenium has been shown to increase intracellular GSH levels (Dalvi and Robbins, 1978) and the activities of γ-glutamylcysteine synthetase, the first and rate limiting enzyme in GSH biosynthesis and of glutathione reductase (Chung and Maines, 1981).

SELENIUM IN INFLAMMATION AND IMMUNITY

Several studies have evaluated the effects of selenium on humoral and cell mediated immunity. Evidence suggests that selenium deficiency in animals generally leads to a less responsive immune system and that nutritional selenium adequacy and moderate selenium supplementation
ensures a viable and responsive immune system in both animals and humans (Spallholz et al., 1990).

Selenium and GSH-Px are demonstrated in many different cells including polymorphonuclear leukocytes, natural killer cells and macrophages (Spallholz, 1990). Antioxidants, including GSH-Px are thought to protect the cell from self-destruction due to free radical damage (Klebanoff, 1980). During phagocytosis, macrophages (Parnham et al., 1983) and polymorphonuclear leukocytes (McCallister et al., 1980) deficient in GSH-Px showed an increased release of \( \text{H}_2\text{O}_2 \) and a reduced ability to reduce excessive oxidants.

**SELENIUM AND ATHEROSCLEROSIS**

Since free radicals are known to play an important role in the initiation of plaque formation, involvement of antioxidants as the preventive agents in atherosclerosis has been implicated. Selenium deficiency is well known to be associated with cardiovascular disorders (Oster and Prellwitz, 1990).

Various investigators have demonstrated statistically significant excess risk of cardiovascular and ischemic heart disease in people with low serum selenium levels (Salonen et al., 1982; Kok et al., 1989; Beaglehole et al., 1990). Studies have reported decrease in total cholesterol and triglycerides levels on selenium supplementation along with high fat diet feeding (Wojcicki et al., 1991; Kang et al., 1998).
Selenium is suggested to induce an antithrombotic effect on the interactions between platelets and endothelial cells by stimulating GSH-Px via a mechanism that is unrelated to the biosynthesis of NO metabolites (Ricetti et al., 1999). The inducible NOS in immunostimulated J774 macrophages has been shown to be inhibited by aminoethylisoselenourea and related compounds (Southan et al., 1996). Sodium selenite, an inorganic form of selenium, is demonstrated to inhibit NO production in asbestos induced alveolar macrophages (Fan et al., 1997) and in human T cells by inhibition of NF-κB DNA binding activity (Kim and Stadtman, 1997).

APOTOPSIS AND ATHEROSCLEROSIS

Cell death has long been recognized within the vessel wall, in particular, in disease states such as atherosclerosis. There are two forms of cell death- apoptosis and necrosis. Necrosis is a passive form of cell death occurring nonspecifically following cellular injury by agents such as toxins, hypoxia, free radicals or oxidized lipids. Apoptosis or programmed cell death (PCD), on the other hand, is a specific, active and regulated form of cell death which produces nuclear and cytoplasmic condensation (Nagata, 1997).

Traditionally, cell death in plaques had been considered to be essentially necrotic- particularly due to hypoxia or toxicity induced by oxidized lipids. However, apoptosis is now increasingly being recognized...
as the mode of cell death in the vessel wall (Bennett et al., 1995; Bjorkerud and Bjorkerud, 1996; Kockx, 1998; Kockx and Hermann, 1998; Kockx et al., 1998). Apoptotic cells have been morphologically demonstrated in atherosclerotic plaques of humans (Geng and Libby, 1995; Isner et al., 1995), rats with arterial wall injury (Han et al., 1995) and cholesterol-fed rabbits (Kockx et al., 1996; Kang et al., 1999). Apoptotic cells were often found to be arranged in clusters and primarily consisted of macrophages and SMCs.

One major clinical implication of apoptotic cell death in atherosclerotic lesions may be reduced plaque stability. Though both SMCs and macrophages can die in the plaques through apoptosis, the significance of macrophage apoptosis is different from that of SMC apoptosis. While increased apoptosis of SMCs can result in the weakening of fibrous cap in the vulnerable regions, increased macrophage apoptosis can lead to plaque stabilization through decreased breakdown of collagen.

**APOPTOSIS AND OXIDATIVE STRESS**

Control of programmed cell death or apoptosis is dependent on a balance between inhibitors and inducers of apoptosis. Oxidative stress, calcium overload, mitochondrial defects or stimulation by proapoptotic factors, each could lead to apoptosis. In recent studies, a potential role of oxidative mechanisms has been suggested in the apoptosis of vascular cells (Dimmeler et al., 1997 a, b).
Reactive oxygen species are principal mediators of cell damage in diverse pathological conditions. Many agents used to induce apoptosis also produce oxygen radicals and are inhibited by antioxidants (Brune et al., 1991; Zhong et al., 1993). ROS and calcium, two entities presumably important for apoptosis, are linked to mitochondria. ROS stimulate a specific Ca$^{2+}$ release from mitochondria, and when mitochondria 'cycle' Ca$^{2+}$ excessively, their ROS production increases (Hennet et al., 1993; Lafon-Cazal et al., 1993).

ROS, such as H$_2$O$_2$ have been seen to induce apoptosis in a variety of cell types (Zhong et al., 1993) thereby directly establishing oxidative stress as a mediator of apoptosis (Buttke and Sandstorm, 1994). It has been reported that addition of ROS or depletion of cellular antioxidants can result in apoptosis (Zhong et al., 1993). Alterations of intracellular redox status may either trigger or block the apoptotic death program, depending on the severity of the oxidative stress (Hampton and Orrenius, 1998). GSH depletion leads to hypercondensation and fragmentation of DNA associated with apoptosis in various cell types (Ratan et al., 1994; Higuchi and Matsukawa, 1999). Glutathione levels determine apoptosis in macrophages (Boggs et al., 1998). Further, compounds with antioxidant abilities were seen to block PCD (Brune et al., 1991; Iwata et al., 1992; Ramakrishnan and Catravas, 1992).
Review of Literature

The mechanism by which oxygen radicals might induce apoptosis is incompletely understood; however, since free radicals induce DNA damage with concomitant upregulation of p53 (Sandstorm and Buttke, 1993), ROS may operate in the same manner.

Cultured endothelial cells undergo apoptosis in response to oxidized LDL (Dimmeler et al., 1997a; Escargueil-Blanc et al., 1997) through a Fas-mediated mechanism (Sata and Walsh, 1998). Apoptosis of vascular SMCs may at least partly be attributable to oxidant damage by hydrogen peroxide (Li et al., 1997). Various investigators have reported induction of apoptosis by oxysterols, particularly the C7 oxysterols in monocytic cell lines (Aupeix et al., 1995), SMCs (Nishio et al., 1996; Nishio and Watanabe, 1996; Lizard et al., 1999), endothelial cells (Lemaire et al., 1998; Lizard et al., 1999) and fibroblasts (Colles et al., 1996).

The above mentioned reports suggest that atherosclerosis has a multifactorial etiology. It has been related to oxidative stress, oxidized LDL and its components, particularly oxysterols and immune system. Now, heat shock proteins are also linked to the pathogenesis of atherosclerosis. In view of the literature presented here, the basic lacuna in the literature seemed to be that expression of hsps at different stages of development of atherosclerosis has not been studied. Further, the regulatory response of hsp70 in an atherogenic model too was found to be unexplored. Though macrophages are a common feature at all stages of
lesion development and play an important role in the initiation and progression of the disease, not much attention has been given to the heat shock protein expression in macrophages subjected to oxidative stress. We, therefore, in the present work, attempted to evaluate the expression and distribution of major hsps at various stages during HFD-induced atherosclerotic progression in rabbits. We also tried to study the regulation of hsp70 expression under these conditions. Further, heat shock protein expression in macrophages exposed to an oxysterol, 7β-hydroxycholesterol, in vitro was also evaluated. Moreover, effect of 7β-hydroxycholesterol on macrophage metabolism has not been studied. So we explored the effect of this oxysterol on cellular redox status and nitric oxide production by macrophages. Also, role of selenium as an antioxidant is well established. An attempt, therefore, was made to study its influence on oxysterol-induced changes. Lastly, status of apoptosis under these conditions in macrophages was also evaluated.